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Fractionation of Silk Fibroin Hydrolysate and Its Protective Function of Hydrogen Peroxide Toxicity

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ABSTRACT: Fractionated components of *Bombyx mori* silk fibroin, which were hydrolyzed with protease, were prepared by preparative recycling HPLC system in order to evaluate the protective effects of molecular weight-controlled *B. mori* silk fibroin components on H₂O₂-injured neuronal cell. Three major fractions having molecular weight less than about 1500 could be first collected using the above recycling techniques. The highest protective effect of molecular controlled *B. mori* silk fibroin components on H₂O₂-injured neuronal cell was obtained when the fraction having molecular weight around 1400 was used. It was suggested that this protective effect of silk fibroin hydrolysate on H₂O₂-injured neuronal cell correlate with content of aromatic amino acids such as tyrosine and phenylalanine.

Keywords: silk fibroin; fibers; peptides, HPLC, hydrogen peroxide toxicity

INTRODUCTION

Silk is well known fibrous protein produced by the silkworm, which has been used traditionally in the form of threads. It is composed of two kinds of protein: a fibrous one (named fibroin) and a gum-like one (named sericin) that surrounds the fibroin fibers to cement them together. One of the most favorable properties is the structural transition from solution to insoluble form, namely the crystallization as a protein. Thus, it is possible to make non-fabric materials from the silk proteins such as a film, gel, powder and solution. Application of silk proteins to biomaterials such as an enzyme-immobilization film for biosensors,¹⁻³ poly vinyl alcohol/ chitosan/ fibroin-blended spongy sheets for regenerative medical materials⁴ and cell-culture matrices⁵, has been widely investigated due to unique structural properties,⁶⁻⁹ and biocompatibility.¹⁰

On the other hands, the hydrolysate of silk fibroin as water-soluble peptides was also investigated to apply foods and dietary supplements.¹¹ However, the biological function of the hydrolyzed peptide is unclear. In the past decade, the antioxidant activity of natural products such as flavonoid species, which are well known as pharmacologically active constituents, has been given much attention because some flavonoid species may be useful to protect neurons from oxidative injury.¹² Previously, evidence for an antioxidant action of the silk sericin onto lipid peroxidation and inhibition of tyrosinase activity in vitro has been reported.¹³ In spite of the impressive usefulness of silk proteins as novel biomaterials, the effect of fractional components of *Bombyx mori* silk fibroin on hydrogen peroxide toxicity of neuron cell activity has been relatively unknown.

In this study, we evaluate fractionation of silk fibroin hydrolysate and the

protective function on hydrogen peroxide toxicity. Molecular weight-controlled hydrolysate of fibroin was prepared using a large-scale recycling HPLC system. The possible mechanism and structural properties were also discussed.

EXPERIMENTAL

Preparation of silk fibroin solution

Raw silk (*Bombyx mori*) cocoons reared on the farm affiliated with Rural Development Administration (RDA) of Korea were used as the raw materials. The raw materials were degummed twice with 0.5% on the weight of fiber (o.w.f) marseilles soap and 0.3% o.w.f. sodium carbonate solution at 100°C for 1h and then washed with distilled water. Degummed silk fibroin fibers (35 g) were dissolved in the mixed solution (700 ml) of CaCl₂, H₂O and ethanol (molar ratio 1 : 8 : 2) at 95°C for 5hs. This calcium chloride-silk fibroin mixed solution was filtered twice using miracloth (Calbiochem, USA) quick filter. For desalting of calcium chloride-silk fibroin mixed solution, the gel filtration column chromatography was performed on a GradiFrac system (Amersham Pharmacia Biotech, Sweden) equipped with a UV-1 detector operating at 210 nm. A commercially available prepacked Sephadex G-25 (800 x 40 mm I.D. column, Amersham Pharmacia Biotech, Sweden) was used. All 100% distilled pure water was used as elution solvent at a flow rate of 25 ml per min, 200 ml of sample injection volume and 30 ml of fraction volume.

