



Research on degradation of silk fibroin by combination of electron beam irradiation and hydrothermal processing

Nguyen Thi Kim Lan*, Dang Van Phu, Le Anh Quoc, Nguyen Quoc Hien

Research and Development Center for Radiation Technology

*Email: lktnguyen345@gmail.com

Abstract: Silk fibers and silk proteins have been demonstrated to be useful to apply in the textile industry, biomedical, cosmetics, pharmaceuticals. In this study, the effects of electron beam (EB) irradiation combined with hydrothermal processing to the solubility of silk fibroin and generation of soluble silk protein were investigated. The solubility of unirradiated and irradiated fibroin samples were greater than 80 % when hydrothermal degradation was performed in the sodium hydroxide solution at an appropriate concentration of 0.05 M. However, the solubility of irradiated fibroin was greater than that of unirradiated sample. The soluble silk protein content increased from 0.462 to 0.653 mg protein/mg silk fibroin when irradiation doses increased from 0 to 200 kGy, respectively. The molecular weight of protein was determined by SDS-PAGE method. The characteristics of silk protein were confirmed by scanning electron microscope (SEM), Fourier transform infrared spectroscopy (FT-IR), thermogravimetric analysis (TGA) and X-ray diffraction (XRD).

Key words: *Silk fibroin, silk protein, electron beam irradiation.*

I. INTRODUCTION

Silkworm (*Bombyx mori*) silk is a natural protein consisting of sericin and fibroin protein. Fibroin has a long history of use as textiles and surgical sutures because of the remarkable properties such as mechanical strength, elasticity, biocompatibility and controlled biodegradation. In addition to, fibroin has been potential material for biomedical applications such as enzyme immobilizing membranes, an oral dosage gel form, scaffolds for tissue engineering and materials with anti-HIV activity or reducing blood glucose and cholesterol levels [1-4]. Proteins and amino acids extracted from silk fibroin have been used as additives in soap production, hair conditioners and body care products because of good moisturizing properties and compatibility with human skin

[2-8]. Many studies have been realized to segment fibroin using proteolytic enzymes. However, the high cost of enzymes themselves has limited industrial production [9-10]. Another method which has been used to recover the silk proteins and amino acids is hydrothermal treatment [11]. The results showed that the use of only water at high temperature and pressure without addition of acid or alkali catalyst would not get products effectively [8]. Research and application of irradiation technology for degradation of silk fibroin have attracted considerable interest. Some of research results showed that gamma irradiation can affect the structures of the fibroin fibers. For example, the high irradiation doses from 500 to 1000 kGy directly affect the solubility of silk fibroin [6, 12]. Every year, the silk industry produces tons of silk wastes from broken down or unreeled cocoons. So, the

application of electron beam irradiation method combined with hydrothermal processing to increase the solubility of fibroin and to induce soluble silk proteins from silk wastes is of great interest.

II. EXPERIMENTAL

Degumming of silk cocoon

Silkworm cocoon was obtained from a local silk farm (Di Linh, Vietnam) and degummed using the hydrothermal degumming method adopted by Yamada et al. [13]. The removal of sericin by hydrothermal reaction was carried out in a SM200 autoclave (Yamato, Japan) at - 120 °C in 30 minutes. The reaction

products consisted of aqueous solution and remaining fibroin residue, which was separated from the soluble product using a filter paper (Satorius, Germany). The fibroin residue was then dried in a forced air oven at 60 °C.

Electron beam irradiation

The irradiation processing of fibroin was done at Research and Development Center for Radiation Technology using the electron beam accelerator (UERL-10-15S2). The doses delivered to different samples were measured by Radiochromic film B3000 (GEX) dosimeter. The samples were subjected to various doses at 0, 50, 100 and 200 kGy. The beam features were given in Table I.

Table I. Specifications of the electron beam accelerator UERL-10-15S2

Beam energy	10 MeV
Beam current	750 μ A
Pulse repetition rate	140 Hz
Pulse width	18.6 μ S
Distance source to sample	50 cm
Dose rate	14.5 kGy \times m/min
Temperature	30 °C

Hydrothermal degradation in NaOH solution of irradiated fibroin

Effect of NaOH concentration

In each experiment, the irradiated fibroin and 0.025-0.075 M NaOH solution (weight ratio = 1:100) were loaded into the autoclave and operated at 120 °C for 1 hour. The solution and the remaining insoluble residue were separated using a filter paper. The insoluble residue was then washed with water until pH = 7, dried to get its net weight. The content of soluble fibroin was calculated following equation (1). The protein content in solution was assayed by Lowry's method [14] using bovine serum albumin (BSA) as a standard and from that the protein content obtained per one weight unit of initial fibroin (mg/mg) was calculated.

$$\text{The content of soluble fibroin (\%)} = 100 \times (m_o - m_r) / m_o \quad (1)$$

Where m_o và m_r are the weight of initial fibroin and the remaining insoluble fibroin residue, respectively.

