Hindawi
Journal of Chemistry
Volume 2018, Article ID 1285823, 9 pages
https://doi.org/10.1155/2018/1285823

Research Article

Application of Food-Grade Proteolytic Enzyme for the Hydrolysis of Regenerated Silk Fibroin from *Bombyx mori*

Ji An Joung, Mi Na Park, Ji Young You, Bong Joon Song, and Joon Ho Choi

Department of Food Science and Biotechnology, Wonkwang University, Iksan 54538, Republic of Korea

Correspondence should be addressed to Joon Ho Choi; jhchoi1124@wku.ac.kr

Received 24 August 2018; Accepted 8 October 2018; Published 8 November 2018

Guest Editor: Sang-Hyun Park

In vitro biodegradation of *Bombyx mori* silk fibroin (SF) was studied using food-grade proteolytic enzymes to replace acid hydrolysis. Based on the residual protein quantity and yield of amino acids (AAs) after enzymatic hydrolysis, we evaluated the proteolytic enzyme process of SF. FoodPro and Alcalase that are classified as alkaline proteases are selected as two of the best candidate enzymes for hydrolysis of SF. The activity of these enzymes exhibits a broad range of pH (6.5 to 9.0) and temperature (50°C to 65°C). The single enzyme treatment of SF using FoodPro exhibited a hydrolytic efficiency of 20–25%, and >2 g/L AAs were released after reaction for 3 h. A 2-stage enzymatic treatment using a combination of FoodPro and Flavourzyme in a sequence for a reaction time of 6 h was developed to enhance the efficiency of the proteolytic process. The yield of AAs and residual protein quantity in the enzymatic hydrolysates obtained from FoodPro-treated regenerated SF in the 1st step was 2,040 ± 23.7 mg/L and 70.6%, respectively. The yield of AAs was > two-fold (4,519 ± 42.1 mg/L), whereas the residual protein quantity decreased to 55.1% after the Flavourzyme treatment (2nd step) compared to those of the single FoodPro treatment. In the mixed treatment by simultaneously using FoodPro and Flavourzyme, approximately 45% of SF was degraded and 4.5 g/L of AAs were released within 3 h of reaction time. The regenerated SF and its enzymatic hydrolysates were characterized by performing UV-visible spectra, gel electrophoresis, and size-exclusion chromatography analyses. In the 2-stage treatment using FoodPro initially and subsequently Flavourzyme, the aggregates and high molecular weight proteins of SF were dissociated and degraded into the low molecular weight proteins/peptides (10–15 kDa and 27 kDa). SF hydrolysates as functional food might be enzymatically produced using the commercial food-grade proteolytic enzymes.

1. Introduction

The silk fibroin (SF) from *Bombyx mori* (silkworm) cocoons present as a double-stranded fibroin fiber that is coated with adhesive sericin proteins. The raw silk fiber is composed of 20–30% sericin and 70–80% SF with trace amounts of waxes and carbohydrates [1]. Pure SF is separated from sericin by the degumming procedure [2], and a variety of aqueous or organic solvent processing methods are used to generate silk biomaterials for a wide range of applications [3–6]. The predominant protein of silk, i.e., fibroin, is a hydrophobic structural protein that is insoluble in hot water and consists of heavy and light chains with 390 kDa and 25 kDa molecular weights (MW), respectively, linked by a disulfide bond [7, 8]. The SF heavy chain is rich in hydrophobic β-sheets that form blocks linked by small hydrophilic linkers or spacers. The predominant regions of SF primarily consist of approximately 76% of amino acids (AAs) with nonpolar side chains. The main AAs of SF are glycine (43.7%) and alanine (28.8%) that form the dominant crystalline β-sheet regions that act as reinforcements and contribute to the strength and stiffness of silk [9, 10]. Although silk is defined as a nondegradable material by the United States Pharmacopeia owing to a negligible loss in tensile strength *in vivo,* the enzymatic degradation behaviors of SF as biomaterials have been reviewed for the medical application *in vivo* [11, 12].

The *in vitro* biodegradation of *B. mori* SF was studied using proteolytic enzymes (collagenase F, α-chymotrypsin I-S, and protease type XXI) to degrade SF fibers and films [13]. The enzymatic hydrolysis of SF was performed using

α-chymotrypsin, collagenase IA, and protease XIV in SF yarns [14], protease XIV in porous SF sheets [15–17], collagenase IA and protease XXIII in 3D-scaffolds SF [18–20], proteinase K, protease XIV, α-chymotrypsin, collagenase, and matrix metalloproteinase in SF hydrogels and films [21], and actinase in SF solution [22]. Due to the unique chemical, mechanical, and biocompatibility properties, *B. mori* SF was investigated as a biomaterial and functional food source for years. Fibroin-derived bioactive proteins/peptides and hydrolysates were developed as the functional components of foods, cosmetics, pharmaceutical preparations, and alternative sources of food additives to improve human health [23]. The applications of SF in processed foods were proposed, and currently, the three forms of SF (solution, gel, and powder) are commercially available [24]. The SF hydrolysates that are prepared by performing acid hydrolysis or enzymatic hydrolysis consist of a mixture of AAs and oligopeptides that are known to exhibit beneficial effects on animal models. The bioactive peptides derived from SF are reported to reduce blood cholesterol level, increase antigenotoxicity, inhibit angiotensin-converting enzyme activity, enhance insulin sensitivity and glucose metabolism, improve alcohol metabolism, and stimulate osteoblastic differentiation [23, 25–28].

