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

Joo-Hong Yeo,[1] Kwang-Gill Lee,[1] Hae-Yong Kweon,[1] Soon-Ok Woo,[1] Sang-Mi Han,[1] Sung-Su Kim,[2] Makoto Demura[3]

[1]*Department of Agricultural Biology, National Institute of Agricultural Science and Technology, Suwon 441-100, Korea*
[2]*Department of Anatomy and Cell Biology, College of Medicine, Chung-Ang University, Seoul 141-730, Korea*
[3]*Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan*

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ABSTRACT: Fractionated components of *Bombyx mori* silk fibroin, which were hydrolyzed with protease, were prepared with a preparative-recycling high-performance-liquid-chromatography system to evaluate the protective effects of molecular-weight-controlled *B. mori* silk fibroin components on H_2O_2-injured neuronal cells. Three major fractions, having molecular weights less than about 1500, were first collected with the recycling techniques. The highest protective effect of the molecular-weight-controlled *B. mori* silk fibroin components on H_2O_2-injured neuronal cells was obtained when a fraction with a molecular weight of approximately 1400 was used. This protective effect of the silk fibroin hydrolysate on H_2O_2-injured neuronal cells was suggested to correlate with the contents of aromatic amino acids such as tyrosine and phenylalanine. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 772–776, 2006

Key words: biocompatibility; fibers; high performance liquid chromatography (HPLC); peptides; proteins

INTRODUCTION

Silk is a well-known fibrous protein produced by the silkworm and has been used traditionally in the form of threads. It is composed of two kinds of proteins: a fibrous one (named fibroin) and a gumlike one (named sericin), which surrounds the fibroin fibers to cement them together. One of the most favorable properties is the structural transition from a solution form to an insoluble form, namely, crystallization as a protein. Thus, it is possible to make nonfabric materials from silk proteins such as films, gels, powders, and solutions. The application of silk proteins to biomaterials such as enzyme-immobilization films for biosensors[1–3] and poly(vinyl alcohol)/chitosan/fibroin blended spongy sheets for regenerative medical materials[4] and cell-culture matrices[5] has been widely investigated because of the unique structural properties,[6–9] and biocompatibility.[10]

On the other hand, hydrolysates of silk fibroin as water-soluble peptides have also been investigated for foods and dietary supplements.[11] However, the biological function of the hydrolyzed peptides 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 attracted much attention because some flavonoid species may be useful in protecting neurons from oxidative injury.[12] Previously, evidence for an antioxidant action of silk sericin for lipid peroxidation and inhibition of tyrosinase activity *in vitro* has been reported.[13] Despite the impressive usefulness of silk proteins as novel biomaterials, the effects of fractional components of *Bombyx mori* silk fibroin on the hydrogen peroxide toxicity of neuron cell activity are relatively unknown.

In this study, we evaluate the fractionation of a silk fibroin hydrolysate and the protective function on hydrogen peroxide toxicity. A molecular-weight-controlled hydrolysate of fibroin was prepared with a large-scale recycling high-performance-liquid-chromatography (HPLC) system. A possible mechanism and structural properties are discussed.

EXPERIMENTAL

Preparation of the silk fibroin solution

Raw silk (*B. mori*) cocoons reared on a farm affiliated with the Rural Development Administration of Korea were used as the raw materials. The raw materials were degummed twice with 0.5% on-the-weight-of-fiber (owf) Marseilles soap and a 0.3% owf sodium carbonate solution at 100°C for 1 h and then washed

Correspondence to: M. Demura (demura@sci.hokudai.ac.jp).
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with distilled water. Degummed silk fibroin fibers (35 g) were dissolved in a mixed solution (700 mL) of $CaCl_2$, H_2O, and ethanol (molar ratio = 1 : 8 : 2) at 95°C for 5 h. This calcium chloride/silk fibroin mixed solution was filtered twice with a Miracloth (Calbiochem, San Diego, CA) quick filter. For the desalting of the calcium chloride/silk fibroin mixed solution, gel filtration column chromatography was performed on a GradiFrac system (Amersham Pharmacia Biotech, Tokyo, Japan) equipped with a UV-1 detector operating at 210 nm. Commercially available and prepacked Sephadex G-25 (800 × 40 mm i.d. column, Amersham Pharmacia Biotech) was used. Pure (100%) distilled water was used as the elution solvent at a flow rate of 25 mL/min; the sample injection volume was 200 mL, and the fraction volume was 30 mL.

