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Memory-Enhancing Effects of Silk Fibroin-Derived Peptides in Scopolamine-Treated Mice

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Although enzyme-hydrolyzed silk fibroin has been reported to enhance cognitive function before, it has been still unknown which peptides can improve memory. Here we report that amino acid sequences of three novel peptides were identified from fibroin hydrolysate. Fibroin hydrolysate was obtained by hydrolysis with protease after partial hydrolysis with 5 M $CaCl_2$. Synthesized peptides derived from these sequences improved scopolamine-induced memory impairments in mice. We confirmed this hydrolysate had effects that improved learning and memory abilities by performing the Rey-Kim test. From this hydrolysate of silk fibroin, amino acid sequences of eight peptides were identified by LC-MS/MS. Three peptides (GAGAGTGSSGFGPY, GAGAGSGAGSGAGAGSGAGAGY, and SGAGSGAGAGSGAGAGSGA) were synthesized to investigate whether they could improve memory. Passive avoidance test and Morris water maze test were performed, and all peptides showed memory-enhancing abilities on scopolamine-induced memory impairments in mice. In this study, we identified three novel peptides that could improve memory, and that silk fibroin hydrolysate was a mixture of various active peptides that could enhance memory.

Keywords: Peptide, memory, fibroin, silk, scopolamine

Introduction

Silk fiber from *Bombyx mori* is composed of 70% fibroin and 30% sericin protein. Recently, silk fibroin (SF) has gained attention as a new biomaterial. Owing to its specific physical and chemical properties [19], SF is being used in various preparations such as cell culture [18], enzyme immobilization [23], and oral medication gel [5]. Many bioactive effects have been reported following the use of silk fiber. For example, blood cholesterol and glucose levels decreased and alcohol absorption was inhibited in SF-fed rats [1,16]. Additionally, when SF reacts with sulfate, it has effects that counteract human immunodeficiency virus (HIV) [4]. Enzymatic or acidic hydrolysate forms of SF have been used as functional food, cosmetics, and medical reagents [2, 9, 13, 26]. It has also been reported that SF hydrolysates (SFH) are effective in decreasing blood glucose levels in patients with type 2 diabetes [21] and in inhibiting absorption of alcohol [13]. More recently, it was revealed that the enzymatic hydrolysate enhances learning and memory function [8, 14].

Since the 1970s, various biologically active peptides have been suggested as nutraceuticals [3, 6, 7, 11, 17]. It has been reported that peptides from silk fibroin hydrolysate, GVGAGY and GVGY, play a role in decreasing blood pressure [6], and peptide GAGAGY stimulated glucose transport in adipocytes. Peptides from silk hydrolysate, GAGA, GAGAGA, GAGAGS, and GAGAGAGS, can suppress the development of picryl chloride-induced atopic dermatitis by reducing IgE production [7].

In this study, we investigated whether enzymatic hydrolysates of SF have memory-enhancing effects and attempted to identify new SFH-derived peptides that exhibit such effects. We then examined whether these peptides can alleviate cognitive impairments in scopolamine-treated mice.

Materials and Methods

Regents and Animals

Scopolamine was obtained from Sigma-Aldrich (St. Louis, MO, USA). Four-week-old, male ICR mice were purchased from Korean BioLink Co. (Chungbuk, Korea). Following a 1-week adaptation period, mice (28–30 g) were divided into 6 groups of 5 mice each: the saline-treated vehicle control group (S); the scopolamine-treated (1 mg/kg) group (SCOP, to lower cognitive function); the scopolamine (1 mg/kg) + SFH-treated (10 mg/kg) group (SFH); and the scopolamine (1 mg/kg) + synthesized peptides-treated (10 mg/kg) group (PY, GY, and GA). All reagents were injected intraperitoneally. Memory impairment was induced by scopolamine injection 30 min prior to trial or training, whereas synthesized peptides or SFH were injected 30 min before the scopolamine injection. Reagents were injected for 8 consecutive days, according to the schedule shown in Fig. 1.