Fibroinase is a native silk digestion enzyme characterized as a cysteine proteinase and digests fibroin and sericin [29, 30]. Alcalase [25–27], actinase [22], and alkaline protease [31] were used to prepare the bioactive peptides and enzymatic hydrolysates (EHs) from SF/silkworm powder. The fibroin solubilization conditions affected the MW distribution of EHs produced using Alcalase supplied from Novozymes [32]. Additionally, actinase was introduced to hydrolyze regenerated silk fibroin (RSF) at 37°C for 12 h [22]. The silkworm powder was modified by enzymatic treatment using alkaline protease (FoodPro), and the MW of silk protein was decreased [31]. To prepare SF hydrolysates, enzymatic hydrolysis is more efficient than acid hydrolysis with aspect to the recovery yield and quality of products. However, very few previous studies reported the production of SF hydrolysates using commercial food-grade enzymes. Therefore, we performed the enzymatic hydrolysis of SF using commercial food-grade enzymes and characterized these EHs.

2. Materials and Methods

2.1. Materials. Degummed SF fiber acquired from a Korean sericulture farm (Kimjae, South Korea) was provided by SSBIO PHARM Co., Ltd. (Cheonan, South Korea). Commercial food-grade proteolytic enzymes, FoodPro® and Alphalase® (DuPont Industrial Biosciences, Brabrand, Denmark), Collupulin™ MG (DSM Food Specialties, Alexander Fleminglaan, Netherlands), Alcalase®, Neutrase®, Protamex®, and Flavourzyme® (Novozymes A/S, Bagsvaerd, Denmark), Promod™ 192P, 279MDP, and 278MDP (Biocatalysts Ltd., Wales, UK), and Bromelain (PT Bromelain Enzyme, Lampung, Indonesia) were purchased and stored at 4°C until their use in experimental reactions. Anhydrous citrate and Na_2HPO_4 to prepare citrate-phosphate buffer (CPB) [33], $CaCl_2$ and 95% ethanol to prepare SF solubilization reagent, and other chemicals were of reagent-grade and purchased from Samchun Pure Chemical Co., Ltd. (Gyeonggi-do, South Korea). Standard protein, i.e., albumin from bovine serum (BSA) and Folin and Ciocalteu's phenol reagent to perform quantification of protein concentration and ninhydrin reagent and glycine to analyze the AAs were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Precision Plus Protein™ standards and Silver Stain Plus™ kit to perform SDS-PAGE and silver staining, respectively, were purchased from BIO-RAD Laboratories (Hercules, CA, USA).

2.2. Preparation of RSF Solution. Degummed SF fiber was dissolved in Ajisawa's reagent [34] composed of $CaCl_2$: ethanol : distilled water in 1 : 2 : 8 molar ratio at 121°C for 15 min, thus yielding a 20% (W/V) solution. This solution was dialyzed against deionized water using a cellulose membrane dialysis tube (MW cut-off 14,000 kDa, Sigma-Aldrich) for >3 days to remove excess salt and ethanol. The RSF solution was centrifuged to remove insoluble materials, and final concentration was determined as 130–180 g/L using the Lowry method [35] with 73–76% recovery yield. RSF solutions at various pH levels were prepared using CPB. The pH-conditioned RSF solutions were stored at room temperature for 48 h to achieve homogeneity. The premature precipitates in RSF solutions were removed by centrifugation at 13,000 rpm for 10 min (Smart-R17, Hanil Scientific Inc., Gimpo, South Korea).

2.3. Estimation of Specific Activities of Proteolytic Enzymes. The proteolytic activity of enzymes was determined using casein as a substrate by performing Sigma's nonspecific protease activity assay [36] with a minor modification. Casein solution (5 mL; 0.65% (W/V)) was mixed with the enzyme solutions (1 mL) of various concentrations. After 10 min incubation at 37°C, 0.4 M trichloroacetic acid (5 mL) was added to terminate the reaction. The reaction mixture was incubated at 37°C for 30 min and centrifuged to remove insoluble precipitates. The supernatant (1 mL) was mixed with 0.55 M sodium carbonate (5 mL) and subsequently 0.5 mM Folin and Ciocalteu's phenol reagent (1 mL). After 30 min incubation at 37°C, the absorbance at 660 nm was measured using a spectrophotometer (Optizen POP, Mecasys Co., Daejeon, South Korea). Based on the standard curve obtained using L-tyrosine, the proteolytic activity was determined in terms of units (U), that is, the quantity of tyrosine equivalents in micromoles released from casein per min. The specific pH of each enzymatic reaction was regulated using 0.25 M CPB at corresponding pH. The protein concentrations of proteolytic enzyme solutions were determined using the Lowry method. The proteolytic activity of enzymes was expressed as specific activity in terms of U/mg-protein.