Enzymatic hydrolysis and fractionation

A proteolytic enzyme, actinase from *Streptomyces griseus* (Kaken Chem Co., Tokyo, Japan), was used for enzymatic degradation. The silk fibroin solution and 5% actinase with respect to the weight of the fibroin were mixed under nitrogen gas at 55°C for 12 h. Then, the solution was heated in a boiling water bath to stop the enzyme reaction and centrifuged at 5000 rpm for 10 min. Then, recycling HPLC was performed to fractionate the enzyme-hydrolyzed silk fibroin on a JAI-908-C60 HPLC apparatus (Japan Analytical Industry Co., Tokyo, Japan) equipped with JAI refractive-index and UV detectors operating at 220 nm. Both a commercially available and prepacked PVA HP-GPC column (JAI-GEL GS-220, 100 cm × 5 cm i.d.) and an ODS-BP column (JAI-GEL, 100 cm × 5 cm i.d.) were employed. Water was used as the eluting solvent at a flow rate of 3 mL/min; the sample injection volume was 20 mL.

NMR measurements

^{13}C-NMR spectra of the silk fibroin and its enzyme-hydrolyzed sample were observed. A silk fibroin solution was prepared by the dialysis of a fibroin solution in 9*M* LiBr against distilled water and by the addition of 10% D_2O at room temperature. The pH value of the sample solution was adjusted to about 7. ^{13}C-NMR experiments operating at 100 MHz were carried out on a JEOL α400 (400-MHz) spectrometer (Tokyo, Japan) at 30°C. The spectral conditions were as follows: 20,000 pulses, a 90° pulse angle (8.70 μs), a 2.00-s delay between pulses, a 27,100.27-Hz spectral width, and 32,768 data points. The chemical shifts were measured with respect to external $(CH_3)_4Si$.[14]

Molecular-weight measurements

The molecular weights of the fractionated components of the silk fibroin were measured by gel permeation chromatography with a TSKgel G2000 SWXL (Tosoh, Tokyo, Japan) column (300 × 7.8 mm). The mobile phase was distilled water. The chromatography was operated at a flow rate of 0.5 mL/min and a column temperature of 37°C and was detected with a refractive index detector (LR-125, Viscotek, Houston, TX). Pullulan P-400, P-200, P-100, P-50, P-20, P-10, and P-5 (Shodex Standard P-82, Showa Denko, Tokyo, Japan) and a poly(ethylene glycol) standard (American Polymer Standards Co., Mentor, OH) were used as standard markers.

Amino acid analysis

The amino acid species were determined by HPLC 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 h. After the hydrolysis, the samples were dried in a vacuum evaporator at 50°C. For amino acid analysis, the samples were diluted with 0.2*M* at a pH 2.2 loading buffer (Biochrom, Ltd., UK). All amino acid compositions were based on daily calibrations to a standard solution (AA-S-18, Sigma, Tokyo, Japan) containing 100 or 125 pmol of each amino acid.

Cell culture and viability assay

PC12 cells were cultured in a common Roswell Park Memorial Institute medium for an immune cell culture supplemented with 5% (v/v) fetal bovine serum and 10% fetal calf serum and were kept at 37°C in humidified 5% CO_2/95% air.[16] For differentiation, retinoic acid was added to a final concentration of 10 μM. The medium was changed every day, and the cultures were allowed to differentiate for 1 week. A number of cells (10^5) were plated on each well of 96-well plates (Corning, Acton, MA) in 100 μL of a medium containing the fibroin peptides and were incubated for 24 h with and without 100 μM of H_2O_2. After the treatment, 10 μL of Alamar Blue was aseptically added. The cells were incubated for 3 h, and the absorbance of the cells was measured at a wavelength of 570 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA). The background absorbance was measured at 600 nm and subtracted. The cell survival was defined[17] as follows: [(test sample count) − (blank count)]/[(untreated control count) − (blank count)] × 100, where *test sample*, *blank*, and *untreated control* mean protected with the fibroin peptides, not protected with the fibroin peptides, and untreated with H_2O_2, respectively. The results for cell viability are given as the mean plus or minus the standard error of the mean. A statistical analysis of the data was carried out with an analysis of variance followed by a Student *t* test, with $p < 0.05$ as the level of significance.