Effect of hydrothermal reaction time

The effect of hydrothermal reaction time of the fibroin samples was determined in the same manner that described as effect of NaOH concentration. However, the experiments were conducted in a reaction time interval of 10-30 minutes and NaOH concentration of 0.05 M. The protein solutions were neutralized to pH = 6-7 by HCl and then dialyzed against deionized water using cellulose tubings (molecular weight cut off 12 kDa) for 18 h with several changes of water to remove salts. Protein

powders were obtained by a Modullyo free-dryer (Thermo Electron Corporation) with operating temperature -50 °C or a ADL311 spray-dryer (Yamato, Japan) with inlet and outlet air temperature of 120 and 60 °C respectively, and liquid flow rate of 5 ml/min.

Analysis protein characterization

SDS-PAGE

Molecular weight (Mw) of the protein was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10 % acrylamide gel using the Mini-PROTEANR 3-cell system. A broad range marker (Bio-rad) was run as a molecular weight marker (7.1-209 kDa). Gels were stained by G250 Coomassie Blue stain and the proteins were detected by dark blue traces on transparent gel background.

Scanning electron microscopy

The morphology of particles of the protein powders were observed by SEM images using S4800 scanning electron microscope (Hitachi, Japan).

FT-IR spectra.

The protein powder samples (0,002 g) were pelleted with KBr (0,2 g) and recorded Fourier Transform Infrared Spectroscopy (FT-IR) by a FTIR-8400S spectrometer (Shimadzu, Japan).

Thermogravimetric spectra

The protein powder samples were put into aluminum pans. Thermogravimetric analysis (TGA) of these samples was done by DTG-60 system (Shimadzu, Japan). The temperature range was scanned from 25 °C to 600 °C at a predetermined rate of 10 °C/min.

X-Ray diffraction measurement

The X-ray diffraction (XRD) analysis of the protein powders were recorded on D8 Advance (Bruker) diffractometer using CuK α radiation. These samples were scanned in the 2 θ range of 10-30 ° with scan rate of 0.4 °/min.

III. RESULTS AND DISCUSSION

Hydrothermal degradation of irradiated fibroin

Table II. Effect of NaOH concentration on the solubility and protein content of irradiated and hydrothermal degraded fibroin

Irradiation dose (kGy)	NaOH concentration (M)					
	0.025	0.05	0.075	0.025	0.05	0.075
	Solubility (%)			Protein content (mg/mg)		
0	44.5 ± 4.2	81.0 ± 3.4	89.4 ± 3.1	0.234 ± 0.061	0.462 ± 0.019	0.471 ± 0.045
50	51.3 ± 1.9	84.6 ± 2.0	92.1 ± 1.6	0.330 ± 0.024	0.541 ± 0.022	0.549 ± 0.027
100	52.1 ± 2.2	86.5 ± 2.5	93.0 ± 2.6	0.364 ± 0.036	0.572 ± 0.044	0.576 ± 0.055
200	53.9 ± 2.8	88.9 ± 2.1	94.6 ± 2.3	0.446 ± 0.039	0.653 ± 0.031	0.661 ± 0.038

The solubility and protein content of irradiated fibroin degraded by hydrothermal

treatment in NaOH solution of 0.025-0.075 M were indicated in Table II. The results showed

that the solubility of unirradiated and irradiated fibroins was more than 80 % after treating by 0.05 M NaOH. The solubility of unirradiated fibroin was 81 % while those of fibroin irradiated at 50, 100 and 200 kGy were 84.6, 86.5 and 88.9 %, respectively. The solubility of 50-200 kGy irradiated fibroins was higher than that of the unirradiation sample from 2-8 %. On the other hand, the solubility of fibroin treated with 0.075 M NaOH increased compared to that of treated with 0.05 M NaOH, but the protein content did not increase significantly. The protein content of 0-200 kGy irradiated fibroins obtained from 0.462 to

0.653 mg protein/mg silk fibroin at the concentration of 0.05 M NaOH while protein contents of these samples treated at 0.075 M NaOH were from 0.471 to 0.661 mg protein/mg silk fibroin. So, the NaOH concentration of 0.05 M was effective to dissolve 1 % irradiated fibroin in the hydrothermal degradation reaction. In the examined dose range of 0-200 kGy, the higher the solubility of fibroin and the protein content was resulted for the higher the radiation dose and the highest protein content was observed for the dose of 200 kGy.

