Doctoral Dissertation (Shinshu University)

The mass production of wild silk from Japanese oak silkmoth (*Antheraea yamamai*) cocoons

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Abbreviations

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ANOVA: analysis of variance
ATR-FTIR: attenuated total reflectance Fourier transform infrared spectroscopy
B_n: backcrossed strains (n represents the number of generations)
DDS: drug delivery system
LUSL: longest unbroken silk length (silk that recorded the longest filament length upon one reeling)
RT: room temperature
SEM: scanning electron microscopy
SD: standard deviation

Chapter 1. General introduction

1. Background

1.1 Domestic silkmoth

The domestic silkmoth, *Bombyx mori*, was literally domesticated by humans and has been used for over 5000 years. Although *B. mori* cannot survive and reproduce without artificial support, it has been an important factor for cultural and economic development because of its high silk productivity and ease of reproduction.[1)]

B. mori exists as a diversity of races or strains. In Japan, various types of *B. mori* are also reared and stored. Approximately 500 races that breed naturally and uniquely from various sericulture regions are maintained at the National Institute of Ago-biological Science. Upon dividing this species into strains including mutants developed by breeding using radiation and chemicals, about 820 strains were reared and stored for research at Kyushu University.[2)] For practical example of developed mutants, a strain called Platina Boy is one mutated strain, in which only male silkworms can hatch, which was established by a genetic approach. This is because the males in this strain are more robust, have higher silk productivity and quality, and show less variation in the fineness of silk filaments compared with the females.[3-5)] Based on this background, *B. mori* has been used for experiments as a major insect model to investigate the function of genes; however, there has been little research about its use as a bioresource.[1,2)]

1.2 Use of the silk

One typical example of the use of *B. mori* as a bioresource is for producing silk. Among the natural fibers, silk is the only filament for which there is no need to apply a spinning process for making yarn, in contrast to the case for staple fibers such as wool, cotton, and other botanical or animal fibers.

Although synthetic fiber has been developed, silk produced by *B. mori* (domestic silk) is still referred to as the “queen of fibers” because of its unique luster, touch, toughness, and dyeability.[6-8)] Although it has been used as a valuable fiber for a long time, the number of silk cocoons produced in Japan is decreasing year by year. However, cocoon production worldwide showed a tendency to increase from 2010 to 2015. This suggests that the demand for silk cocoons is not decreasing (**Table 1**).[9)]

Table 1. Fluctuation of *B. mori* cocoon production in Japan and top 5 cocoon-producing countries

	B. mori cocoon production in each year [1000 t]					
Country	2010	2011	2012	2013	2014	2015
China	621.00	661.00	655.00	643.00	641.00	628.00
India	131.92	139.87	144.43	142.49	164.54	157.52
Uzbekistan	23.94	24.67	25.01	26.46	25.00	27.24
Iran	9.78	10.21	57.9	11.07	11.49	12.54
Thailand	4.65	4.58	77.6	4.76	4.84	4.88
Japan [a)]	265	220	202	168	149	135
Total [b)]	799.95	847.33	846.24	834.25	853.16	836.51

a) For Japan, the unit for each year is t.

b) Values for the total cocoon production include other countries, such as Brazil, Vietnam, and North Korea.

Regarding the silk products made with imported cloth, contrary to the decreasing silk cocoon productivity in Japan, imports of silk cloth in Japan were maintained or increased, especially for garments for women, from 2010 to 2017. This suggests that silk products are still attractive to consumers (**Table 2**).[9)]

Table 2. Fluctuation of level of silk products made from imported cloth in Japan

Items	Raw silk value estimation in year [1000 bales (60 kg each)]							
	2010	2011	2012	2013	2014	2015	2016	2017
Garments (for men)	2.8	2.2	2.4	3.1	3.4	2.4	2.2	1.7
Garments (for women)	38.4	51.2	50.0	63.5	53.4	65.6	73.9	81.2
Underwear / bedding (for men)	1.0	1.4	1.5	1.9	2.0	1.3	1.9	2.2
Underwear / bedding (for women)	13.9	15.5	15.5	20.5	21.9	18.8	20.3	22.1
Handkerchiefs	0.3	0.5	0.3	0.4	0.3	0.3	0.2	0.3
Shawls / scarves	2.2	2.2	2.3	2.2	2.0	1.9	1.5	1.4
Ties	18.1	14.5	12.8	11.9	9.7	7.8	7.9	10.5
Total [a)]	133.0	141.9	141.6	163.8	144.9	144.7	160.5	161.9

a) Values for the total imported silk products include other products, such as knitting, sash worn with Japanese clothes.

Silk is also used as a material for medical purposes. For example, it has been used as a suture thread for a long time because of its biodegradability and high biocompatibility. Currently, the properties of silk are utilized to develop applied biomaterials such as scaffolds for cells or wound-covering materials to enhance wound regeneration.[10,11] Besides medicine, there are many applications for silk, such as in interior decoration, food, and cosmetics. Examples of the application of silk products are summarized in **Table 3**.[9]

Table 3. Examples of the application of silk products

Present circumstances	Category	Examples
Actualized	Fabric	Japanese kimono, sweater, blouse, stocking
	Household goods	Glass wiper, mask, insole, sheet
	Interior / crafts	Tapestry, tablecloth, lampshade, embroidery
	Food	Confectionery, health food, supplement
	Cosmetics	Foundation, face pack, conditioner, face lotion
	Industrial goods	Filter, fishing line, sewing machine thread
	Medical	Suture thread, bandage, gauze
Developing	Industrial goods	Bio-reactor, insulator
	Medical	Artificial blood vessel, drug delivery system, scaffold,

Biopolymers that are not produced by *B. mori*, such as spiders' webs, are also regarded as silk.[12,13]. These types of fiber are also attractive for use as biomaterials. For example, orb-weaving spiders have seven types of silk gland and secrete different types of silk proteins. Each of the spider silk proteins is employed for particular purposes. One type of spider silk secreted from major ampullate silk glands, called drag-line silk, is used by the spider itself to hang from its web. Given this use, drag-line silk has superior tensile strength and strain compared with domestic silk.[14] Another example is from the aquatic insects caddisflies, *Stenopsyche marmorata*, which synthesize silk webs that have adhesive properties, enabling the collection of food underwater; this is also anticipated to be useful as a biomaterial.[15] Although these examples have promising functions that are lacking for domestic silk, these types of silk are difficult to commercialize via production from their original species. For this reason, some research has been performed to create transgenic *B. mori* that can commercially produce useful silk from other species by using *B. mori*'s high productivity of silk protein.[14] Against this background, there have been dramatic developments in gene editing technology. Such technology including TALEN and CRISPR/Cas9 has already been applied to *B. mori*.[16,17]

Some transgenic *B.mori* were practicalized to produce recombinant proteins which are used for cosmetics or medical applications. The research for producing large amounts of recombinant proteins by using *B.mori* was also taken. For producing high performance silk by transgenic *B.mori* was still not practicalized. However, the research of generate transgenic *B.mori* which produce high performance silk was also taken.[18,19] As such, it is becoming increasingly feasible to create *B. mori* that produces other silk proteins commercially.