2.4. Proteolytic Hydrolysis of RSF. Enzymatic reaction was performed using RSF solution along with food-grade

enzymes at specific conditions. Each sample acquired from the reaction mixture was boiled at 100°C for 10 min to inactivate the enzyme and centrifuged at 13,000 rpm for 10 min to remove the insoluble precipitate. The supernatant of each sample was used to measure the protein and AAs concentrations by performing the Lowry method and ninhydrin reaction, respectively [37]. The protein and AAs concentrations were expressed in terms of BSA and glycine equivalent, respectively. The protein concentration of SF solution used for enzymatic reaction was approximately 30 g/L. The efficiency of protein degradation by enzymatic hydrolysis was expressed as the percentage of remaining protein using the following equation: residual protein (%) = $(P_f/P_i) \times 100$ where P_i is the initial concentration of the sample and P_f is the protein concentration of reaction mixture at a specific condition. To screen the proteolytic enzymes to perform SF hydrolysis, each enzyme (6 U) was incubated with 330 mg of RSF for 3 h. The effects of temperature and pH on the reactions involving the candidate enzymes (FoodPro and Alcalase) were determined by performing a combination reaction using enzyme (5 U) and SF (400 mg) for 5 h. The effect of substrate-to-enzyme ratio was determined using the initially selected enzyme, i.e., FoodPro, and an enzyme concentration of 5 U was used during the subsequent enzymatic treatment experiments. To enhance the efficiency of enzymatic hydrolysis by performing the 2-stage treatment, SF was initially allowed to react with FoodPro and then with any other selected proteolytic enzyme.

2.5. Gel Electrophoresis. The MW of SF and its EHs were determined by performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% Mini-PROTEAN® TGX™ precast protein gel (BIO-RAD). The total protein samples of RSF, initial EHs obtained using FoodPro, and final EHs obtained using Flavourzyme were loaded at protein concentration values of 28–84, 19–57, and 15–45 μg/well, respectively. The samples were resolved in SDS-PAGE with Precision Plus Protein standards as MW markers and stained using a Silver Stain Plus kit (BIO-RAD).

2.6. Size-Exclusion Chromatography (SEC). The high-performance liquid chromatography system combined with a photodiode array detector (Atlus™, PerkinElmer, Waltham, MA, USA) and 5Diol-300-II size-exclusion column (COSMOSIL, 7.5 mm ID × 300 mm, 300 Å pore size, Nacalai Tesque, Inc., Kyoto, Japan) was used to perform SEC. The protein quantities used during analyses were 700 μg RSF, 250 μg EHs obtained after 1st step using FoodPro, and 200 μg EHs obtained after 2nd step using Flavourzyme. 0.25 M CPB (pH 6.0) was used as the mobile phase at a flow rate of 1 mL/min, and the eluted protein concentration was measured at 280 nm at 30°C.

2.7. Statistical Analysis. All the experiments were performed in triplicates, and results were represented as mean value ± standard deviation. Analysis of variance was conducted, and the mean variations were analyzed using Duncan's multiple range test ($p < 0.05$). The statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) for Windows 12.0 software (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. pH Dependency of RSF Solubility in Aqueous Solution. pH affects the charges of carboxyl as well as amino groups in RSF, enables electrostatic interactions, and promotes intramolecular or intermolecular interactions such as hydrophobic interactions and hydrogen bonding. The effect of pH on the RSF solubility in aqueous solution was determined using 0.25 M CPB solutions with pH range 3.0–9.0. The protein concentration of each sample equilibrated at a specific pH for 48 h was determined before and after centrifugation (Figure 1). The protein concentration in RSF solution prior to centrifugation was relatively stable (26.8 ± 0.81 g/L) at pH between 5.0 and 9.0. After centrifugation, the stable protein concentration (26.7 ± 0.14 g/L) was obtained at a narrow pH range 5.0–6.5. Some of the soluble RSF was coagulated in alkaline condition; therefore, the soluble protein concentration after centrifugation decreased at pH > 7. In pH < 5 condition, the soluble protein concentration after centrifugation decreased owing to colloidal precipitation. The stable protein concentration was maintained at pH between 5.0 and 6.5 that might be associated with the spinning process (decrease from 6.9 to 4.8 along the path) in the silk glands [38]. A similar effect of pH on RSF solubility was reported in a study that indicated pH dependency between the shear sensitivity of SF and β-sheet crystallization [39].

3.2. Screening of Candidate Proteolytic Enzyme to Hydrolyze RSF. The specific activities of food-grade proteolytic enzymes and results of enzymatic hydrolysis at particular pH and temperature conditions are summarized in Table 1. The reaction conditions such as pH and temperature of each enzyme were selected based on the manufacturer's recommendations. FoodPro and Alcalase are classified as endotype and alkaline proteases that exhibit higher specific activity values than other proteolytic enzymes. An equal unit of each enzyme was used to screen the candidate proteolytic enzyme to perform RSF hydrolysis. FoodPro, Alcalase, Protamex, and/or Alphalase were potential candidates to efficiently hydrolyze SF owing to the concentration of AA in the EHs which was >2,000 mg/L. Flavourzyme is classified as mixed endotype/exotype and was not selected as the SF started to precipitate after 1 h reaction time. With an increase in the AA concentrations in EHs, the residual protein (%) decreased to approximately 80% in Alcalase-, FoodPro-, Protamex-, and Alphalase-treated SF. The AA concentrations in EHs represent not only free AAs but also peptides because the ninhydrin reaction is used to determine free amino and amine groups in AAs, peptides, and proteins [40]. Alcalase and FoodPro were selected as candidate proteases to hydrolyze SF. These two enzymes are produced by *Bacillus* strains, known as serine proteases, and classified