Peptide Synthesis

Synthesized peptides were obtained from Anygen Co. Ltd. (Jangseong, Korea). Peptides were prepared *via* traditional F-moc peptide synthesis and purified by high-performance liquid chromatography. The peptide composition was confirmed by mass spectrometry (AXIMA CFR Kratos; Shimadzu, Tokyo, Japan).

Volunteers for Clinical Trial

Volunteers ranged from 13 to 70 years of age, and included individuals who represented the public in terms of cognitive variability. Volunteers included those who were in early teens to 20s and demonstrated good memory and learning ability, those who were in 30–40s and had started to exhibit mild memory impairment, and those who were in their 50–60s and had a larger degree of memory decline. Volunteers were excluded from this study if they were receiving medication and/or functional food, had neuropsychiatric problems, had any serious disease, had neurodegenerative disease, and/or were deemed not suitable for participation. Thirty-eight volunteers were randomly divided into 2 groups: 19 in the placebo group and 19 in the SF group. Since 31 volunteers completed the experiment, the final analysis included 15 in the placebo group and 16 in the SF group.

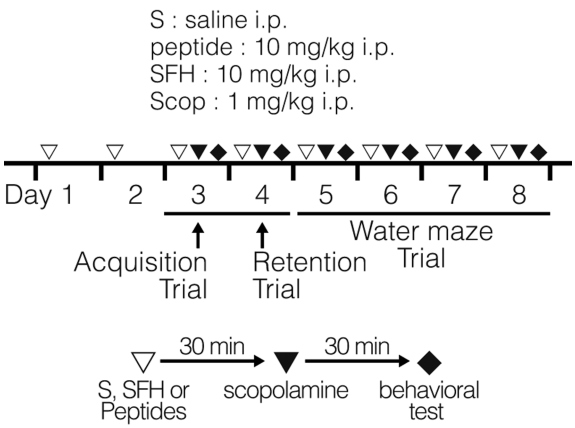

Fig. 1. Schedule of injections.
Peptides were injected for 2 days before the acquisition trial. On the day of trial, peptides and scopolamine were injected. In the water maze experiment, injections were performed in the same manner until the end of the trials. As a positive control, SFH was injected instead of peptides.

Preparation of SFH

We prepared SFH according to a previously described method [27]. Briefly, silk cocoons were boiled in a solution of 0.03% sodium carbonate and 0.05% marseilles soap for 30 min to remove dirt and sericin. Cocoons were then washed with distilled water and dried at room temperature. Dried fibroin fibers were dissolved and partially hydrolyzed by adding them to a 5 M $CaCl_2$ solution and boiling the fibers for 1 h. The dissolved fibroin solution was cooled to 30°C and filtered with Whatman No. 1 filter paper. The filtrate was then transferred to a cellulose dialysis membrane. Salts were completely removed by dialysis, and the solution was then mixed with protease, followed by separation and purification of the protein portion (MW 500–5,000 Da) using Sephadex G-25 gel-filtration chromatography.

Determination of Amino Acid Sequences

Using an Accela UPLC and LTQ-Orbitrap XL (Thermo Fisher Scientific, Franklin, USA) equipped with a BEH130 C18 column (1.7 µm, 2.1 × 100 mm), liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed at the GyeongGi Bio Center (Suwon Korea) to determine the amino acid sequences of peptides present in the SF hydrolysate. HPLC and instrument conditions were as follows: Buffer A, water; Buffer B, acetonitrile; gradient, 1–25% Buffer B for 50 min; flow rate, 0.3 µl/min; detection ion mode, positive ion ($[M + H]^+$); scan range, Ms: *m/z* 300–1,800; spray voltage, 4.0 kV; capillary voltage, 35 V; capillary temperature, 300°C; resolution, FWHM (@400) = 30,000.