Table III. Effect of time of hydrothermal reaction on solubility and protein content of irradiated fibroin before and after dialysis

Dose (kGy)	Time (min.)								
	10	20	30	10	20	30	10	20	30
	Solubility (%)			Protein content before dialysis (mg/mg)			Protein content after dialysis (mg/mg)		
0	53.8 ± 3.2	62.6 ± 2.8	67.3 ± 2.0	0.390 ± 0.020	0.427 ± 0.030	0.436 ± 0.013	0.233 ±0.012	0.244 ±0.011	0.227 ±0.023
50	56.9 ± 4.0	64.5 ± 2.2	69.2 ± 3.1	0.425 ± 0.028	0.494 ± 0.027	0.533 ± 0.032	0.237 ±0.010	0.247 ±0.018	0.231 ±0.017
100	60.6 ± 2.5	67.3 ± 2.7	71.7 ± 4.5	0.452 ± 0.031	0.539 ± 0.034	0.564 ± 0.015	0.242 +0.016	0.254 ±0.020	0.238 ±0.017
200	66.4 ± 2.3	72.3 ± 4.1	76.9 ± 4.5	0.498 ± 0.040	0.585 ± 0.093	0.622 ± 0.056	0.251 ±0.010	0.263 ±0.008	0.249 ±0.032

Changes in the solubility and protein content before and after dialysis of irradiated fibroin upon time of hydrothermal degradation reaction in 0.05 M NaOH solution were presented in Table 3. The solubility of unirradiated and irradiated fibroin increased by the increase of hydrothermal reaction time. The analysis results of the protein content showed that the protein content obtained before dialysis also increased by the increase of reaction time. However, after the protein solutions were

dialysed by cellulose membranes with Mw cut off 12 kDa, the remaining protein content at hydrothermal reaction time of 30 minutes was less than those at reaction time of 10 and 20 minutes. This might due to lost of protein fragments whose -Mw was less than 12 kDa were created during the reaction time of 30 minutes and so these fragments were exchanged against in dialysis process. This result suggested that, at the conditions employed, the hydrothermal degradation

reaction time of 20 minutes was effective to get the pure protein content.

Characteristics of silk protein

Molecular weight measurment

The results of gel electrophoresis analysis of silk protein were showed in Fig. 1. The Mw of protein was in range from 13 to 200 kDa and the sizes dominant lied between 34 and 50 Da that indicated the dagradation of peptide linkage. The result here is consistent

with other studies. In the research of effects of gamma irradiation on biodegradation of silk fibroin [6], the fibroin fibers irradiated in range 0-1000 kGy were hydrolyzed by enzymatic methods in 7 days to prepare proteins whose Mw were about 33-37 kDa. Meanwhile, the research results of Lamoolphak et al. [11] showed that the protein obtained after hydrothermal decomposition of fibroin at temperature over 140 °C had Mw less than 10 kDa.

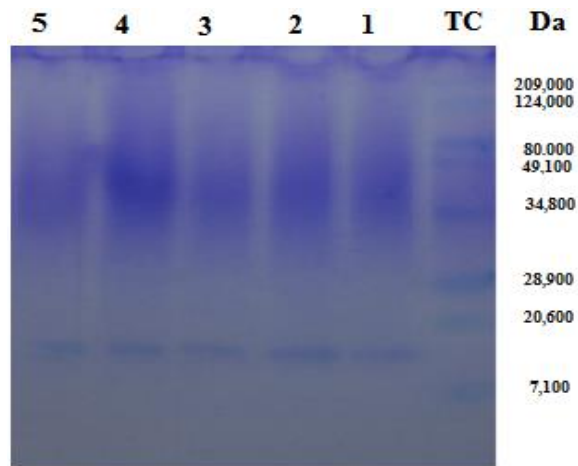


Fig.1. SDS-PAGE of the marker (lane TC); spray dried sample: 50 kGy - (lane 1); free-dried samples: 0 kGy (lane 2), 50 kGy (lane 3), 100 kGy (lane 4) and 200 kGy (lane 5)