Enzymatic hydrolysis and fractionation

Proteolytic enzyme, actinase from *Streptomyces griseus* (Kaken Chem. Co., Japan) was used for enzymatic degradation. The silk fibroin solution and 5% of actinase to the weight of fibroin was mixed under nitrogen gas, at 55°C for 12 hours. Then, the

solution was heated in a boiling water bath to stop the enzyme reaction and centrifuged at 5,000 rpm for 10 min. Then, recycling HPLC was performed to fractionate the enzyme hydrolyzed silk fibroin on a JAI-908-C60 HPLC (Japan Analytical Industry Co., Tokyo, Japan) equipped with a JAI RI and JAI UV detector operating at 220 nm. Both a commercially available prepacked PVA HP-GPC column (JAI-GEL GS-220, 100 cm x 5 cm I.D.) and ODS-BP column (JAI-GEL 100 cm x 5 cm I.D.) were employed. Water was used as the eluting solvent at a flow rate of 3 ml/min, 20 ml of sample injection volume.

NMR measurement

^{13}C NMR spectra of the silk fibroin and its enzyme hydrolyzed sample were observed. Silk fibroin solution was prepared by dialyzing fibroin solution in 9 M LiBr against distilled water and adding 10 % D_2O at room temperature. The pH value of sample solution was adjusted to about 7. ^{13}C NMR experiments operating at 100 MHz were carried out on a JEOL α 400 (400 MHz) spectrometer at 30 °C. Spectral conditions were the following: 20000 pulses, 90° pulse angle (8.70 μs), 2.00 s delay between pulse, 27100.27 Hz spectral width, 32768 data points. Chemical shifts were measured relative to external $(\text{CH}_3)_4\text{Si}$.¹⁴

Molecular weight measurement

Molecular weights of the fractionated components of silk fibroin were measured by gel permeation chromatography (GPC) with a TSK-gel G2000 SWXL column (300 x 7.8 mm). The mobile phase was distilled water. The chromatography was operated with a flow rate of 0.5 ml/min, column temperature at 37°C and detected with a refractive index (Viscotek, LR-125, USA) detector. Pullulan P-400, P-200, P-100, P-50, P-20, P-10, and P-5 (Shodex Standard P-82, Showa Denko, Japan) and

polyethylene glycol standard (American Polymer Standards Co., Mentor, USA) were used as standard markers.

Amino acid analysis

The amino acid species were determined by high performance liquid chromatography analysis with a Biochrom 20 Plus Amino Acid Analyzer (Amersham Pharmacia Biotech, Cambridge, UK)¹⁵. The peptide fractions were hydrolyzed in excess 6 N hydrochloric acid under standard conditions at 110°C for 22 hours. After hydrolysis, samples were dried in a vacuum evaporator at 50°C. For amino acid analysis, samples were diluted with 0.2 M at pH 2.2 loading buffer (Biochrom Ltd, UK). All amino acid compositions are based on daily calibrations to a standard solution (Sigma AA-S-18) containing 100 or 125 picomoles of each amino acid.

Cell culture and viability assay

PC12 cells were cultured in a common Roswell Park Memorial Institute (RPMI) medium for immune cell culture supplemented with 5% (v/v) foetal bovine serum (FBS) and 10% foetal calf serum (FCS) and were kept at 37°C in humidified 5% CO₂/95% air¹⁶. For differentiation, retinoic acid was added to a final concentration of 10 μM. Medium was changed every day and cultures were allowed to differentiate for 1 week. A number of 10⁵ cells were plated on a well of 96-well plates (Corning, NY, USA) in 100 μl of media containing the fibroin peptides and incubated for 24 hrs with and without 100 μM of H₂O₂. After the treatment, 10 μl of alamarBlue™ was aseptically added. The cells were incubated for 3 hrs and the absorbance of the cells was measured at a wavelength of 570 nm using an ELISA Reader (Molecular Devices, CA, USA). The background absorbance was measured at 600 nm and subtracted. The cell survival was defined¹⁸ as $[(\text{test sample count}) - (\text{blank count})] / [(\text{untreated control}$

count)-(blank count)] \times 100, where test sample, blank and untreated control mean protecting with the fibroin peptides, no protecting with the fibroin peptides, and untreated with H₂O₂, respectively. Results for cell viability are given as mean \pm S.E.M. Statistical analysis of the data was carried out using analysis of variance (ANOVA) followed by Student's t-test, using P<0.05 as the level of significance.