Clinical Experiments

During the course of the study period, the volunteers were instructed to continue their usual meal and smoking patterns, but were not allowed to drink alcohol. Volunteers were randomly assigned to the placebo or test group. The Rey-Kim Memory Test [12] was then performed in a double-blind manner before administration of SFH. Volunteers in the test group were administered 200 mg of SFH b.i.d., p.o., (400 mg/day) for 3 weeks, while the control group received a placebo. On completion of the 3-week clinical trial, the Rey-Kim Test was performed again to evaluate changes in memory.

Rey-Kim Memory Test

During the Rey-Kim Memory Test, verbal memory performance was assessed using the K-Auditory Verbal Test (KAVLT), a Korean version of the Rey Auditory Verbal Learning Test [24]. Nonverbal

memory performance on the K-Complex Figure Test (KCFT), a Korean version of the Rey Complex Figure Test [25] was also assessed. The KCFT is essentially identical to the standard Rey Complex Figure Test [15]. All KCFT tests were scored according to the standard 36-point scoring system [15].

Passive Avoidance Test

Passive avoidance tests were performed in identical illuminated and non-illuminated boxes [22]. The floors of each compartment were composed of 2 mm stainless-steel rods spaced 1 cm apart. The illuminated compartment (20 × 20 × 20 cm) was equipped with a 100 W bulb. These compartments were connected with a guillotine door (5 × 5 cm).

For the acquisition trial, a mouse was placed into the illuminated compartment after the injection of reagents, and a door was opened 10 sec later. When the mouse had completely entered into the dark compartment, the door was closed and a 0.5 mA electric shock was given for 3 sec duration. The retention trial was performed 24 h after the acquisition trial where the mouse was placed into the illuminated compartment. Latency time for both acquisition and retention trials was measured as the time taken to enter the dark compartment of the box after the door was opened.

Morris Water Maze Test

The Morris water maze test was carried out in a pool 100 cm in diameter and 30 cm in height [22]. The pool was filled with milky water and kept at 22–25°C. An escape platform (6 cm in diameter) was submerged 1 cm below the surface of water in one of the pool quadrants. During the navigation test (performed on days 5–8), the mouse was placed in the maze and was allowed to search for the platform for 60 sec. If the mouse was able to locate the platform, it was allowed remain on the platform for 10 sec. If the mouse could not find the platform within 60 sec, the mouse was placed on the platform for 10 sec. Over the 4 subsequent days, mice were given four trial sessions per day. The time interval between each trial session was 1 min. Scopolamine was injected 30 min before the trials, while synthetic peptides or SFH was injected 30 min prior to scopolamine.

Statistical Analysis

Numerical data are presented as means ± standard error of the mean (SEM). For the clinical trial, a paired *t*-test was used to analyze the relationship of scores between tests before and after SFH administration.

For the passive avoidance test, statistical analyses were performed separately for the acquisition and retention trials. We first tested whether there was a mean difference in latency time between reagent types in the acquisition trial. A one-way ANOVA was performed and the model formula is

$$Y_{ij} = \beta_0 + \beta_i + \varepsilon_{ij},$$

where i denotes the received reagent and j denotes j th mouse. Y_{ij} and ε_{ij} are the log-transformed latency time and error term, respectively. When β_i was significant ($p < 0.05$) using the ANOVA, we were interested in determining which pair was different. In this case, so-called multiple correction was desirable because we tested several hypotheses simultaneously. We used Tukey's multiple comparison for our *post hoc* test. If the adjusted *p*-value for each pair was below 0.05, the corresponding pair was declared to have a significant different mean. We also applied the same method of analyses to the retention trial.

For the Morris water maze test, there was no simple trend between escape latency time and day or trial. Thus, we treated day and trial session as categorical variables. To incorporate individual variability, the following linear mixed model was used:

$$Y_{ij} = \beta_0 + \beta_j + v_i + \beta_{trt} + \varepsilon_{ij}$$

where i denotes i th mouse and j denotes the order of the experiment (day and trial session). Y_{ij} and ε_{ij} are the escape latency time and error term, respectively. The parameter of interest, β_{trt} (the effect of each reagent), was calculated. Note that v_i is a random effect used to explain the correlation between sequential observations of i th mouse and is assumed to follow $N(0, \sigma_v^2)$. When β_{trt} was found significant, multiple comparison of the means were performed as before. If the adjusted *p*-value for each pair was below 0.05, the corresponding pair was declared to have a significant different mean.