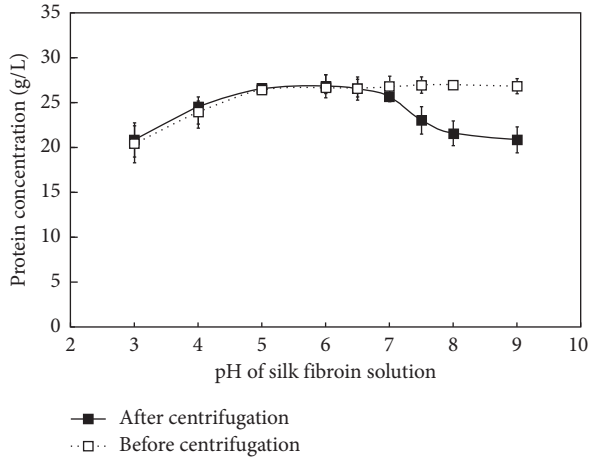

Figure 1: Effect of pH on the solubility of regenerated silk fibroin in 0.25 M citrate-phosphate buffer.

as alkaline proteases. Although fibroinase is the native silk-digesting enzyme, it was identified as a cysteine protease [29, 30]; Collupulin and Bromelain are classified as cysteine proteases, and they exhibited low activities in enzymatic hydrolysis of SF in our study.

3.3. Effects of Temperature, pH, and Substrate-to-Enzyme Ratio on RSF Hydrolysis. The effect of temperature on the candidate enzymes was determined by performing the reactions at range 50°C–65°C and pH 8.0 (Table 2). The proteolytic activities of Alcalase and FoodPro were efficient at a broad temperature range between 50°C and 60°C and drastically decreased at >65°C. The optimal temperature to conduct RSF hydrolysis was determined as 60°C. The effect of pH on the efficiency of enzymatic hydrolysis of SF was determined at pH range 6.5–9.0 and 60°C (Table 3). The proteolytic activities of Alcalase and FoodPro were effective at a broad pH range 6.5–9.0, and the optimal pH was determined as 8.0. Finally, FoodPro was selected as the proteolytic enzyme to perform the 1st step of 2-stage RSF hydrolysis. To determine the efficiency of enzymatic hydrolysis, the substrate-to-enzyme ratio was estimated using a fixed quantity of RSF (400 mg) and FoodPro (1, 5, 10, or 25 U) at pH 8.0 and 60°C (Figure 2). A decrease in residual protein (%) and an increase in AAs of EHs were proportional to an increase in FoodPro concentration ranging from 1 to 10 U per RSF (400 mg).

3.4. Enhancement of Efficiency in Enzymatic Hydrolysis. The enzymatic hydrolysis using FoodPro was almost saturated after 3 h of reaction and did not extend during a prolonged reaction time. To improve the efficiency of enzymatic hydrolysis in RSF, a 2nd enzyme (5 U) was introduced after 3 h of reaction initiation using FoodPro. The 2nd enzymatic treatment was conducted for additional 3 h of reaction time at specific conditions, and the results are summarized in Table 4. The AAs in the EHs obtained from 1st step FoodPro-treated RSF was 2,040 ± 23.7 mg/L, and it increased > two-fold (4,519 ± 42.1 mg/L) during the subsequent Flavourzyme treatment (2nd step). The drastic increase in AA concentration in EHs might be associated to the characteristics of Flavourzyme that exhibits mixed activities of endotype and exotype enzymes. The residual protein was 70.6 ± 5.39% after FoodPro treatment (1st step), and it further decreased to 55.1 ± 5.28% during Flavourzyme treatment (2nd step). Therefore, RSF was efficiently hydrolyzed by the 2-stage enzymatic treatment by sequentially using FoodPro and Flavourzyme at the 1st and 2nd step, respectively, within 6 h of reaction time. To reduce the enzymatic reaction time, the AAs and protein yields of mixed proteolytic enzyme treatment by simultaneously using the combination of FoodPro and Flavourzyme were compared to those of the 2-stage enzymatic treatment (Figure 3). In the mixed proteolytic enzyme treatment, the enzymatic hydrolysis was conducted at 60°C and pH 7.0. The residual protein and AAs concentration in EHs during the mixed proteolytic enzyme treatment for 3 h were 54.4 ± 2.35% and 4,663 ± 23.0 mg/L, respectively. Additionally, enzymatic hydrolysis in the mixed proteolytic enzyme treatment was not extended at prolonged reaction time up to 6 h. Therefore, the RSF in aqueous solution was hydrolyzed by mixed proteolytic enzyme treatment which reduced the reaction time compared to that by the 2-stage enzymatic treatment by sequentially using FoodPro and Flavourzyme.