RESULTS AND DISCUSSION

¹³C NMR and fractionation of enzyme-treated silk fibroin

Silk fibroin fibers were dissolved with high concentrated calcium chloride aqueous solution with adequate additive agent (ethanol). After this preparation, we attempted to separate salts from the regenerated silk protein solution by size exclusion chromatography using a Sephadex G-25 as described in Experimental. Recovery of the protein during the desalting process was in the 85 to 90% range. Then, silk fibroin solution was treated with proteolytic enzyme to prepare peptide fragments. Figure 1 shows ¹³C NMR spectra of *B. mori* silk fibroin treated with and without enzymatic digestion. Expanded region between 40-44 ppm is shown. These ¹³C $^{\alpha}$ peaks are attributed to glycine residues. These peaks are convenient for monitoring digestion of the fibroin amino acid sequence because the sequential information of Gly-X-Gly in the silk fibroin heavy (390 kD) and light chain (26 kDa) is 89 % of residues¹⁷. ¹³C chemical shift of glycine residues which consists of silk fibroin (Fig.1.A) indicated one major peak at 42.6 ppm with a distribution less than 0.2 ppm. This result agrees with the previous report¹⁴. By the enzymatic digestion (Fig.1.B), the glycine-C $^{\alpha}$ peaks of silk fibroin peptides split into four major peaks at 43.1-43.2, 42.1-42.2, 41.3 and 40.3 ppm, respectively and there were no remaining peaks at 42.6 ppm (native silk fibroin).

To fractionate the peptide fragments, the recycling preparative HPLC system was applied. As shown in Fig. 2, the first cycle did not show separated peaks. However, with increasing the recycling number the peak separation increased at interval of 20 min. Three individual peaks could be observed more than 4 recycling steps. Complete baseline separation of the clear single peak was obtained in cycle 7-steps. The isolated components were 25 mg (fraction-1), 70 mg (fraction-2) and 90 mg (fraction-3), respectively. Recovery of the loaded peptides to the recycling preparative HPLC system was 88.1%. Advantages in the recycling technique in HPLC demonstrated here were the separation efficiently for a large scale, and the relatively short separation time.

Molecular weight distribution of fractional components

The molecular weight calibration curve of pullulan and polyethylene glycol standard vs. weight-averaged molecular weight (M_w) of fractional components of silk fibroin is shown in Fig. 3. The fractional components 1, 2 and 3 correspond to averaged M_w 430, 800 and 1400, respectively. Yamada *et al.* have been reported that chemical degradation of silk fibroin during degumming and dissolving processes.¹⁹ Native fibroin solution extracted from silk gland tissue has molecular masses of about 350 and 25 kDa, which correspond to the heavy and light chains of native fibroin molecules. However, by degumming and following dissolving treatment with CaCl_2 of fibroin fibers, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the regenerated solution showed a broad smeared band at lower molecular weight. In the present study, it is suggested that the native fibroin molecule was also degraded to a mixture of polypeptides of various sizes during the preparation of the fibroin solution because similar dissolving method with CaCl_2 was used. Interestingly, the following protease treatment and the fractionation using the recycling HPLC gave us three major

fractions as shown in Fig. 3. There has been no report about separation of the fibroin peptides using the recycling HPLC method. In our present work, three kinds of fibroin peptides having lower molecular weight less than 1500 were first obtained by the enzymatic degradation of the regenerated silk fibroin solution.

The influence of silk fibroin fractional component on cell viability

The protective effect of silk fibroin fractional component against H₂O₂ (100 μM) induced neuronal cell death was determined (Figure 4). The cell survival was defined as $[(\text{test sample count}) - (\text{blank count})] / [(\text{untreated control count}) - (\text{blank count})] \times 100$, where test sample, blank and untreated control correspond to protecting with the fibroin peptides, no protecting with the fibroin peptides, and untreated with H₂O₂, respectively. The silk fibroin fractional components were treated with two different concentrations, as 10 and 100 μg/ml. Compared with the case of the blank (no protective with the silk fibroin fractional components), the cell viability was significantly increased in a dose dependent manner.