Results

SFH Enhanced Memory Quotient

The memory quotient (MQ) is the most direct index reflecting memorization ability. The average MQ of the test

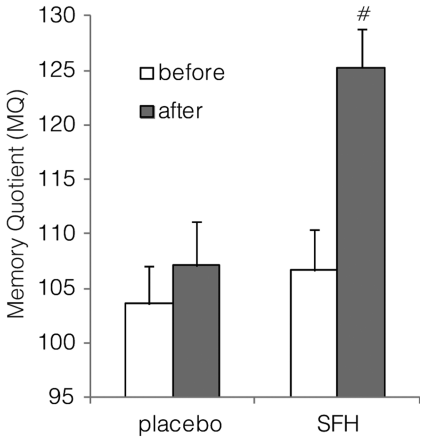

Fig. 2. Enhanced memory quotient in the SFH group.
SFH (400 mg/day) was administered for 3 weeks. Scores before and after administration of SFH were compared using a paired *t*-test. MQ scores are expressed as the mean ± SEM ($^{\#}p < 0.05$ as compared with the placebo group).

group was around 106.6, and it increased significantly to about 125.2 after administration of SFH for 3 weeks (Fig. 2, $p < 0.001$), but not in the placebo group. These results are similar to those of previous studies [8, 14] and suggest that our SFH contains active peptides that enhance memorization ability in normal individuals. To find novel peptides enhancing memory, separation of peptides and amino acid sequencing were performed with this SFH by LC-MS.

Determination of Amino Acid Sequences

Although lots of peak were separated from SFH, only eight peaks could be sequenced in the LC-MS chromatogram (Fig. 3), shown in Table 1. There were three common sequences at the C-terminal regions among the peptides; therefore, these peptides were divided into 3 groups: Pep 1, 2, and 3 were grouped as PY, Pep 4 and 5 were grouped as GY, and Pep 6, 7, and 8 were grouped as GA. The common sequences of the three groups are marked as shadowed characters in Table 1. The common sequence of the PY group was identical to sequence Pep 3. In the GY group, Pep 5 was the same as Pep 4, except for two amino acids. In the GA group, Pep 8 had an extra amino acid than those in the common sequence of group GA. Therefore, Pep 3, 5, and 8 were synthesized as representative peptides of each group and used in animal tests.

Effect of Peptides on the Passive Avoidance Test

Retention latency in the passive avoidance test reflects long-term memory functions in rodents [20]. Therefore, we tested the effects of the silk fibroin peptides on scopolamine-induced memory deficits using the step-through passive avoidance test. In the retention trial, the latency time for saline-treated mice reached 180 sec (our maximum cut off time). The mean step-through latency of the scopolamine-injected group was significantly lower than that of the saline group (Fig. 4, $p < 0.01$ *vs.* S group). SFH and synthesized peptides of the PY and GY groups significantly improved the scopolamine-induced memory defects (Fig. 4, $p < 0.05$ *vs.* scopolamine group). The peptide of the GA group also increased in latency, but it was not statistically significant. Latency times during the acquisition trials remained unaffected by scopolamine, SFH, or synthesized peptide injections (Fig. 4, black bar). These data suggest that SFH, peptide PY, and peptide GY improve impairments of long-term memory.

Table 1. Amino acid sequences of peptides in SFH.