RESULTS AND DISCUSSION

^{13}C-NMR and fractionation of the enzyme-treated silk fibroin

Silk fibroin fibers were dissolved in a high-concentration calcium chloride aqueous solution with an adequate additive agent (ethanol). After this preparation, we attempted to separate salts from the regenerated silk protein solution by size exclusion chromatography with a Sephadex G-25, as described in the Experimental section. The recovery of the protein during the desalting process was in the 85–90% range. Then, the silk fibroin solution was treated with a proteolytic enzyme to prepare the peptide fragments. Figure 1 shows ^{13}C-NMR spectra of *B. mori* silk fibroin treated with and without enzymatic digestion. An expanded region between 40 and 44 ppm is shown. These $^{13}C^{\alpha}$ peaks are attributed to glycine residues. These peaks are convenient for monitoring the digestion of the fibroin amino acid sequence because the sequential information of Gly-X-Gly in the silk fibroin heavy (390 kD) and light chains (26 kDa) is 89% of the residues.[18] The ^{13}C chemical shift of glycine residues, which consist of silk fibroin [Fig. 1(A)], indicated one major peak at 42.6 ppm, with a distribution of less than 0.2 ppm. This result agrees with a previous report.[14] By enzymatic digestion [Fig. 1(B)], the glycine C^{α} 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 preparative-recycling HPLC system was applied. As shown in Figure 2, the first cycle did not show separated peaks. However, as the recycling number increased, the peak separation increased at an interval of 20 min. Three individual peaks could be observed for more than four recycling steps. Complete baseline separation of the clear, single peak was obtained in a cycle of seven steps. The isolated components were 25 (fraction 1), 70 (fraction 2), and 90 mg (fraction 3), respectively. The recovery of the loaded peptides for the preparative-recycling HPLC system was 88.1%. The advantages of the recycling technique in HPLC demonstrated here were the separation efficiently for a large scale and the relatively short separation time.

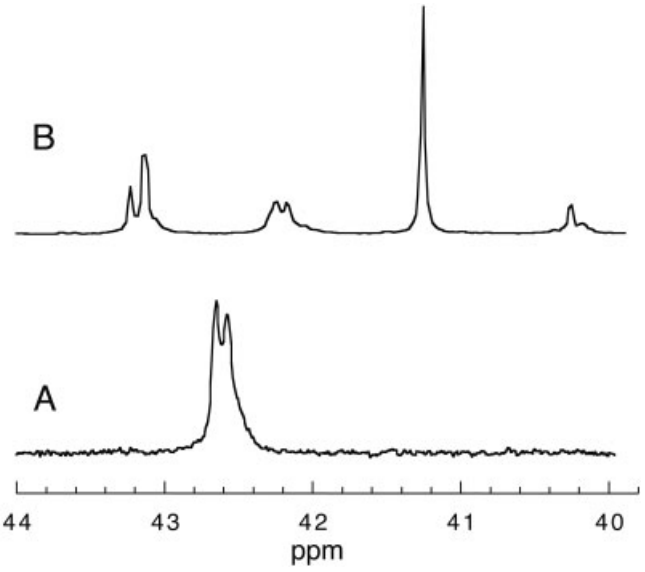

Figure 1 Expanded ^{13}C-NMR spectra of *B. mori* silk fibroin treated (A) without and (B) with enzymatic digestion. Only $^{13}C^{\alpha}$ peaks attributed to glycine residues are shown (see the text).

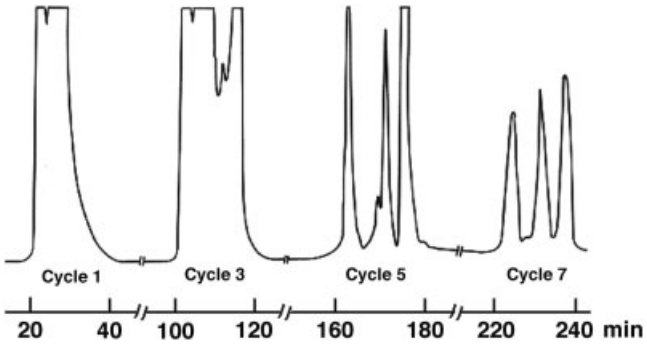

Figure 2 Preparative-recycling HPLC chromatography of the enzyme-treated silk fibroin. Three peaks are obtainable through a total of four to seven cycling steps. The left, middle, and right peaks are fractions 1, 2, and 3, respectively.