1.3 Wild silkmoth

In contrast to domesticated *B. mori*, there are also other silkmoths that live in the wild. A range of species of wild silkmoth living in various regions are used to produce wild silk worldwide. For example, about 80 species of wild silkmoth living in Asia and Africa are known to produce wild silk.[20] Regarding silk production, dominant species include *Antheraea pernyi*, *A. yamamai*, *A. assama*, *A. mylitta*, and *Samia cynthia*. Among these species, *A. yamamai*, commonly known as the Japanese oak silkmoth, is a wild silkmoth in Japan the silk of which is the one of the most valuable because of its natural coloration.[21,22] Among the genus *Antheraea*, these are distributed widly in the temperate and tropical zone. Genus *Antheraea* have been mass produced especially in India, China

and Southeast Asia for use as yarns, clothes and pupae for foods. Moreover, *A. mylitta* was diversifying to 44 races. Among the Indian oak Tasar silkworm, there is a semi domesticated silkworm (*Antheraea proylei*) which is also used for producing silk.[23,24)]

For the production of wilk silkmoth, *A. pernyi* cocoon was produced over 60,000 tons in year since 2006 in China.[25)] The productions of wild raw silks from *A. assama, A. mylitta, S. cynthia* tended to increase in India from 2010 to 2017 (**Table 4**). As such, it could be considered that the demand of wild silks is also increasing.[26)]

Table 4. Fluctuation of level of wild raw silks production in India [a)]

	Raw silk value estimation in year [t]							
Silkmoth	2010	2011	2012	2013	2014	2015	2016	2017
A. assama	124	126	119	148	158	166	170	192
A. mylitta	1,166	1,590	1,729	2,619	2,434	2,819	3,268	2,988
S. cynthia	2,760	3,072	3,116	4,237	4,726	5,060	5,637	6,661

a) Values for the *A. assama, A. mylitta, S. cynthia* total were reported from the Central Silk Board, Bengaluru, India.

In Japan, there is a history of using wild silk from the *A. yamamai*. Kawahara *et al.* reported the mass spectrometry analysis to the fabric which found in the ruins of Makimuku site, located on Sakurai, Nara, Japan. The result showed that the fabric was made of silk fibroin from the *A. yamamai*. This also means that *A. yamamai* silk was already used in the third centuries.[27)]

Rearing the *A. yamai* was started between 1781 to 1789 in Azumino, Nagano, Japam. However, rearing rearing *A. yamamai* had been stopped once in 1915 due to the irruption of Mt. Yakedake. After World War II, restarting the rearing *A. yamamai* had occurred in various areas in Japan and assignment of reconstruction was conducted by a Nagano Sanngyou Shikennjou in 1947.[28)]

For another example, organization of rearing and using *A. yamamai* started in Date, Fukushima, Japan for regional development in 2005. In the year of starting, approximately 3000 *A. yamamai* cocoons were harvested. However, the production of *A.*

yamamai cocoons was increased to approximately 7000 cocoons in 2010.[29] According to the Japanese wild silk conference held on September 29th in 2018, approximately 100,000 *A. yamamai* cocoons were harvested in 2017 from three organizations, one farmer and one University. As such, it is considerable that the demand of *A. yamamai* silk also has a possibility to increase.

Compared with the amount of active research on *B. mori* and its silk, given its beneficial traits and great promise, relatively little research about wild silkmoths has been performed. Although numerous types of wild silk have been used since ancient times as valuable fibers, research on their use as biomaterials has been limited compared with that on domestic silk.

Owing to the wild types of silk having their own coloration, luster, and other properties that differ from those of domestic silk, some reports have described the possibility that wild silkmoths could be used as a source of new biomaterials. For example, *Cricula trifenestrata* makes a cocoon with a beautiful gold color and its yellow pigments could be extracted from this cocoon. When these yellow pigments were used for dying woolen fabrics, they were shown to have an effect of protecting woolen fabrics from insect damage.[30] *A. pernyi* sericin and spider egg sac silk have also been investigated for their biodegradability, compatibility, and association with high viability of cells when used for regenerative medical scaffolds.[31,32] For *A. yamamai*, the amino acid sequence, motif, crystal structure, mechanical properties, and morphology of its silk fibroin have been analyzed, with anticipation of its use as a biomaterial.[33]

2. Objective

Wild silks including *A. yamamai* silk are produced less and used less for practical purposes compared with domestic silk because of substantial variation in their fineness and the difficulty of reeling them. Therefore, efforts to make wild silks practically useful could make a major contribution to the fabric market because of their unique color, luster, and touch, which are not shared by domestic silk. Moreover, increasingly active efforts are being made in researching and developing silk material for medical use, which may involve the need for progress in silk production. The more intense this research and development becomes, the more wild silk might be used for the investigation or production of materials. Thus, improving the productivity of rare wild silks is important.

This study aims to improve the mass production of wild silk and contribute to its development as a natural resource. This paper describes an investigation of the differences among the products of different numbers of backcrosses by comparing bred wild silkmoth strains and suggests appropriate numbers of backcrosses to breed strains that can improve the productivity of wild silk using *A. yamamai*. Given that wild silk is regarded as valuable and expensive fibers, especially *A. yamamai* silk, improvements to obtain more wild silk as yarn are also important. This paper thus also describes a method of cocoon boiling and reeling to acquire a greater quantity of valuable silk as filament yarn.

Chapter 2. Backcross breeding

1. Abstract

Backcrossing of the Japanese oak silkmoth *A. yamamai* was undertaken to breed strains with greater silk productivity by hybridizing a strain with a high fertilized female rate (SUB-52) and a strain with heavy cocoons (SUB-11). Via repeated backcrossing from 2010 to 2018, we acquired and stored backcrossed strains B_n (n represents the number of generations) from the third to seventh generations. Upon comparing SUB-52, SUB-11, and each B_n statistically, we showed that B_6 is superior to the other strains in terms of silk productivity. Although the cocoon weight and cocoon shell weight in the last backcrossed strain B_7 were significantly higher than those of SUB-52, there were no significant differences in these variables between B_7 and its recurrent parent SUB-11. We thus considered that B_7 approaches the characteristics of SUB-11, so backcrossing should be limited to six generations. For future work, my colleagues and I plan to improve the fertilized female rate by using B_n for hybridization.

2. Introduction

Silk produced by nonmulberry silkmoths is regarded as being useful as biomaterial because of its differences, such as in shape and amino acid composition, compared with silk produced by the domestic silkmoth *B. mori*. The Japanese oak silkmoth *A. yamamai* is one of the nonmulberry, saturniid silkmoths native to East Asia.[22,33] *A. yamamai* is known to make cocoons that contain blue pigment and yellow pigment, the combination of which makes its cocoon shell bright green in color.[34,35]

Silk from *A. yamamai* has been used in Japan as a material for fabric for a long time; however, it has also been investigated for use as a biomaterial given its characteristic amino acid motifs.[33] Therefore, we consider that increasing the demand for silk from *A. yamamai* would be contribute to research about *A. yamamai's* silk as a biomaterial. However, even though the body of *A. yamamai* is much larger than that of *B. mori*, its silk productivity is lower.

In pursuit of the mass production of *A. yamamai* silk, we consider that crossbreeding is one possible approach to improve the efficiency of silk productivity per cocoon weight or the total amount of silk produced by enhancing cocoon weight itself or increasing the number of harvestable cocoons. In our laboratory, we collected *A. yamamai* samples from various regions and reared them as strains classified by their regions of origin. To select the strains for crossbreeding, the characteristics of each strain were investigated. As a result, we selected two strains for crossbreeding. One strain showed a high fertilized

female rate (SUB-52), which would be expected to increase the number of harvestable cocoons, and the other had heavy cocoons (SUB-11), which would be expected to contribute to the mass production of silk.

In this research, backcrossing was used as the method of crossbreeding. For the genomics, *A. yamamai* has 31 bivalent chromosomes and WZ (female) / ZZ (male) sex chromosome system. Therefore, homologous recombination has not occurred in the female which has a heterozygous sex chromosome with W and Z.[36] We thus decided to approach backcrossing by hybridizing with males which had a possibility of homologous recombination and females as recurrent parent. The largest females in the SUB-11 group were used for hybridization with SUB-52 to breed their hybrids as the first generation. Male hybrids were hybridized with the largest female in group SUB-11 as a recurrent parent to breed backcrossed strains B_n (n represents the number of generations). B_n that inherit the characteristics of interest are used for the next hybridization to obtain the next generations B_{n+1}. By repeated backcrossing, we stored backcrossed strains from B_3 to B_7 in 2018.