3.5. Characterization of RSF and Its EHs Using UV-Visible Spectra. To characterize RSF and its EHs, UV-visible spectra analysis was performed using the same concentrations of samples, and results are presented in Figure 4. All the samples with 2.0 g/L concentration exhibited a characteristic protein absorption band in UV-visible spectra. The absorption intensities at 280 nm increased after enzymatic treatments, indicating that the hydrophobic and self-assembled regimes in RSF protein were initially relaxed, and subsequently the degradable protein/peptide content increased compared to those of untreated RSF. Moreover, similar changes were reported using alkaline protease-treated silkworm powder in UV-visible spectra analysis [31].

3.6. MW Determination of RSF and Its EHs Using SDS-PAGE. The MWs of RSF and its EHs were analyzed by performing SDS-PAGE using 4–20% gradient gel (Figure 5). The RSF exhibited a broad MW distribution ranged from 60 to 150 kDa, and the protein bands were visible at approximately 10–22 kDa. The broad protein bands of RSF at approximately 100 kDa might be related to the intermolecular interaction between the SF molecules, and similar results were reported in previous reports [2, 31]. After enzymatic treatment using FoodPro in the 1st step, the broad protein bands of RSF disappeared and distinct protein bands at 350–420 kDa and 60–70 kDa MW were observed. In the EHs obtained from FoodPro-treated RSF, a novel protein band at 27 kDa was detected and the intensities of several bands at approximately 10–12 and 15 kDa were enhanced. In enzymatic treatment using FoodPro at the 1st step, the RSF aggregates were dissociated and gradually degraded into low MW proteins at

Table 1: The specific enzyme activities of food-grade enzymes and the results of enzymatic hydrolysis of regenerated silk fibroin during 3 h reaction.

Food-grade enzyme	Specific activity (U/mg)	Conditions		Enzymatic hydrolysis	
		pH	Temperature (°C)	Residual protein (%)	Amino acid (mg/L)
Alcalase® AF 2.4 L	14.0 ± 0.66	8.0	60	71.8 ± 1.07^{b}	2,209 ± 37.7^{ab}
Alphalase® NP	4.86 ± 0.18	7.0	60	84.4 ± 0.53^{d}	2,074 ± 25.8^{c}
Bromelain BR1200	0.10 ± 0.00	7.0	50	98.2 ± 2.39^{h}	580 ± 87.4^{f}
Collupulin™ MG	2.09 ± 0.02	6.0	60	92.4 ± 0.71^{f}	1,228 ± 40.8^{e}
Flavourzyme® 500 MG	2.38 ± 0.02	7.0	50	$^{(1)}$54.0 ± 1.67^{a}	2,011 ± 90.7^{c}
FoodPro® alkaline protease	15.0 ± 0.80	8.0	60	78.0 ± 0.19^{c}	2,271 ± 51.8^{a}
Neutrase™ 0.8 L	1.72 ± 0.02	7.0	50	97.1 ± 0.16^{g}	489 ± 69.6^{f}
Promod™ 192P	$^{(2)}$0.59 ± 0.01	5.0	50	NA	NA
Promod™ 278MDP	0.88 ± 0.01	7.0	60	90.8 ± 0.40^{f}	1,461 ± 12.4^{d}
Promod™ 279MDP	$^{(2)}$2.62 ± 0.05	5.0	60	NA	NA
Protamex®	3.11 ± 0.03	7.0	50	81.8 ± 2.51^{e}	2,118 ± 89.6^{bc}

$^{(1)}$The silk fibroin started to precipitate during the enzymatic reaction with Flavourzyme. $^{(2)}$Enzymatic reaction was performed at pH 7 instead of pH 5 as the substrate precipitated at acidic pH 5. NA: enzymatic reaction was not observed as these enzymes are classified as exotype proteases. a–g: means in the same column followed by different letters differ significantly ($p < 0.05$).

Table 2: Effects of temperature on the enzymatic hydrolysis of regenerated silk fibroin using FoodPro and Alcalase at pH 8.0.

Enzyme		FoodPro		Alcalase	
Temperature (°C)	Time (h)	Residual protein (%)	Amino acid (mg/L)	Residual protein (%)	Amino acid (mg/L)
50	0	100.0 ± 1.16^{d}	62 ± 1.5^{h}	100.0 ± 1.14^{f}	62 ± 1.5^{h}
	3	86.1 ± 3.65^{c}	2,456 ± 4.5^{e}	86.9 ± 1.67^{e}	2,417 ± 15.7^{e}
	5	86.3 ± 0.60^{c}	2,671 ± 19.5^{a}	87.3 ± 2.97^{e}	2,662 ± 26.2^{c}
55	0	100.0 ± 1.16^{d}	64 ± 1.6^{h}	100.0 ± 1.14^{f}	64 ± 1.6^{h}
	3	80.5 ± 1.29^{b}	2,441 ± 13.1^{e}	84.5 ± 3.92^{d}	2,426 ± 19.3^{e}
	5	80.9 ± 1.09^{b}	2,602 ± 30.6^{c}	83.1 ± 3.40^{c}	2,536 ± 38.7^{d}
60	0	100.0 ± 1.13^{d}	62 ± 1.5^{h}	100.0 ± 1.10^{f}	62 ± 1.5^{h}
	3	77.2 ± 2.17^{a}	2,537 ± 18.9^{d}	75.8 ± 0.72^{b}	2,769 ± 17.4^{b}
	5	75.9 ± 2.41^{a}	2,645 ± 4.5^{b}	74.2 ± 0.37^{a}	2,891 ± 23.4^{a}
65	0	100.0 ± 1.14^{d}	67 ± 1.6^{h}	100.0 ± 1.13^{f}	67 ± 1.6^{h}
	3	98.0 ± 1.50^{d}	1,843 ± 10.7^{g}	100.5 ± 3.24^{f}	1,787 ± 23.0^{f}
	5	98.2 ± 1.12^{d}	1,876 ± 5.4^{f}	99.2 ± 2.93^{f}	1,750 ± 4.7^{g}