With increasing concentrations of fractional component-1, neuronal survival ratio in the oxidative stress paradigm of cells increased from 15.1% to 21.6%. Similar increasing was observed with other two fractions. The highest survival ratio (32%) was obtained when 100 μg/ml of fraction-3 was used as shown in Figure 4. These results indicate that the fractional component of silk fibroin is associated with the protective role of superoxide anion (O₂⁻) against reactive oxygen species in the oxidative stress paradigm. A characteristic feature of the fibroin is the high proportion of the smaller side group amino acids, glycine, alanine and serine.²⁰ Amino acid composition of three fractional components, which were separated using the recycling HPLC was analyzed. The higher mol% of fraction-1 had the order of glycine > alanine > serine > tyrosine.

This order agrees with that of native silk fibroin. Contrary, fractions 2 and 3 exhibited statistically different rate of change, indicating larger amounts of aromatic amino acids, tyrosine and/or phenylalanine. Thus, it may be suggested that the fractional components of amino acid composition which has hydroxyl group or aromatic ring amino acids, such as serine, tyrosine and phenylalanine are concerned to protective role of superoxide anion. In this particular experimental model of neurotoxicity; the fractional component of silk fibroin may play a relevant role on the generation of reactive oxygen species. Therefore, these results suggest that the fractional component of silk fibroin, especially fraction 2 and 3, attenuated the levels of superoxide anion (O_2^-) production in H_2O_2 -induced cell viability signaling. These effects may represent an additional property of these peptides to antioxidative disease such as Alzheimer's or Parkinson's disease.²¹⁻²³ Recently, it has been reported that the recombinant silk sericin, which contains repeats of serine- and threonine-rich amino acid residues, protects against cell death caused by acute serum deprivation in insect cell culture.²⁴ This report support importance of hydroxyl group or aromatic ring amino acids on the protection of cell death. Amino acid sequential analysis of the fractionated hydrolysates is in progress in order to evaluate effect of the sequential specificity and aromatic amino acid residues of fibroin peptides on the cell viability.

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Figure Captions

Figure 1. Expanded ^{13}C NMR spectra of *B. mori* silk fibroin treated with (B) and without (A) enzymatic digestion. Only $^{13}\text{C}^\alpha$ peaks attributed to glycine residues are shown (see text).

Figure 2. Preparative recycle HPLC chromatography of enzyme-treated silk fibroin. Three peaks are obtainable through total 4-7 cycling steps; left, middle and right peaks are fractions- 1, 2, and 3, respectively.

Figure 3. Calibration plot of weight average molecular weight (Mw) with pullulan and polyethylene glycol standard (open circle) vs. retention time on fractional components. The marks of Mw, 430, 800 and 1400 correspond to fraction-1, 2 and 3, respectively.

Figure 4. Effect of fractional components of silk fibroin peptides on survival of H_2O_2 -injured neuronal cells. White and gray boxes correspond to 10 and 100 $\mu\text{g}/\text{ml}$ of the fibroin peptides added in the medium, respectively. Error bars are also shown.