Theoretically, the more backcrossing that is performed when breeding a strain, the closer it becomes to its parent from the previous generations[37]. As such, we needed to investigate the appropriate number of backcrosses to breed a backcrossed strain with highly efficient silk production by comparing SUB-52, SUB-11, and $B_{3–7}$. We also reeled cocoon filaments from each strain group and measured their fiber properties to investigate the differences in this variable among the strains.

3. Materials and methods

3.1. Materials

A. yamamai was captured from various regions in Japan and reared at Shinshu University Faculty of Textile Science and Technology. Two strains were used for backcrossing: one showing a high fertilized female rate (SUB-52) (**Table 5**) and the other exhibiting heavy cocoons (SUB-11) (**Table 6**). The fertilized female rate was defined as the percentage of fertilized pairs which their eggs were successfully hatching relative to the hybridized pairs which included fertilized pairs and other pairs that could not lay eggs or their eggs could not hatch.

Chlorinated lime granules (with the main component of calcium hypochlorite trihydrate) and sodium hydroxide (NaOH) were used for the disinfection or sterilization of eggs, oak trees *Quercus acutissima* in the oak groves managed by Shinshu University Faculty of Textile Science and Technology and instruments (Wako Pure Chemical Industries, Ltd.,

Osaka, Japan).

Branches of the *Q. acutissima* were harvested as food from oak groves managed by Shinshu University Faculty of Textile Science and Technology. Rubber boots were worn by all staff working in this field, which were disinfected by spraying with NaOH solution (0.001% vol./vol.) after use to diminish the risk of death by diseases transmitted from the oak grove to other oak groves.

Table 5. Numbers of hybridized pairs, fertilized pairs, and fertilized female rate of SUB-52, SUB-11, and other strains in 2013

Strain	Hybridized pairs	Fertilized pairs	Fertilized female rate [a] [%]
Others 1	21	3	14.3
SUB-11	71	15	21.1
Others 2	38	11	28.9
Others 3	38	22	57.9
SUB-52	49	38	77.6

a) Fertilized female rate = (Fertilized pairs) × 100 / (Hybridized pairs) [%]

Table 6. Cocoon weights of SUB-52, SUB-11, and other strains for the females in 2013

Strain	Samples	Cocoon weight [a] [g]
Others 1	9	9.021 ± 0.910
SUB-11	24	12.513 ± 1.004
Others 2	5	10.517 ± 0.787
Others 3	15	8.039 ± 0.650
SUB-52	13	9.348 ± 1.238

a): Cocoon weights are shown as the mean (left) and standard deviation (right)

3.2. Methods

3.2.1. Disinfection

Chlorinated lime granules were dissolved with tap water to prepare them at a concentration of 1% (w/v) as antiseptic solution (3 l). Eggs of *A. yamamai* were put in the empty tea bag in groups sorted by their mother moth. Eggs in these bags were immersed in the solution and stirred by a magnetic stirrer (15 min) to bleach and disinfect their surface. Eggs were then rinsed under tap water until the smell of chloride had been removed from the egg bags. They were subsequently air-dried at room temperature (RT) and stored in a Petri dish in each group until hatching.

To diminish the risk of diseases such as nuclear polyhedrosis virus infection, soil in the oak groves was scorched for disinfection by flamethrowers in winter. *Q. acutissima* trees were also disinfected by spraying with NaOH solution (0.001% vol./vol.). Moreover, slaked lime was spread on the oak groves and covered by agricultural mulch to protect the trees from natural enemies and to prevent weed growth in the field before spring.

3.2.2. Rearing[38)]

Hatched larvae were moved from the Petri dishes to fresh *Q. acutissima* branches in the rearing cases. Branches were wrapped with wet paper at the cut portion to ensure the maintenance of their moisture and freshness. Rearing cases were kept clean by removing residues from *A. yamamai* larvae, disinfection by spraying with 70% EtOH, and exchanging old *Q. acutissima* branches for new ones every morning. A group of *A. yamamai* were released onto the *Q. acutissima* in the oak groves when the fourth-instar larvae first appeared in the group, to reduce the labor-intensiveness of the rearing.

Cocoons attached to *Q. acutissima* were harvested 10 days after the first spinning at the stage of fifth-instar larvae. Individuals that were still not making cocoons in the group were also collected and reared in the rearing case until they made cocoons.

3.2.3. Weighing and sex determination

Cocoons for which more than 2 weeks had passed since their shells had formed were weighed. Floss of the cocoons that had been spun as a scaffold for their creation was peeled off carefully before these measurements. After weighing the cocoons, cocoon shells were cut with a razor blade and the pupae and exuviae were removed. Cutting of the cocoon shells was performed carefully so as not to damage the pupae inside. Cocoon shells without pupae and exuviae were weighed and the weight of cocoon shells as a percentage of the total cocoon weight was determined. The pupae taken out from the cocoons were evaluated and their sex was determined.

3.2.4. Backcrossing

For hybridization, a pair of male and female moths was placed into a bamboo basket, which was then sealed with paper to prevent escape or hybridization with other moths. Laid eggs and the body of the female moth were collected after the moths had died. The body of the female moth was examined to determine the possibility of transovarial transmission of infectious diseases.

Pairs of male SUB-52 and female SUB-11, which produced particularly heavy cocoon shells, were used for the first backcross to obtain the first generation of backcrossed strain B_1. Both reared male B_1 and female SUB-11 were selected as being associated with heavy cocoon shells and used for backcrossing to obtain the next generation, namely, backcrossed strain B_2. By repeating backcrossing with the latest generation male B_n and female SUB-11 as recurrent parents to obtain the next generation of backcrossed strain B_{n+1}, we obtained and stored backcrossed strains B_3 to B_7. To rear the strain of SUB-52, SUB-11 and all B_n the next year, pairs of the same strain or pairs of siblings were hybridized and their eggs were collected to rear as the same strains as in previous generations.

4. Result and discussion

The variables of cocoon weight, cocoon shell weight, and percentage of cocoon shell weight of females (**Table 7**) and males (**Table 9**) in each strain were compared by ANOVA (Tukey–Kramer method, significance level: 0.05) (**Tables 8, 10**). In the male group of B_6, two individuals exhibited outlying cocoon shell weight of over 1.0 g (1.225 g and 1.323 g) and percentage of cocoon shell of nearly 20% (19.266% and 18.219%). To evaluate the characteristics of other B_6 males, the variables of cocoon weight, cocoon shell weight and percentage of cocoon shell weight in each strain of males were reanalyzed upon excluding the two outlying individuals in B_6 (**Table 11**).

Cocoon weight: In the females, all B_n and SUB-11 had significantly heavier cocoons than SUB-52 (comparing between B_6 and SUB-52: $\rho < 0.05$, comparing between other strains and SUB-52: $\rho < 0.01$). Otherwise, there were no significant differences between all combinations of B_n and SUB-11 ($\rho > 0.05$). We consider that the mass of female *A. yamamai* was improved from SUB-52 since the third-generation B_3 had bred as well as SUB-11. In males, there were no significant differences between all combinations of SUB-52, SUB-11, and B_n ($\rho > 0.05$). Upon excluding outlying individuals from the B_6 group in males, there were also no significant differences between all combinations of SUB-52, SUB-11, and B_n ($\rho > 0.05$).