a–h: means in the same column followed by different letters differ significantly ($p < 0.05$).

Table 3: Effects of pH on the enzymatic hydrolysis of regenerated silk fibroin using FoodPro and Alcalase at 60 °C.

Enzyme		FoodPro		Alcalase	
pH	Time (h)	Residual protein (%)	Amino acids (mg/L)	Residual protein (%)	Amino acids (mg/L)
6.5	0	100.0 ± 0.92^{c}	27 ± 0.7^{i}	100.0 ± 2.55^{c}	27 ± 0.7^{f}
	3	84.9 ± 0.88^{b}	1,857 ± 10.0^{h}	86.4 ± 2.38^{b}	1,826 ± 42.8^{e}
	5	83.4 ± 4.87^{b}	2,058 ± 13.2^{g}	87.2 ± 2.53^{b}	1,895 ± 2.4^{d}
7.0	0	100.0 ± 0.49^{c}	27 ± 0.7^{i}	100.0 ± 1.62^{c}	27 ± 0.7^{f}
	3	80.6 ± 5.74^{ab}	2,223 ± 2.4^{f}	81.3 ± 0.69^{a}	2,072 ± 16.4^{c}
	5	80.7 ± 2.30^{ab}	2,254 ± 19.0^{e}	82.0 ± 2.45^{a}	2,156 ± 55.7^{c}
8.0	0	100.0 ± 1.92^{c}	27 ± 0.7^{i}	100.0 ± 1.83^{c}	27 ± 0.7^{f}
	3	79.7 ± 1.55^{ab}	2,355 ± 10.0^{d}	79.2 ± 2.59^{a}	2,300 ± 50.8^{b}
	5	77.4 ± 0.20^{a}	2,515 ± 25.5^{a}	79.0 ± 1.83^{a}	2,435 ± 6.4^{a}
9.0	0	100.0 ± 1.51^{c}	27 ± 0.7^{i}	100.0 ± 3.18^{c}	27 ± 0.7^{f}
	3	79.4 ± 4.45^{ab}	2,372 ± 14.0^{c}	79.5 ± 0.85^{a}	2,291 ± 148^{b}
	5	76.2 ± 1.50^{a}	2,442 ± 32.7^{b}	80.2 ± 1.24^{a}	2,397 ± 29.1^{a}

a–i: means in the same column followed by different letters differ significantly ($p < 0.05$).

10–27 kDa range. After Flavourzyme treatment (2nd step), the protein bands at 420 kDa of EHs obtained from FoodPro-treated RSF (1st step) gradually disappeared and a novel band at 315 kDa was detected. The intensities of bands at 10–12, 15, and 27 kDa were enhanced after Flavourzyme treatment (2nd step). Therefore, the SDS-

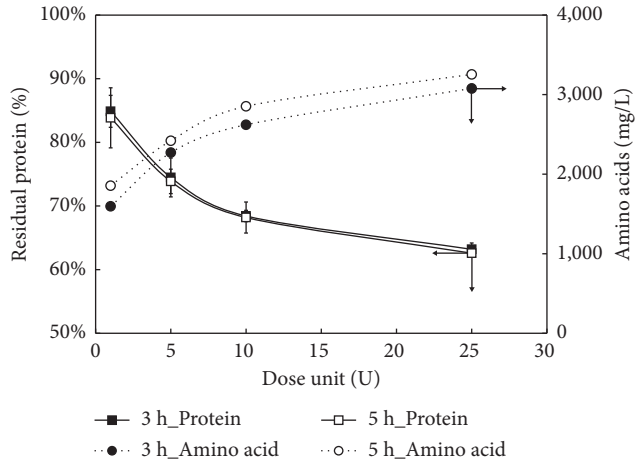

Figure 2: Effect of substrate (fibroin) : enzyme ratio on the proteolytic activity of FoodPro in 0.25 M citrate-phosphate buffer.

Table 4: Selection of the second proteolytic enzyme to enhance the efficiency of enzymatic hydrolysis of FoodPro-treated silk fibroin.