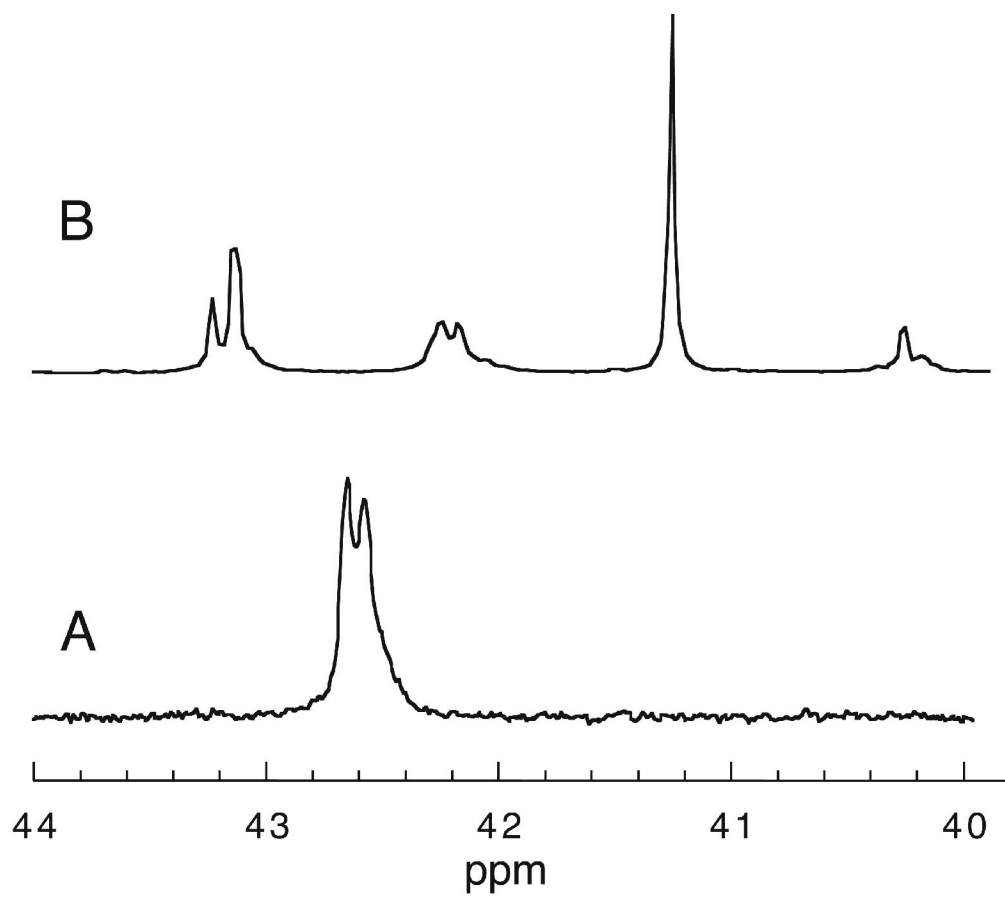


Figure 1 Yeo et al.

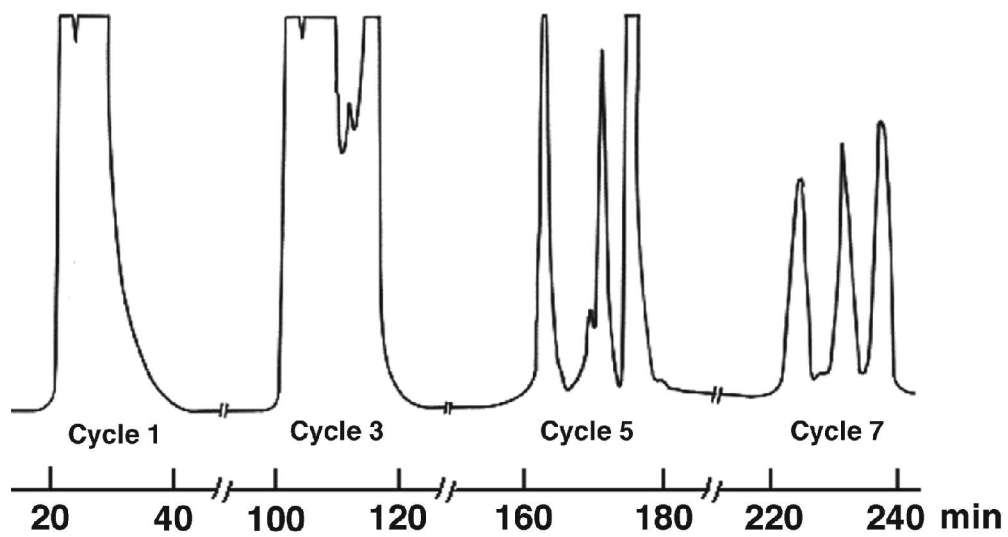


Figure 2 Yeo et al.