Cocoon shell weight: In the females, all B_n and SUB-11 produced significantly more silk filaments than SUB-52 (comparing between B_3 and SUB-52: $\rho < 0.05$, comparing between other strains and SUB-52: $\rho < 0.01$). Otherwise, there were no significant differences between all combinations of B_n and SUB-11 ($\rho > 0.05$). According to the ρ value in cocoon shell weight comparing with SUB-52, cocoon shell weights in backcrossed strains B_4 to B_7 and SUB-11 ($\rho < 0.01$) were far superior to that of B_3 ($\rho < 0.05$). We consider that the mass of silk filaments was improved from SUB-52 since the third-generation B_3 was bred; however, it still improved since fourth-generation B_4 as well as SUB-11. In the males, B_4 produced significantly more silk filaments than SUB-52 ($\rho < 0.05$). B_6 also produced significantly more silk filaments than SUB-52, B_3, and SUB-11 (comparing between B_6 and SUB-52: $\rho < 0.01$, comparing between B_6 and B_3 or SUB-11: $\rho < 0.05$). Upon excluding outlying individuals from the B_6 group in males, B_4, B_6, and B_7 produced significantly more silk filaments than SUB-52 ($\rho < 0.05$).

Percentage of cocoon shell weight: In the females, the percentage of cocoon shell weight in B_6 was significantly higher than in SUB-52, B_3, B_7, and SUB-11 (comparing between B_6 and SUB-52: $\rho < 0.05$, comparing between B_6 and B_3, B_7, or SUB-11: $\rho < 0.01$). The value for B_7 was also significantly higher than for B_3 ($\rho < 0.05$). In the males, only B_6 showed a significantly higher value than the other strains ($\rho < 0.01$). In the case when outlying individuals from the B_6 group in males were excluded, B_6 showed significantly higher values than SUB-52 and B_3 ($\rho < 0.01$). Despite outlying individuals being excluded, the B_6 group in males showed comparatively higher mass of produced silk and higher production efficiency than the other strains.

Table 7. Cocoon weight, cocoon shell weight, and percentage of cocoon shell weight in each strain of females [a)]

Strain	Number of samples	Cocoon weight [g]	Cocoon shell weight [g]	Percentage of cocoon shell weight [b)] [%]
SUB-52	8	7.858 ± 1.147	0.625 ± 0.065	8.012 ± 0.685
B_3	33	10.274 ± 0.978	0.802 ± 0.089	7.805 ± 0.421
B_4	24	10.188 ± 2.040	0.842 ± 0.191	8.263 ± 0.786
B_5	14	10.394 ± 1.820	0.871 ± 0.157	8.428 ± 1.056
B_6	19	9.796 ± 0.994	0.869 ± 0.119	8.864 ± 0.779
B_7	88	10.504 ± 1.434	0.868 ± 0.135	8.262 ± 0.654
SUB-11	40	10.274 ± 1.359	0.822 ± 0.133	7.983 ± 0.589

a) The variables of cocoon weight, cocoon shell weight, and percentage of cocoon shell weight are shown as the mean (left) and standard deviation (right).

b): Percentage of cocoon shell weight = (Cocoon shell weight) × 100 / (Cocoon weight) [%]

Table 8. Significant differences determined by ANOVA (Tukey–Kramer method) in cocoon weight, cocoon shell weight, and percentage of cocoon shell weight in each strain of females [a)]

Combination		Cocoon weight	Cocoon shell weight	Percentage of cocoon shell weight
Strain 1	Strain 2			
SUB-52	B_3	0.0005**	0.017*	0.99
SUB-52	B_4	0.0017**	0.002**	0.97
SUB-52	B_5	0.0016**	0.001**	0.80
SUB-52	B_6	0.025*	0.0005**	0.047*
SUB-52	B_7	0.00002**	0.00004**	0.95
SUB-52	SUB-11	0.00039**	0.0039**	1.0
B_3	B_4	1.0	0.92	0.15
B_3	B_5	1.0	0.67	0.061
B_3	B_6	0.91	0.61	0.0000026**
B_3	B_7	0.99	0.21	0.018*
B_3	SUB-11	1.0	1.0	0.92
B_4	B_5	1.0	1.0	0.99
B_4	B_6	0.97	1.0	0.061
B_4	B_7	0.96	0.98	1.0
B_4	SUB-11	1.0	1.0	0.68
B_5	B_6	1.0	1.0	0.52
B_5	B_7	1.0	1.0	0.98
B_5	SUB-11	1.0	0.90	0.34
B_6	B_7	0.45	1.0	0.0089**
B_6	SUB-11	0.89	0.88	0.000096**
B_7	SUB-11	0.98	0.57	0.32

a) "*" and "**" indicate significant differences between the strains.

*: $\rho < 0.05$, **: $\rho < 0.01$

Table 9. Cocoon weight, cocoon shell weight, and percentage of cocoon shell weight in each strain of males [a)]

Strain	Number of samples	Cocoon weight [g]	Cocoon shell weight [g]	Percentage of cocoon shell weight [c)] [%]
SUB-52	5	6.202 ± 0.792	0.539 ± 0.035	8.786 ± 1.142
B_3	30	7.154 ± 1.101	0.673 ± 0.095	9.522 ± 1.524
B_4	29	7.344 ± 0.796	0.746 ± 0.104	10.147 ± 0.837
B_5	16	7.162 ± 0.773	0.700 ± 0.094	9.796 ± 0.938
B_6 [b)]	16 (14)	6.703 ± 0.651 (6.687 ± 0.674)	0.806 ± 0.198 (0.739 ± 0.081)	12.038 ± 2.755 (11.080 ± 0.900)
B_7	113	7.062 ± 0.864	0.712 ± 0.128	10.058 ± 1.257
SUB-11	75	6.920 ± 0.986	0.697 ± 0.143	10.031 ± 1.299

a) The variables of cocoon weight, cocoon shell weight, and percentage of cocoon shell weight are shown as the mean (left) and standard deviation (right)

b) In the case of B_6, number of samples, cocoon shell weight, and its percentage were recalculated upon excluding two outlying individuals, as shown in parentheses.

c) Percentage of cocoon shell weight = (Cocoon shell weight) × 100 / (Cocoon weight) [%]

Table 10. Significant differences determined by ANOVA (Tukey–Kramer method) in cocoon weight, cocoon shell weight, and percentage of cocoon shell weight in each strain of males [a)]

Combination		Cocoon weight	Cocoon shell weight	Percentage of cocoon shell weight
Strain 1	Strain 2			
SUB-52	B_3	0.31	0.33	0.92
SUB-52	B_4	0.13	0.019*	0.39
SUB-52	B_5	0.37	0.19	0.78
SUB-52	B_6	0.93	0.001**	0.0001**
SUB-52	B_7	0.37	0.058	0.40
SUB-52	SUB-11	0.60	0.12	0.44
B_3	B_4	0.98	0.32	0.58
B_3	B_5	1.0	0.99	1.0
B_3	B_6	0.67	0.018*	0.00000019**
B_3	B_7	1.0	0.77	0.48
B_3	SUB-11	0.89	0.98	0.60
B_4	B_5	1.0	0.92	0.98
B_4	B_6	0.26	0.75	0.00027**
B_4	B_7	0.74	0.86	1.0
B_4	SUB-11	0.33	0.60	1.0
B_5	B_6	0.78	0.25	0.0001**
B_5	B_7	1.0	1.0	0.99
B_5	SUB-11	0.96	1.0	1.0
B_6	B_7	0.75	0.096	0.0000029**
B_6	SUB-11	0.98	0.04*	0.0000045**
B_7	SUB-11	0.94	0.99	0.32

a) "*" and "**" indicate significant differences between the strains.