Enzyme treatment		Stage	Reaction condition			Residual protein (%)	Amino acids (mg/L)
			Time (h)	Temperature (°C)	pH		
Silk fibroin		—	0	60	—	100.0 ± 1.54^{c}	17 ± 0.7^{j}
1st step: FoodPro (FP)		1	3	60	8.0	70.6 ± 3.81^{b}	2,040 ± 23.7^{i}
2nd step	FP ⟶ Alcalase	2	+3	60	8.0	65.5 ± 5.67^{b}	2,611 ± 22.0^{d}
	FP ⟶ Alphalase	2	+3	60	7.0	65.6 ± 6.83^{b}	2,505 ± 28.7^{e}
	FP ⟶ Bromelain	2	+3	50	7.0	68.7 ± 9.73^{b}	2,455 ± 16.2^{fg}
	FP ⟶ Collupulin	2	+3	60	6.0	68.5 ± 8.32^{b}	2,227 ± 4.7^{h}
	FP ⟶ Flavourzyme	2	+3	50	7.0	55.1 ± 2.91^{a}	4,519 ± 42.1^{a}
	FP ⟶ Neutrase	2	+3	50	7.0	65.4 ± 8.68^{b}	2,453 ± 16.2^{fg}
	FP ⟶ Pomod 192	2	+3	50	5.0	67.1 ± 7.39^{b}	2,689 ± 54.7^{c}
	FP ⟶ Promod 278	2	+3	60	7.0	67.7 ± 7.61^{b}	2,438 ± 35.7^{g}
	FP ⟶ Promod 279	2	+3	60	5.0	65.0 ± 7.38^{b}	2,746 ± 18.7^{b}
	FP ⟶ Protamex	2	+3	50	7.0	66.6 ± 6.11^{b}	2,453 ± 25.7^{f}

+3: additional reaction time. a–j: means in the same column followed by different letters differ significantly ($p < 0.05$).

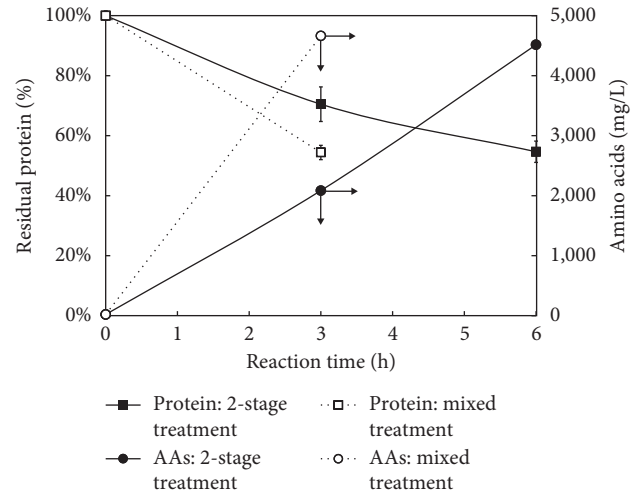

Figure 3: Comparison between the 2-stage and mixed treatments using FoodPro and Flavourzyme during the enzymatic hydrolysis of silk fibroin.

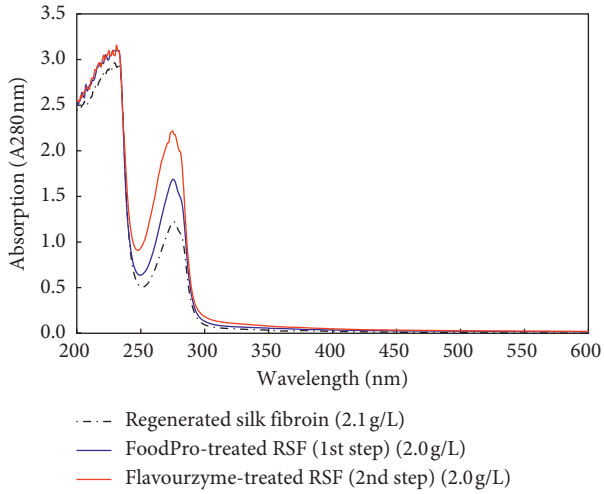

Figure 4: UV-visible spectra of regenerated fibroin and its enzymatic hydrolysates obtained using FoodPro in the 1st step and Flavourzyme in the 2nd step.

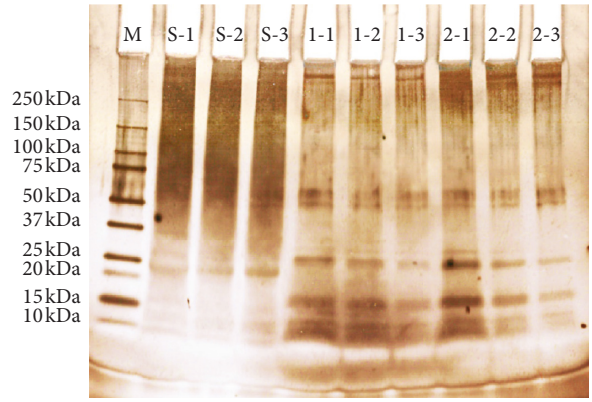

Figure 5: SDS-PAGE of the regenerated silk fibroin and its enzymatic hydrolysates obtained using FoodPro in the 1st step and Flavourzyme in the 2nd step. M: molecular weight marker. S-1: regenerated silk fibroin, 84 μg/well. S-2: regenerated silk fibroin, 42 μg/well. S-3: regenerated silk fibroin, 28 μg/well. 1-1: enzymatic hydrolysate obtained using FoodPro at the 1st step, 57 μg/well. 1-2: enzymatic hydrolysate obtained using FoodPro at the 1st step, 29 μg/well. 1-3: enzymatic hydrolysate obtained using FoodPro at the 1st step, 19 μg/well. 2-1: enzymatic hydrolysate obtained using Flavourzyme at the 2nd step, 45 μg/well. 2-2: enzymatic hydrolysate obtained using Flavourzyme at the 2nd step, 23 μg/well. 2-2: enzymatic hydrolysate obtained using Flavourzyme at the 2nd step, 15 μg/well.