Molecular weight distribution of the fractional components

The molecular-weight calibration curve of pullulan and poly(ethylene glycol) standards versus the weight-average molecular weight (M_w) of fractional components of the silk fibroin is shown in Figure 3. Fractional components 1, 2, and 3 correspond to M_w values of 430, 800, and 1400, respectively. Yamada et al.[19] reported the chemical degradation of silk fibroin during degumming and dissolving processes. A native fibroin solution extracted from silk gland tissue had molecular masses of about 350 and 25 kDa, which corresponded to the heavy and light chains of native fibroin molecules. However, through the degumming and dissolution treatment with $CaCl_2$ of the fibroin fibers, sodium dodecyl sulfate/polyacrylamide gel electrophoresis analysis of the regenerated solution showed a broad, smeared band at a lower molecular weight. This study 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 a similar dissolving method with $CaCl_2$ was used. Interestingly, the following protease treatment and fractionation with recycling HPLC

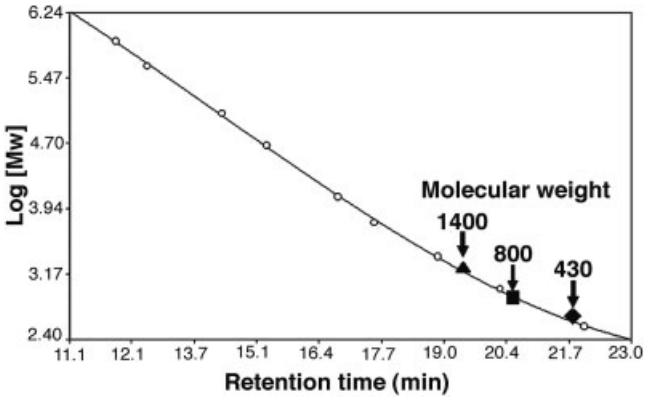

Figure 3 Calibration plot of M_w with pullulan and poly(ethylene glycol) standards (marked by open circle) versus the retention time on fractional components. The marks for 430, 800, and 1400 correspond to fractions 1, 2, and 3, respectively.

gave us three major fractions, as shown in Figure 3. There has been no report about the separation of fibroin peptides with the recycling HPLC method. In this work, three kinds of fibroin peptides with molecular weights less than 1500 were first obtained by the enzymatic degradation of the regenerated silk fibroin solution.

Influence of the silk fibroin fractional component on cell viability

The protective effect of the silk fibroin fractional component against H_2O_2 (100 μM)-induced neuronal cell death was determined (Fig. 4). The cell survival was defined as follows: [(test sample count) − (blank count)]/[(untreated control count) − (blank count)] × 100, where *test sample*, *blank*, and *untreated control* correspond to protected with the fibroin peptides, not protected with the fibroin peptides, and untreated with H_2O_2, respectively. The silk fibroin fractional components were treated with two different concentrations (10 and 100 μg/mL). Compared with the case of the blank (not protected with the silk fibroin fractional components), the cell viability was significantly increased in a dose-dependent manner.

With an increasing concentrations of fractional component 1, the neuronal survival ratio in the oxidative stress paradigm of cells increased from 15.1 to 21.6%. A similar increase was observed with the other two fractions. The highest survival ratio (32%) was obtained when 100 μg/mL fraction 3 was used, as shown in Figure 4. These results indicate that the fractional component of the silk fibroin is associated with the protective role of the superoxide anion (O_2^-) 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] The amino acid composition of the three fractional components, which were separated with recycling HPLC, was analyzed. The higher molar percentage of fraction 1 had the order of glycine > alanine > serine > tyrosine. This order agreed with that of native silk fibroin. In contrast, fractions 2 and 3 exhibited statistically different rates of change, indicating larger amounts of aromatic amino acids (tyrosine and/or phenylalanine). Thus, it can be suggested that the fractional components of amino acid compositions with hydroxyl groups or aromatic ring amino acids, such as serine, tyrosine, and phenylalanine, are involved in the protective role of O_2^-. In this particular experimental model of neurotoxicity; the fractional component of the silk fibroin may play a relevant role in the generation of reactive oxygen species. Therefore, these results suggest that the fractional components of the silk fibroin, especially fractions 2 and 3, attenuated the levels of O_2^- production in the H_2O_2-induced cell viability signaling. These effects may represent an additional property of these peptides against antioxidative diseases such as Alzheimer's and Parkinson's.[21–23] Recently, it has been reported that 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 cultures.[24] This report supports the importance of hydroxyl groups or aromatic ring amino acids in protection against cell death. An amino acid sequential analysis of the fractionated hydrolysates is in progress to evaluate the effects of