*: $\rho < 0.05$, **: $\rho < 0.01$

Table 11. Significant differences determined by ANOVA (Tukey–Kramer method) in cocoon weight, cocoon shell weight, and percentage of cocoon shell weight in each strain of males when outlying individuals from the B_6 group were excluded [a)]

Combination		Cocoon weight	Cocoon shell weight	Percentage of cocoon shell weight
Strain 1	Strain 2			
SUB-52	B_3	0.31	0.27	0.88
SUB-52	B_4	0.13	0.0102*	0.26
SUB-52	B_5	0.37	0.14	0.69
SUB-52	B_6	0.95	0.031*	0.0075**
SUB-52	B_7	0.37	0.036*	0.27
SUB-52	SUB-11	0.61	0.081	0.31
B_3	B_4	0.98	0.25	0.45
B_3	B_5	1.0	0.99	0.99
B_3	B_6	0.69	0.64	0.0022**
B_3	B_7	1.0	0.72	0.35
B_3	SUB-11	0.90	0.97	0.47
B_4	B_5	1.0	0.90	0.97
B_4	B_6	0.28	1.0	0.23
B_4	B_7	0.75	0.83	1.0
B_4	SUB-11	0.33	0.53	1.0
B_5	B_6	0.78	0.99	0.07
B_5	B_7	1.0	1.0	0.99
B_5	SUB-11	0.96	1.0	0.99
B_6	B_7	0.77	0.99	0.056
B_6	SUB-11	0.98	0.90	0.057
B_7	SUB-11	0.94	0.98	1.0

a) "*" and "**" indicate significant differences between the strains.

*: $\rho < 0.05$, **: $\rho < 0.01$

Chapter 3. Method of cocoon boiling and reeling using proteinase

1. Abstract

Japanese oak silkmoth (*Antheraea yamamai*) cocoons were treated with proteinase K solution (dissolved in phosphate buffer) or heated in 0.05% (w/v) $NaHSO_3$ solution as a conventional method. Cocoons were reeled using an automatic reeling machine to obtain silk fibers. Fibers and silk that could not be reeled (due to a thin inner shell layer that could no longer be reeled, debris that arose due to brushing to pull out the cocoon filaments from the cocoon surface, and feeding to the automatic reeling machine) were weighed and the ratio of silk fiber to the total weight of cocoon shells was calculated to determine the reeling efficiency (recovery). According to a *t*-test, there was a significant difference in this efficiency between the stage at the recovery of silk fiber treated with proteinase K [mean ± standard deviation (SD): 72.4% ± 3.8%] and that subjected to the conventional method (mean ± SD: 63.0% ± 7.9%). Reeled fibers were also subjected to tensile testing and a *t*-test. The results showed that there was no significant difference between the mean tensile strength [N] per denier [d] of fibers treated with proteinase K (mean ± SD: 46.1 ± 5.2 mN/d) and the conventional method (mean ± SD: 44.9 ± 4.8 mN/d). These results suggest that the method of using proteinase K solution on cocoons of *A. yamamai* for reeling makes it possible to obtain silk fibers more efficiently with the same mechanical properties as in the conventional method. This approach can thus be expected to be useful as an efficient method for reeling silk from wild silkmoth cocoons.

2. Introduction

Japanese oak silkmoth (*A. yamamai*) is a nonmulberry silkmoth belonging to the Saturniid family, which is native to East Asia. This silkmoth can also produce silk like the domestic silkmoth *Bombyx mori*, which can be used for fabric. Silk from *A. yamamai* has a unique green color and luster that is lacking in that of *B. mori* and is regarded as a valuable material for woven fabric.[22,33,39] Regarding the cocoons of *B. mori*, this silkmoth is highly domesticated and it is easy to obtain silk from its cocoons by reeling. In contrast, for *A. yamamai*, it is difficult to perform reeling to obtain silk from its cocoon.[40]

Cocoon boiling is performed by soaking in heated water or exposure to chemicals before reeling to soften the cocoon shell and facilitate reeling. In the case of most wild silkmoth cocoons including those of *A. yamamai*, the adhesion between cocoon filaments is higher and there is poorer ventilation of cocoon shells than in *B. mori*, which complicate reeling.[41]

These characteristics are caused by the presence of calcium oxalate monohydrate, which

is lacking from the cocoons of *B. mori*. Calcium oxalate monohydrate is an organic mineral crystal. These organic crystals are distributed on the surface of wild silkmoth cocoons and adhere to each cocoon filament. In *A. yamamai*, the silk adopts a flat rectangular cross-sectional shape, which also enhances the adhering area. This also covers gaps between cocoon filaments and prevents permeation and softening of sericin at the inner layer of cocoon shells to promote reeling.[39,41-44)] Because of its unique luster and color that are lacking in silk produced by *B. mori*, there is a possibility of expanding sericulture and the silk industry by developing reeling cocoons of *A. yamamai*.

For degumming, methods of using proteinases to increase sericin degradation and improve silk properties in *B. mori* were reported.[45,46)] To promote reeling, Devi *et al.* and Singh *et al.* reported cocoon boiling using proteinase for the oak tasar (*Antheraea proylei J.*).[47,48)] Moreover, Gellynck *et al.* reported the biodegradability of silk proteins, with results showing that the linear density did not diminish upon treatments with proteinase K and trypsin.[32)] As such, there is a possibility of using proteinase K and trypsin to degrade silk proteins of *A. yamamai* that peel off with compounds that adhere the cocoon filaments and make it easier and more efficient to achieve reeling.

In this research, we treated cocoon shells of *A. yamamai* with phosphate buffer and with enzyme solutions of proteinase K and trypsin to investigate whether calcium oxalate monohydrate could be removed and how proteinases affect its elimination. We also investigated the reeling and fiber properties of *A. yamamai* silk upon exposure to proteinase and compared these with the equivalents upon exposure to the conventional method of cocoon boiling.

3. Materials

The desiccated *A. yamamai* cocoons were provided by Shinshu University Faculty of Textile Science and Technology. Proteinase K and trypsin, as proteinase enzymes that degrade proteins, disodium hydrogen phosphate 12-water ($Na_2HPO_4 \cdot 12H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), sodium hydrogen sulfite ($NaHSO_3$), and sodium hydrogen carbonate ($NaHCO_3$) were used for treatment or cocoon boiling (Wako Pure Chemical Industries, Ltd., Osaka Japan). Solvent for treating cocoon shells and boiling with proteinase solutions, pH 7.8 phosphate buffer, was prepared from $Na_2HPO_4 \cdot 12H_2O$ and $NaH_2PO_4 \cdot 2H_2O$. Moreover, 0.05% (w/v) $NaHSO_3$ solution and 0.05% (w/v) $NaHCO_3$ solution were prepared for boiling and reeling in the conventional method.

4. Methods: Removability of calcium oxalate monohydrate from Japanese oak silkmoth cocoon by using proteinase for cocoon boiling

4.1. Cocoon treatment

Cocoon shells of *A. yamamai* were cut into 1 cm^2 (1 cm × 1 cm) square pieces as samples. Each sample was treated with various types of enzyme solution, with different concentrations and times. Types of solution were pH 7.8 phosphate buffer, 0.1 mg/ml proteinase K solution, 1.0 mg/ml proteinase K solution, 0.1 mg/ml trypsin solution, and 1.0 mg/ml trypsin solution. Times for treatment were set as 4, 8, 16, and 24 h. Temperature was fixed at 55 °C using an aluminum block bath, ALB-301 (SCINICS, Tokyo, Japan). After treatment, samples were rinsed with sterile water (three times) and autoclaved (121 °C, 20 min) to inactivate the enzyme. To maintain other variables as constant as possible, samples treated with phosphate buffer were also autoclaved. Samples were stored in a zippered pack after air-drying and kept in the dark at room temperature until use. Three samples were prepared in each condition.