Group	Peptide	Amino acid sequence
PY	Pep 1	ASGAGAGAGAGAGTGSSGFGPY[a]
	Pep 2	GAGAGAGAGTGSSGFGPY
	Pep 3	GAGAGTGSSGFGPY
GY	Pep 4	GSGAGAGSGAGAGSGAGAGSGAGAGY[a]
	Pep 5	GAGAGSGAGSGAGAGSGAGAGY
GA	Pep 6	SGAGAGSGAGAGSGAGAGSGAGAGSGA[a]
	Pep 7	SGAGAGSGAGAGSGAGAGSGA
	Pep 8	SGAGSGAGAGSGAGAGSGA

[a]Consensus sequences are represented with shadowed characters between or among sequences within each group.

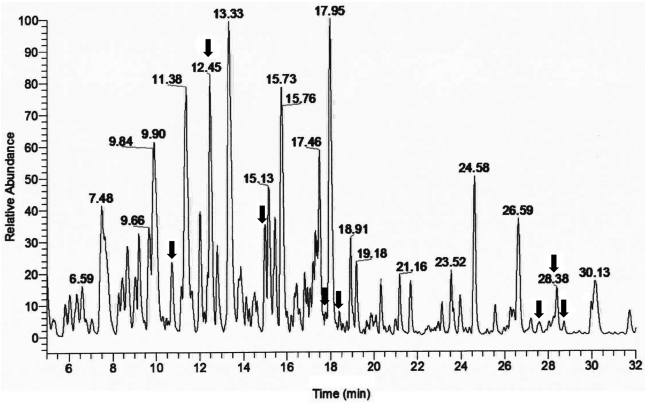

Fig. 3. Qualitative analysis of the SFH by LC-MS.
The mobile phase consisted of distilled water (solvent A) and acetonitrile (solvent B). Solvent B gradient was 1–25% for 50 min with a linear gradient. The injection volume was 3 µl, and the flow rate was 0.3 µl/min. Amino acids sequences of peaks (marked with arrows) were determined.

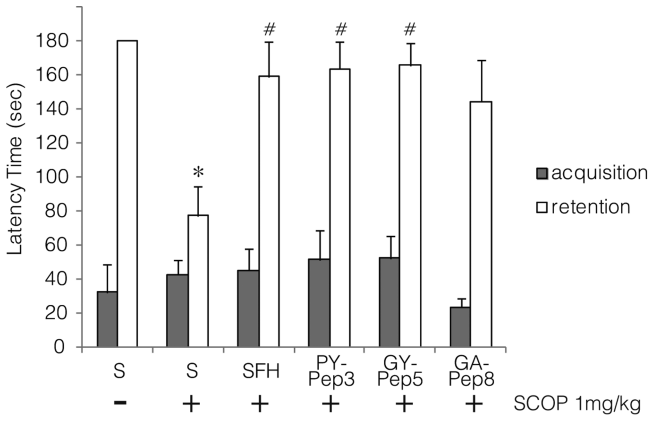

Fig. 4. Effect of peptides on scopolamine-induced memory deficits in the passive avoidance test.
Peptides or SFH (10 mg/kg, i.p.) were injected 60 min prior to the acquisition trial. Scopolamine (1 mg/kg, i.p.) was injected 30 min after the peptide injections. Data represent means ± SEM. (*$p < 0.05$ as compared with the control group, #$p < 0.05$ as compared with the scopolamine-treated group).

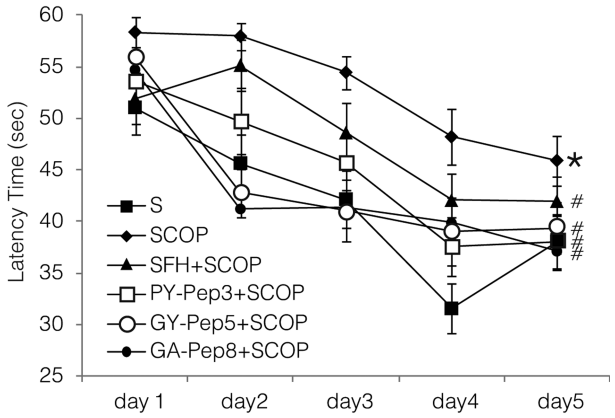

Fig. 5. Effect of peptides on scopolamine-induced memory deficits in the Morris water maze.