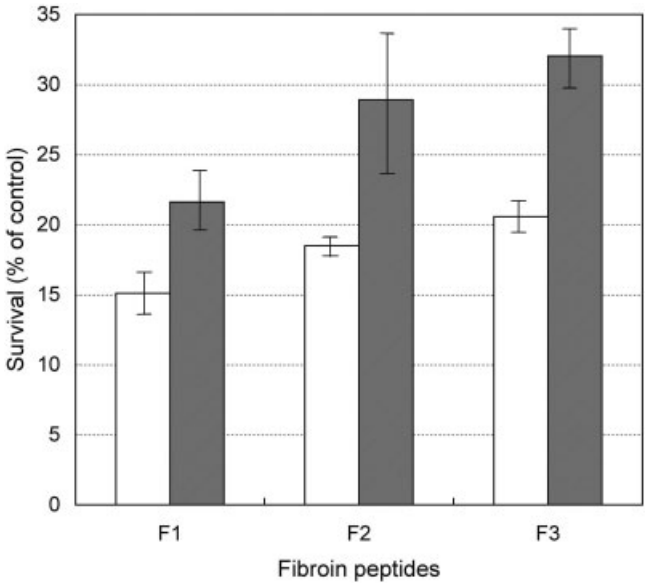

Figure 4 Effect of the fractional components of silk fibroin peptides on the survival of H_2O_2-injured neuronal cells. The white and gray boxes correspond to 10 and 100 μg/mL concentrations of the fibroin peptides added to the medium, respectively. Error bars are also shown.

the sequential specificity and aromatic amino acid residues of fibroin peptides on cell viability.

References

1. Demura, M.; Asakura, T.; Kuroo, T. Biosensors 1989, 4, 361.
2. Demura, M.; Asakura, T. Biotechnol Bioeng 1989, 33, 598.
3. Demura, M.; Asakura, T. J Membr Sci 1991, 59, 39.
4. Yeo, J. H.; Lee, K. G.; Kim, H. C.; Oh, Y. L.; Kim, A. J.; Kim, S. Y. Biol Pharm Bull 2000, 23, 1220.
5. Minoura, N.; Aiba, S.; Gotho, Y.; Tsukada, M.; Imai, Y. J Biomed Mater Res 1995, 29, 1215.
6. Ishida, M.; Asakura, T.; Yokoi, M.; Saito, H. Macromolecules 1990, 23, 88.
7. Asakura, T.; Yoshimizu, H.; Yoshizawa, F. Macromolecules 1998, 21, 2038.
8. Demura, M.; Kitamura, A.; Shibamoto, A.; Asakura, T. J Appl Polym Sci 1988, 36, 535.
9. Asakura, T.; Kitaguchi, M.; Demura, M.; Sakai, H.; Komatsu, K. J Appl Polym Sci 1992, 46, 49.
10. Yeo, J. H.; Lee, K. G.; Kim, S. Y. Eur Polym J 2003, 39, 1159.
11. Chen, K.; Umeda, Y.; Hirabayashi, K. J Seric Sci Jpn 1996, 65, 131.
12. Gao, Z.; Huang, K.; Xu, H. Pharmacol Res 2001, 43, 173.
13. Kato, N.; Sato, S.; Yamanaka, A.; Yamada, H.; Fuwa, N.; Nomura, M. Biosci Biotechnol Biochem 1998, 62, 145.
14. Asakura, T.; Murakami, T. Macromolecules 1985, 18, 2614.
15. Franchi-Gazzola, R.; Visigalli, R.; Bussolati, O.; Dall'Asta, V.; Gazzola, G. C. J Biol Chem 1999, 274, 28922.
16. Righi, M.; Tongiorgi, E.; Cattaneo, A. J Neurosci 2000, 20, 3165.
17. Shimoke, K.; Chiba, H. J Neurosci Res 2001, 63, 402.
18. Zhou, C. Z.; Confalonieri, F.; Medina, N.; Zivanovic, Y.; Esnault, C.; Yang, T.; Jacquet, M.; Janin, J.; Duguet, M.; Perasso, R.; Li, Z. G. Nucleic Acids Res 2000, 28, 2413.
19. Yamada, H.; Nakao, H.; Takasu, Y.; Tsubouchi, K. Mater Sci Eng C, 2001, 14, 41.
20. Asakura, T.; Sakaguchi, R.; Demura, M.; Manabe, T.; Uyama, A.; Ogawa, K.; Osanai, M. Biotechnol Bioeng 1993, 41, 245.
21. Fahn, S.; Cohen, G. Ann Neurol 1992, 32, 804.
22. Mecocci, P.; MacGarvey, U.; Beal, M. F. Ann Neurol 1994, 36, 747.
23. Markesbery, W. R. Biol Med 1997, 23, 134.
24. Takahashi, M.; Tsujimoto, K.; Yamada, H.; Takagi, H.; Nakamori, S. Biotechnol Lett 2003, 25, 1805.