Observation of SEM

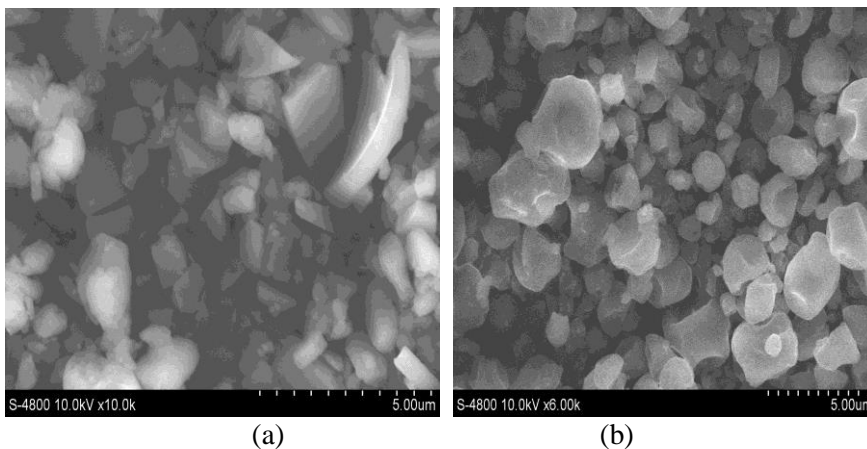


Fig.2. SEM image of protein powder (a) freeze drying and (b) spray drying-

The results on Fig. 2 showed that the freeze drying process of protein solution created the protein powder with long flake-like

particles while spray drying process formed protein powder with circular particles whose size was in range between 2 and 5 μm.

FTIR characterization

The results of infrared spectra of the protein powders from unirradiated and irradiated fibroin were indicated in Fig. 3. The results showed that, the infrared spectra of irradiated samples presented characteristic absorption peaks were similar that of unirradiated sample. The absorption peak of C=O stretching mainly occurred at the

wavelength of 1652-1654 cm^{-1} . The vibration of N-H bonding was at position of 1539-1542 cm^{-1} . And the phase combinations of C-N stretching and C=O bending vibration fell in wavelength of about 1242 cm^{-1} [3, 15]. It was found that there was no difference in the FT-IR spectral results of the protein powders obtained from fibroin irradiated and unirradiated.

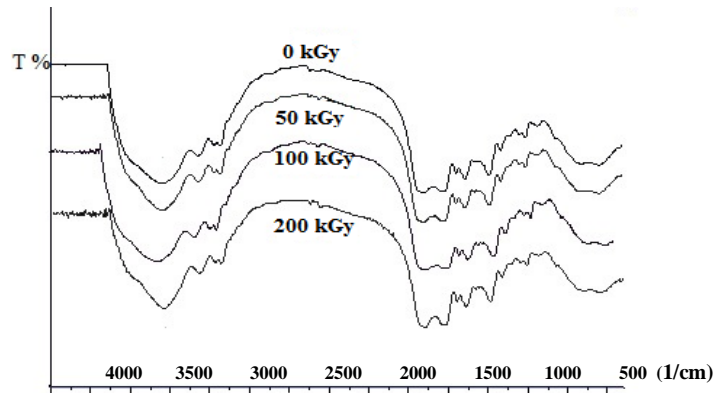


Fig. 3. Infrared spectrum (FTIR) of protein powders from 0-200 kGy irradiated fibroin

TGA Characterization

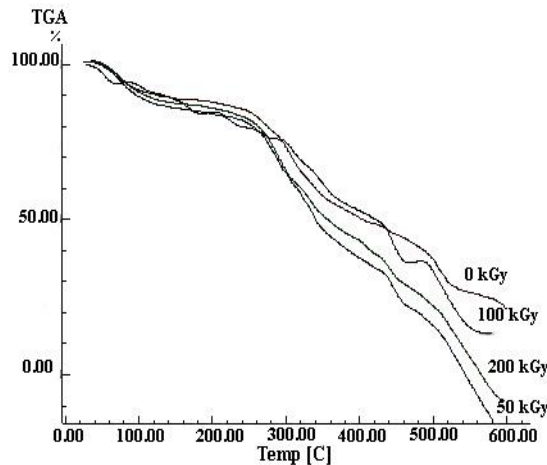


Fig. 4: TGA thermograms of protein powders

The thermogravimetric analysis (TGA) results of the protein powders from fibroin unirradiated and irradiated 50-200 kGy were presented in Fig. 4. The thermogram of all samples indicated the division into four distinct