Peptides or SFH (10 mg/kg, i.p.) were injected 60 min prior to the trial. Scopolamine (1 mg/kg, i.p.) was injected 30 min after the peptide injections. Data represent means ± SEM. (*$p < 0.05$ as compared with the control group, #$p < 0.05$ as compared with the scopolamine-treated group).

Effect of Peptides on the Morris Water Maze Test

Saline-treated mice exhibited a normal decrease in escape latency throughout the training trials. However, the escape latency of the scopolamine-treated mice was significantly longer than that of the S group (Fig. 5, $p < 0.001$). SFH could reduce the escape latency time in scopolamine-treated mice (Fig. 5, $p < 0.001$ *vs.* scopolamine group) as has been shown in previous studies [10]. Additionally, all three peptides significantly reduced the escape latency time when compared with that of the scopolamine-treated group (Fig. 5, $p < 0.001$). Lastly, the escape latency of the peptide GA-treated group was significantly shorter than that of the SFH-treated group (Fig. 5, $p < 0.05$). There were no significant differences among the peptides in the PA, GY, and SFH groups. These data suggest that SFH, peptide PY, peptide GY, and peptide GA improve impairments of long-term memory, and peptide GA shows the best improvement.

Discussion

Because of its pharmaceutical properties, interest in silk fibroin has increased ever since it was developed as a functional food. Silk fibroin hydrolysate can be obtained by hydrolyzing silk fibers with an acid or enzymes. The hydrolysate contains hundreds of peptide fragments, and many investigations have been performed to determine which peptide fragments have pharmaceutical properties, and several peptides with pharmaceutical activities have been determined. For example, GVGY and GVGAGY have blood pressure reducing activities [6], GAGAGY stimulates glucose transport in adipocytes [11], and GAGAGAGS, GAGAGS, GAGAGA, and GAGA suppress IgE production [7]. However, no peptide has been identified that has memory-enhancing effects, although it was reported that SFH could enhance cognitive function.

In this study, mass spectrometric analysis revealed that the SFH was composed of various peptide mixtures (Fig. 2). We identified eight peptides (Table 1), and demonstrated three synthetic peptides (GAGAGTGSSGFGPY, GAGAGS GAGSGAGAGSGAGAGY, and SGAGSGAGAGSGAGA GSGA), which exhibited memory-enhancing effects on cognitively impaired mice.

These peptides have some common features: a tyrosine on the C-terminal, GA and/or GS repeat sequence(s), and a glycine-rich structure. Because silk fibroin protein has a highly repetitive structure, it is very likely that similar peptides are produced by hydrolysis. Some studies have assumed that the tyrosine on the C-terminus [6] and GAGA repeat [7] might be key structures for the pharmaceutical properties of silk fibroin. However, it is difficult to explain the variety of pharmaceutical actions if they have such similar amino acid structures.

Although peptide of only group GA showed significant difference compared with SFH in the Morris water maze, all peptide-treated mice had more reduced mean values of escape latency time than that of SFH-treated mice (Fig. 5). This suggests that compared with SFH, peptides were more effective to improve scopolamine-induced memory impairment. Although the same amount was injected into all mice (10 mg/kg), SFH contained various non-effective peptides, whereas the contents of the functional peptides were all active. Further studies are necessary to investigate the relationship between efficacy and dosage.

In conclusion, the results of this study indicate that the enzyme hydrolysate of silk fibroin can enhance the cognitive function of normal individuals. This hydrolysate is a mixture of functional peptides. Among these peptides, we first identified three memory-enhancing peptides (GAGAGT GSSGFGPY, GAGAGSGAGSGAGAGSGAGAGY, and SGA GSGAGAGSGAGAGSGA) from enzyme hydrolysate of silk fibroin. These three synthetic peptides alone can improve memory impairments in mice. The amino acid structure of these three peptides might aid in the ongoing investigation into the pharmaceutical capabilities of silk fibroin.

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