4.2. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Nicolet iS50 (Thermo Fisher Scientific, Waltham, MA, USA) was used for analyzing the surface of samples (No. of scans: 32, resolution: 4 cm^{-1}, data space: 0.482 cm^{-1}, spectral range: 400–4000 cm^{-1}). Both outer and inner surfaces of samples were measured three times each. Untreated cocoon shell pieces were also measured as a control. From the spectra of the outer layer of cocoon shells, the absorbances at the peak of oxalate (1312 cm^{-1}) and the peak of the amide II region (1590–1504 cm^{-1}) were measured.[42,43] The ratios of absorbance at the peaks of oxalate and the amide II region in samples were calculated to investigate the degree of coating with calcium oxalate.[42]

$$\text{Ratio of absorbance} = (A_{1312cm-1}/A_{1590–1504cm-1})^{42)}$$

Mean of the ratios of absorbance in samples were calculated and compared by F-test to determine the significance of differences among untreated cocoon shells, cocoon shells treated only with phosphate buffer, and cocoon shells treated with proteinase solutions.

4.3. Scanning electron microscopy (SEM)

Samples were observed by SEM (JSM-5600LV; JEOL, Ltd., Tokyo, Japan) to investigate the removal of calcium oxalate (treated for 4 h with 0.1 mg/ml proteinase K solution, 1.0 mg/ml proteinase K solution, and 1.0 mg/ml trypsin solution). In addition,

untreated cocoon shells and cocoon shells treated for 4 h with phosphate buffer were observed. Moreover, samples treated for 24 h by phosphate buffer, 1.0 mg/ml proteinase K solution, and 1.0 mg/ml trypsin solution were observed as excessively treated samples.

5. Results and discussion

5.1. Appearance of treated cocoons

Figure 1 shows the appearance of cocoon shell pieces treated with phosphate buffer, proteinase K, and trypsin for various times or with various concentrations of proteinase. Samples were attached to graph paper using double-coated adhesive tape. Because the cocoon shells were stiff and oval in shape, samples naturally exhibited curved surfaces. When attaching the samples to the graph paper, samples were flattened. However, in the case of treatment with phosphate buffer and short treatment with trypsin at both high and low concentrations, the samples remained stiff and returned to a curved state. In contrast, samples treated with protein K and with trypsin for a long period were soft and did not return to a curved state. Especially upon treatment with a high concentration of proteinase K solution, samples became fluffy and bulky upon 4 h of treatment. Even with the low-concentration proteinase K solution, parts of the cocoon shells were separable after 4 h of treatment. We thus consider that proteinase made the cocoon shells soft, so it could be expected that cocoon filaments would be easily separated in this experiment.

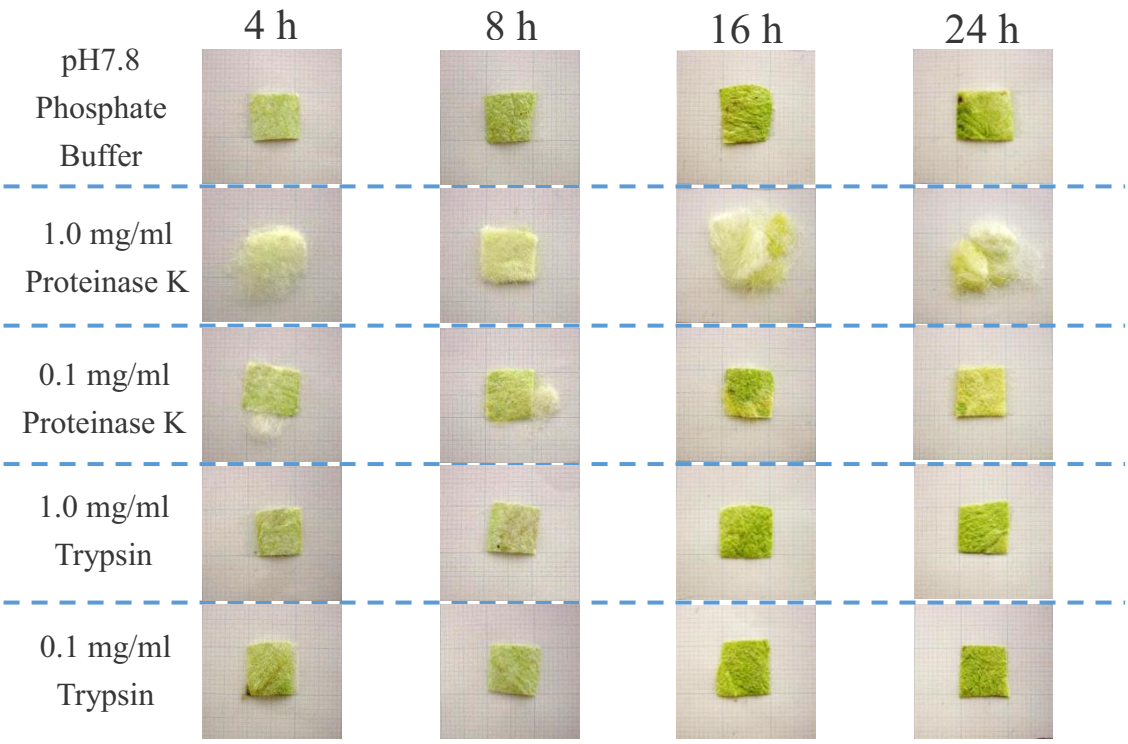

Figure 1. Appearance of treated sample cocoons. For samples treated with only phosphate buffer and samples treated with trypsin for a short period at both concentrations, sample stiffness remained, with a curved shape. For the samples treated with low-concentration proteinase K solution, the cocoon shells softened and parts of them were separable after 4 h of treatment. For the samples treated with high-concentration proteinase K solution, the samples became fluffy and bulky after 4 h of treatment. In this case, filaments were excessively separated, which may have caused difficulty in reeling.

5.2. Removability of calcium oxalate determined by FTIR

Figure 2 shows the FTIR spectra for untreated outer layer of cocoon shells, untreated inner cocoon shells, outer layer of cocoon shells treated with phosphate buffer (4 h), and outer layer of cocoon shells treated with 0.1 mg/ml proteinase K solution (4 h). In the spectra, the absorbance at the peak of oxalate (1312 cm^{-1}) is higher for the untreated outer layer of cocoon shells.

For the peak of the amide II region, absorbance for the untreated outer layer of cocoon shells tended to be lower than that for the peak of oxalate. In the case of treatment with phosphate buffer, absorbance tended to be higher or closer to the absorbance at the peak of oxalate. In the cases when inner cocoon shells were treated with proteinase K solution or left untreated, absorbance tended to be higher than for the peak of oxalate. We thus consider that, upon treatment with phosphate buffer or proteinase solution, cocoon filaments were exposed, instead of the removal of calcium oxalate crystals from the outer layer of cocoon shells.