sections. From room temperature to 100 $^{\circ}\text{C}$, the weight loss was due to water evaporation of about 9 %. In the second, from 100 to 280 $^{\circ}\text{C}$, the samples began to decompose in range from 20 to 30 %. In the third, there was a difference

in thermal decomposition of the irradiated fibroin samples and the original sample. The protein powder of unirradiated fibroin was decomposed 60 % at temperature of 520 °C. While the protein powder of irradiated samples lost weight over 70 % at the same temperature. At the end, about 70 % weight of the protein powder of unirradiated fibroin and 90 % weight of the protein powder of 50-200 kGy irradiated fibroin were decomposed to volatiles at 600 °C. These results showed that the thermal stability of the protein powder decreased with increasing irradiation dose for silk fibroin. According to the study of Nogueira et al. [16], the thermal decomposition of fibroin membranes is influenced by the internal structure and physical properties of the sample with the degree of molecular orientation being one of the most important parameters. Well-oriented silk materials will have decomposition temperature of above 300 °C, no oriented silk fibers with β -sheet structure usually decompose in about 290-295 °C and amorphous silk fibroin occurs at a temperature lower than 290 °C. The thermal stability of the protein powder obtained from the spray drying process was less than that of the free dried protein powders. The results of TGA in this study showed that the protein powders had dominant amorphous structure.

X-ray diffraction analysis

As well know that, silk fibroin has two regions of structure that are crystalline and amorphous. Three types of crystal structure are α -helix (silk I), β -sheet (silk II) and helical structure (silk III) [2, 11]. The main diffraction peaks of silk I are present at $2\theta = 12.2^\circ, 19.7^\circ, 24.3^\circ$ and 28.2° while that of silk II are present at about $2\theta = 9.1^\circ, 18.9^\circ, 20.7^\circ$ [2]. In addition to, the amorphous structure displays a broad diffraction peak [11]. XRD spectra of protein powder of fibroin irradiated 0-200 kGy were recorded in the 2θ range of 0° to 30° (Fig. 5). A sharp peak at $2\theta = 13^\circ$ and a broad peak at $2\theta = 28^\circ-31^\circ$ were displayed on the XRD spectra of the protein powder from unirradiated fibroin. This result indicated that there was presence of both crystal and amorphous structure in the protein powder. On the other hand, XRD of protein powder prepared of fibroin irradiated 100 and 200 kGy showed two broad peaks which shifted to position of $2\theta = 19^\circ-22^\circ$ and $2\theta = 28^\circ-30^\circ$. This could be a result of essential presence of amorphous structure. In the same condition of irradiation, protein obtained from spray dried solution showed the XRD result being the same as that of free dried samples.

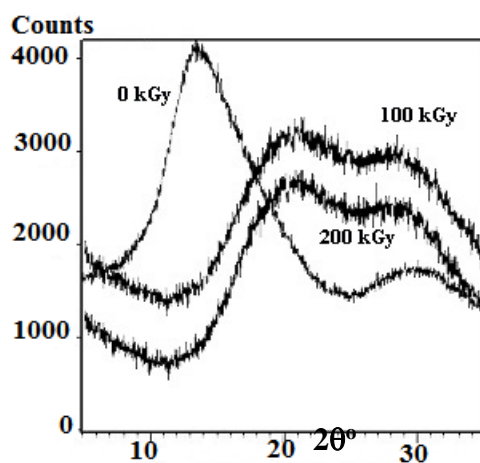


Fig. 5. XRD spectra of protein powder of fibroin unirradiated and irradiated at 100 and 200 kGy

IV. CONCLUSIONS

The research on degradation of silk fibroin by electron beam irradiation combined with hydrothermal processing to prepare silk protein was carried out. The solubility of unirradiated and irradiated fibroin was higher than 80 % when hydrothermal reaction was performed in NaOH solution of 0.05 M. The solubility of irradiated fibroin was higher than that of the unirradiated sample. The hydrothermal degradation reaction time of 20 minutes was effective to get a pure protein content. The protein content increased from 0.462 to 0.653 mg protein/mg silk fibroin when irradiation doses increased from 0 to 200 kGy, respectively. The highest protein content was observed for a dose of 200 kGy. The Mw of proteins were mainly in the range from 34-50 kDa. The process of freeze-drying or spray-drying formed protein powders whose particle size was 2-5 μm . It was found that the protein powder had essential amorphous structure and the EB irradiation process affected the structure of silk fibroin.

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