PAGE results indicated that the aggregates and high MW proteins of RSF were dissociated and hydrolyzed in the 2-stage enzymatic treatment using FoodPro initially and then Flavourzyme.

3.7. MW Characteristics of RSF and Its EHs Using SEC. RSF samples (700 μg) and EHs (200–250 μg) were effectively separated using a 5Diol-300-II size-exclusion column with a good resolution (Figure 6). The untreated RSF exhibited

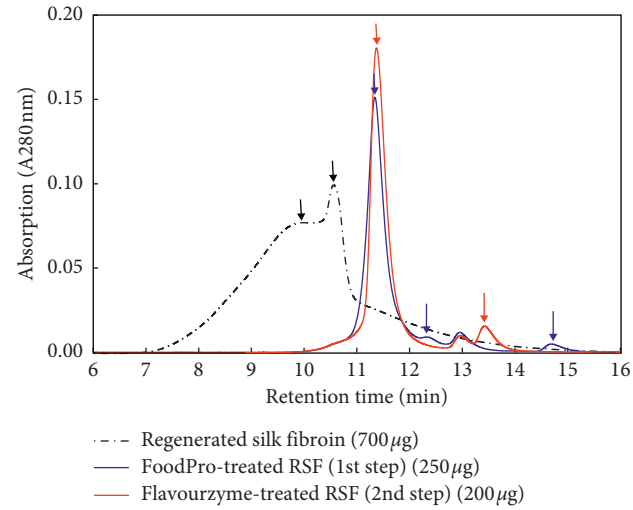

Figure 6: Size-exclusion chromatography of the regenerated silk fibroin and its enzymatic hydrolysates obtained using FoodPro in the 1st step and Flavourzyme in the 2nd step.

two peaks in the chromatogram. The first peak might be strongly associated to the intermolecular interaction, whereas the second peak represented the SF molecules. A previous report indicated that the elution profile of silk obtained after SEC exhibits an early-eluted peak that might represent the silk aggregates and the late-eluted peak represents the nonaggregated silk molecules [41]. This previous report suggested that Ajisawa's reagent produces more number of β-sheet structures and aggregates during dialysis owing to the high exposure of hydrophobic residues compared to the LiBr dissolution [41] and caused higher degradation of the SF heavy chains than other solvents [42]. In the SEC profile of EHs obtained from FoodPro-treated RSF, the peaks that represented untreated RSF disappeared and novel peaks were generated at low MW with enhanced intensities. In this study, the variations in absorption intensities during SEC were similar to those in UV-visible spectra analysis. These results were well matched to those of actinase-treated SF as the absorption intensity of the early peak of aggregated SF decreased with an increase in the later peak of nonaggregated SF molecules [22]. In the SEC profile of EHs, several peaks at 12.5, 13.0, and 14.8 min retention time points were observed after FoodPro treatment (1st step). After Flavourzyme treatment (2nd step), the peaks at 12.5 and 14.8 min retention time points were decreased and a novel peak at 13.5 min retention time was generated. The variations in the elution profiles of soluble SF were similar to those during the biodegradation of silk films using protease from *Streptomyces griseus* [13].

4. Conclusion

B. mori SF and its hydrolysates were investigated as functional materials in foods and alternative sources of food additives. To prepare the SF hydrolysates, enzymatic hydrolysis using commercial food-grade enzymes can replace the acid hydrolysis. The RSF solubility was relatively stable at

a narrow range of pH (5.0–6.5). FoodPro and Alcalase were selected as two of the best candidate enzymes to hydrolyze SF at pH range 6.5–9.0 and temperature between 50°C and 65°C. In a single enzyme treatment using either FoodPro or Alcalase, approximately 20–25% of SF was hydrolyzed and 2 g/L AAs were released from SF. In the 2-stage enzymatic treatment using the combination of FoodPro and Flavourzyme in a sequence, approximately 45% of SF was degraded and 4.5 g/L AAs were released within 6 h of reaction time. The RSF and its EHs were characterized by performing UV-visible spectra, SDS-PAGE, and SEC analyses. In the 2-stage enzymatic treatment using FoodPro initially and then Flavourzyme, the aggregates and high MW proteins of SF were dissociated and biodegraded into the low MW proteins/peptides (10–15 kDa and 27 kDa). SF hydrolysates that are used as functional food can be produced through environment-friendly enzymatic hydrolysis using commercial food-grade proteolytic enzymes.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this study.

Acknowledgments

This work was supported by Wonkwang University in 2017.

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