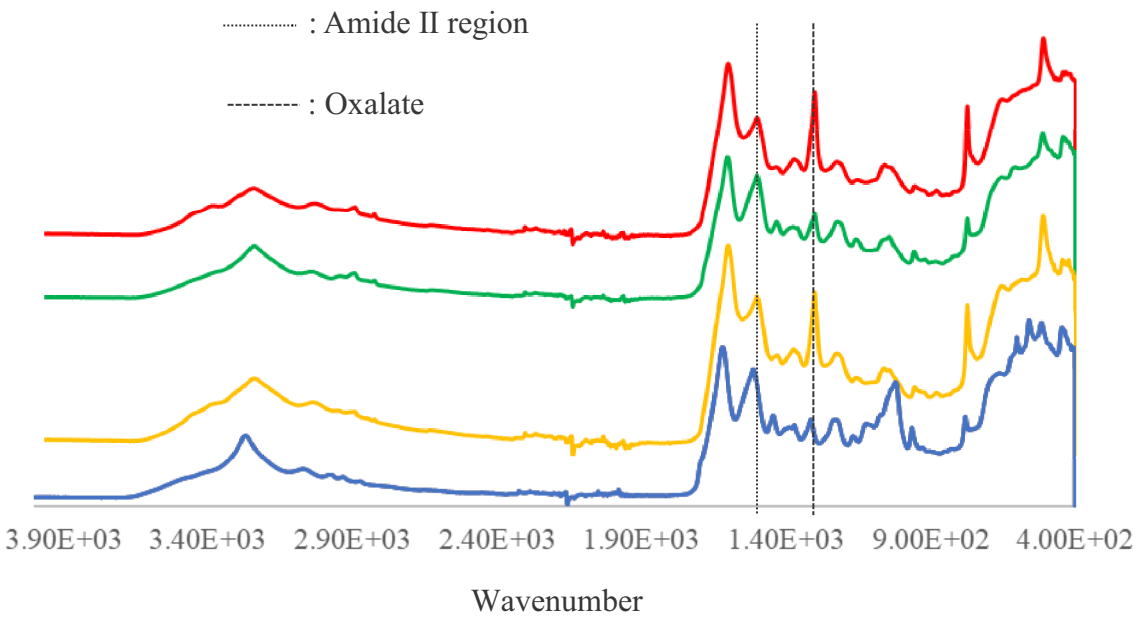

Figure 2. FTIR spectra of *A. yamamai* cocoon shells. Red line: Untreated outer layer of cocoon shells (n = 6). Green line: Untreated inner layer of cocoon shells (n = 6). Yellow line: Outer layer of cocoon shells treated for 4 h with phosphate buffer (n = 6). Blue line: Outer layer of cocoon shells treated for 4 h by 0.1 mg/ml proteinase K solution (n = 6). Vertical dotted line on the left indicates the peak corresponding to the amide II region. Vertical dotted line on the right indicates the peak corresponding to oxalate.

According to the F-test (significance level: 0.05), samples treated with phosphate buffer had sufficient removal of calcium oxalate upon treatment for 8 h ($\rho < 0.05$) (data not shown). We consider that immersion in phosphate buffer itself has an effect of removing calcium oxalate. Regarding the treatment with proteinase, 0.1 mg/ml proteinase K solution, 1.0 mg/ml proteinase K solution, and 1.0 mg/ml trypsin solution were sufficient to remove calcium oxalate upon treatment for 4 h ($\rho < 0.05$) (data not shown). **Table 12** shows the results of F-test comparing among untreated cocoon shells, and shells treated for 4 h with phosphate buffer or for 4 h with 0.1 mg/ml proteinase K solution. Although the use of 0.1 mg/ml proteinase K solution was proven to remove oxalate ($\rho = 0.0067$), there was only a tendency for a difference between phosphate-buffer-treated samples and proteinase-treated samples ($\rho = 0.081$). We consider that the combination of phosphate buffer and proteinase was more efficient and quicker for removing oxalate compounds than treatment with only phosphate buffer.

Table 12. Ratio of absorbance for outer layer of cocoon shells of *A. yamamai*: (A) untreated, (B) treated for 4 h with phosphate buffer, and (C) treated for 4 h with 0.1 mg/ml proteinase K solution, with corresponding ρ-values. [a)]

Treatment	Ratio of absorbance	ρ-value
A. Untreated	1.041 ± 0.223	$0.22^{A–B)}$
B. Phosphate buffer (4 h)	0.898 ± 0.234	$0.081^{B–C)}$
C. 0.1 mg/ml proteinase K (4 h)	0.689 ± 0.091	$0.0067^{A–C)}$

a) Values of "ratio of absorbance" are shown as the mean (left) and standard deviation (right); the number of samples was 6 for each case.

A–B): ρ-value upon comparing untreated and phosphate-buffer-treated cases by F-test.

B–C): ρ-value upon comparing phosphate-buffer- and proteinase-K-treated cases by F-test.

A–C): ρ-value upon comparing untreated and proteinase-K-treated cases by F-test.

5.3. Surface of cocoon determined by SEM

According to the SEM observation, cocoon filaments were practically covered with adherent substances in the case of untreated cocoon surfaces [(a) in **Fig. 3**]. For cocoon shells treated with phosphate buffer, cocoon filaments at the inner layer of cocoon shells could be seen; however, calcium oxalate crystals were still present [(b) in **Fig. 3**]. Upon immersion for 24 h in 1.0 mg/ml proteinase K solution [(d) in **Fig. 3**], filaments were excessively separated, which may impede reeling. According to these results, treatment with 0.1 mg/ml proteinase K solution for more than 4 h had detrimental effects [(c) in **Fig. 3**]. From SEM images and F-test, we consider that a reaction time of less than 4 h is sufficient to remove calcium oxalate from cocoons using 0.1 mg/ml proteinase K solution.

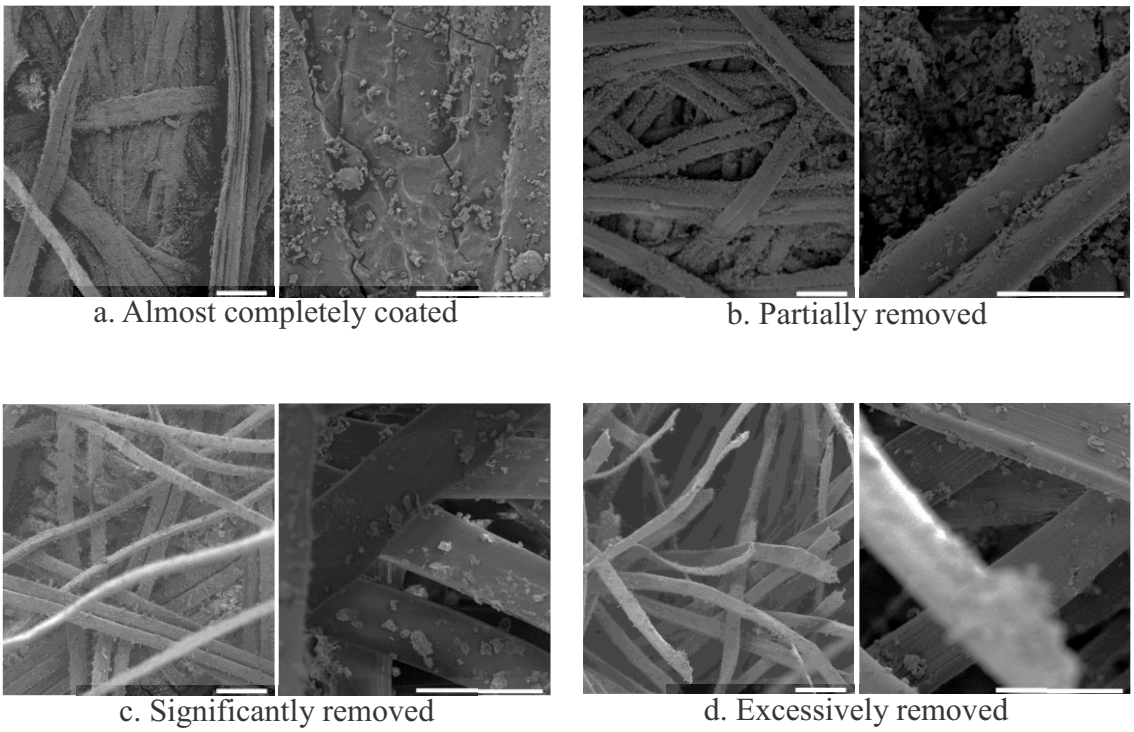

Figure 3. Pairs of low-magnification (left, ×100, scale bar: 100 μm) and high-magnification SEM images (right, ×500, scale bar: 50 μm) of *A. yamamai* cocoon shells. (a) Untreated: Filaments were almost completely coated by adherent substances. (b) Treatment with phosphate buffer for 4 h: Adherent substances were partially removed, so that the filament could be seen more clearly than for untreated cocoon shells; however, plenty of calcium oxalate crystals (cubical substances in the image) and other adherent substances remained. (c) Treatment with 0.1 mg/ml proteinase K solution for 4 h: Calcium oxalate crystals were significantly reduced, so that filaments were separable from cocoon shells for reeling. (d) Treatment with 1.0 mg/ml proteinase K solution for 24 h: Almost all adherent substances were reduced and filaments were excessively separated from each other, which impedes reeling.