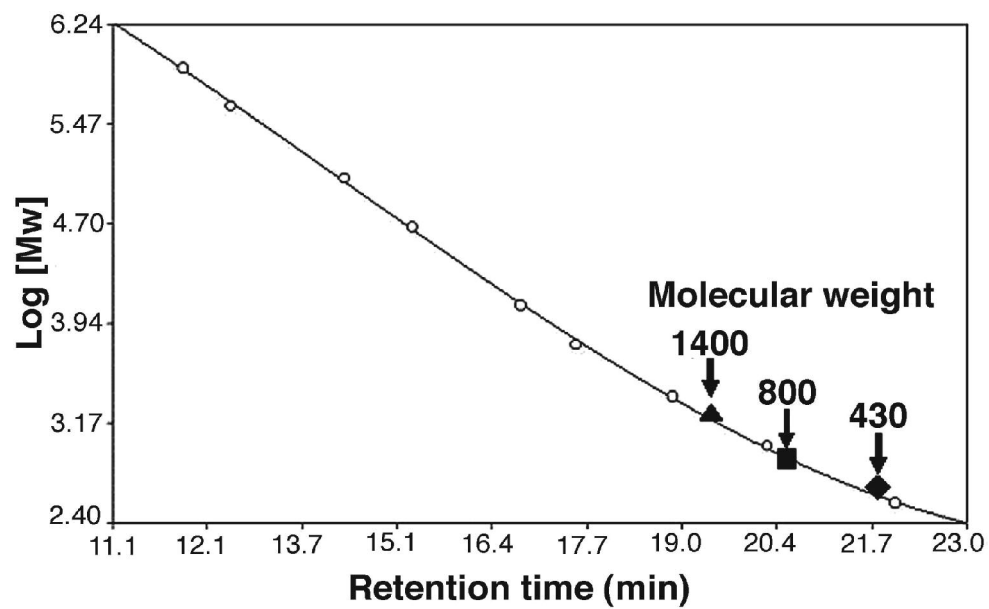


Figure 3 Yeo et al.

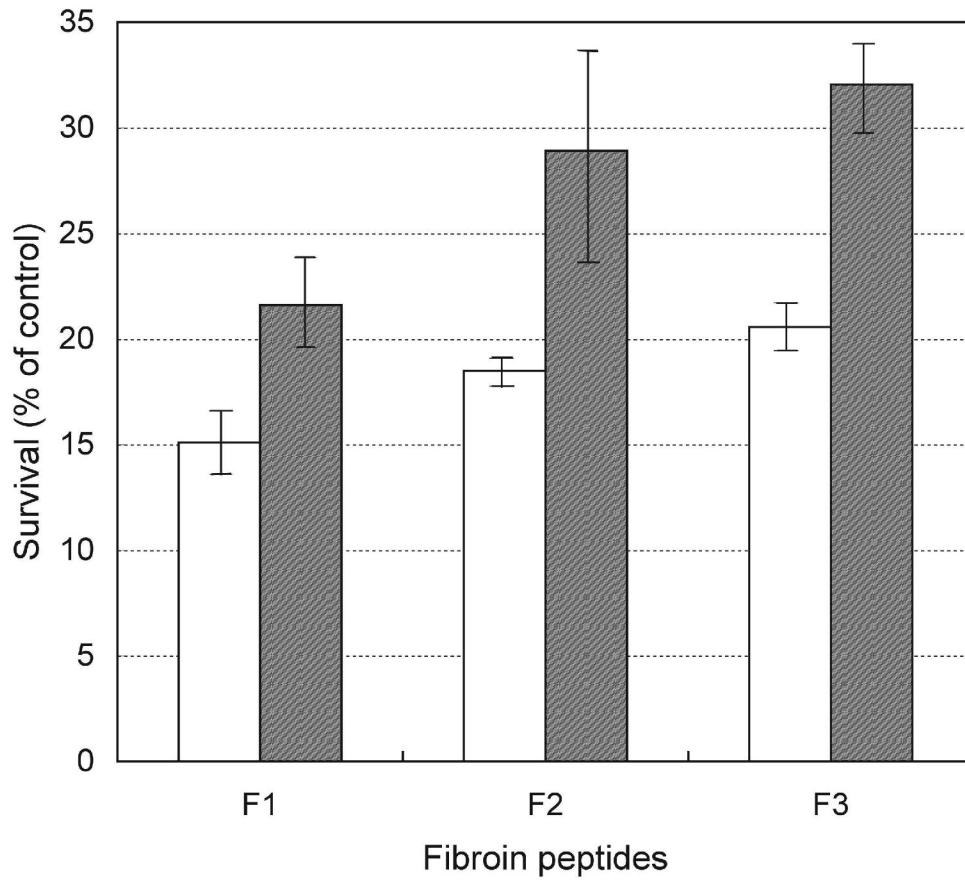


Figure 4 Yeo et al.