6. Method: Improvement of boiling by using proteinase and effect on fiber properties

In the previous chapter showed that proteinase K solution can eliminate calcium oxalate monohydrate from the cocoon surface of *A. yamamai* effectively and quickly in low concentration. It can be expected that there is a possibility to use proteinase K to degrade silk protein of *A. yamamai* that peel off with compounds that adhering the cocoon filaments and make it easier and more efficient for reeling (**Fig. 4**).

We weighed 50 cocoons that could be put into a 50-ml centrifuge tube, which has space for 25-mm-wide cocoons. and calculated their mean weight (2.43 g) and standard deviation (SD; 0.27 g). Because there was a possibility that the size of a cocoon or cocoon shell weight may affect the result of reeling, we determined the range of cocoon weights for the experiment for the mean ± SD (2.16–2.70 g) to diminish the error caused by cocoon shell weight.

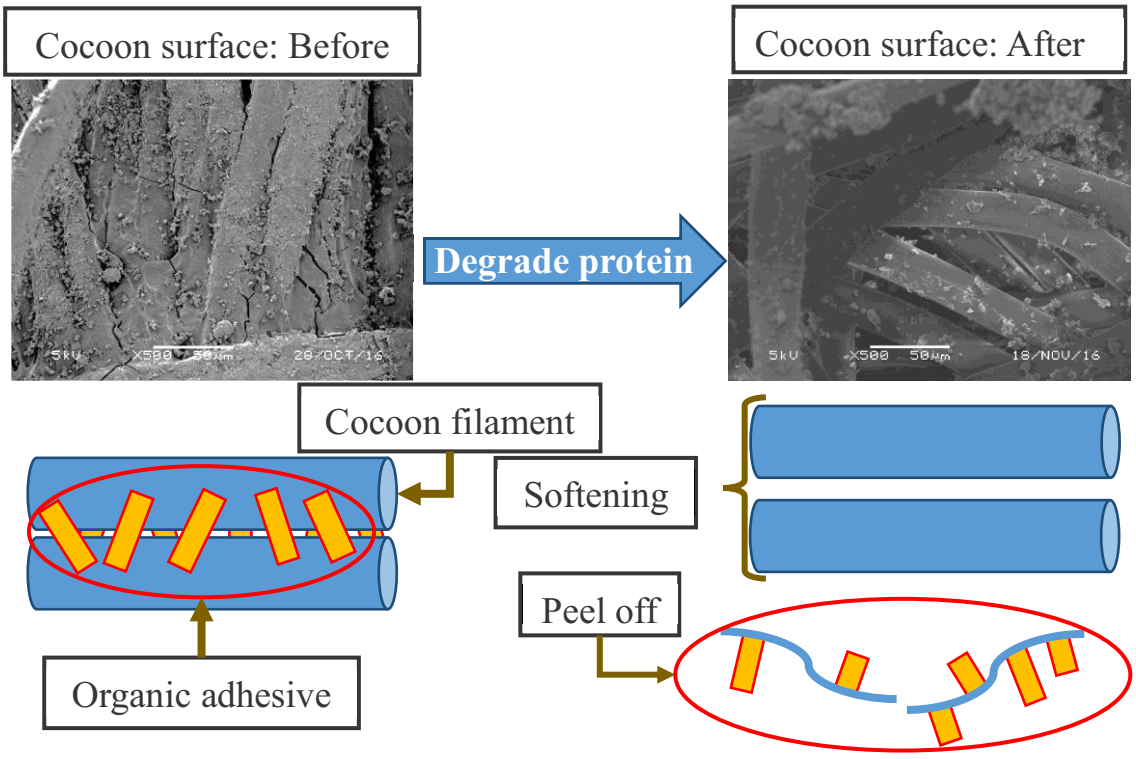

Figure 4. Concept of enzyme degradation for reeling. At the layer of the cocoon shell, cocoon filaments are adhered by organic adhesive (mainly calcium oxalate monohydrate), which hardens the cocoon shell and makes it difficult to reel (left side of figure). By using the proteinase to degrade protein, the surface of silk proteins is peeled off with organic compounds (right side of figure), which enhances softening of the silk filaments, facilitating reeling.

6.1. Cocoon boiling

The weight, width, and length of cocoons were measured before boiling.

Boiling using proteinase K: In accordance with the results of preliminary experiments, the combination of 2 h of treatment with 0.1 mg/ml proteinase K solution and heating in a solvent for boiling was the most efficient method for obtaining cocoon filaments. Proteinase K was thus weighed (2.0–2.3 mg) and dissolved in a 50-ml centrifuge tube with pH 7.8 phosphate buffer (20–23 ml) to prepare 0.1 mg/ml proteinase K solution. Moreover, pH 7.8 phosphate buffer was also decanted at approximately 30 ml into another 50-ml centrifuge tube. Proteinase K solution was heated at 55–60 °C in a water bath. Tap water was poured sufficiently into the pot and heated to prepare boiling water. Decanted pH 7.8 phosphate buffer was heated to 90–95 °C by boiling water in the pot. Cocoons were immersed in the proteinase K solution and treated for 2 h in the water bath. For thorough treatment, clingfilm in the shape of a stick was also added to the tube to prevent the cocoons for floating out from the solution. After enzyme treatment, cocoons were moved into heated pH 7.8 phosphate buffer. Here, another stick formed from clingfilm was also used to ensure thorough immersion, along with continued heating in boiling water (90–95 °C, 3 min). The tube with cocoons was moved to a water bath and its temperature was gently decreased (55–60 °C, 5 min) to ensure permeation of the solution inside the cocoon shells.[49] Phosphate ions could also remove the calcium oxalate.[42] To confirm the effect of the phosphate buffer itself, the same process of boiling without proteinase K was also undertaken.

Boiling by the conventional method[40]: Here, 0.05% $NaHSO_3$ solution was decanted to approximately 30 ml in two 50-ml centrifuge tubes. One of these 0.05% $NaHSO_3$ solutions was heated at 60–65 °C by a water bath, while the other was heated at over 90 °C by boiling water in the pot. Cocoons were immersed in 0.05% $NaHSO_3$ solution heated by boiling water (90–95 °C, 60 s). Here, the clingfilm in the form of a stick was also used to ensure thorough immersion. Cocoons were also immersed in 0.05% $NaHSO_3$ solution heated at a lower temperature (60–65 °C, 40 s). After heated at a lower temperature, cocoons were immersed into 0.05% $NaHSO_3$ solution heated by boiling water (90–95 °C, 3 min). Then, the clingfilm and cap of the tube were removed for high-temperature steam treatment (98 °C, 4 min).

6.2. Reeling

Cocoons after boiling were immediately used as samples for reeling. Reeling was performed on single cocoons using an automatic reeling machine (Nissan, Kanagawa, Japan). The solvent was poured into a small bowl and put on warm water in the reeling

machine. Each cocoon was then put into the solvent in the bowl. Cocoon filament was pulled out from the cocoon by brushing and fed into the reeling machine. Such brushing and feeding were performed carefully to ensure that as little silk as possible was wasted (brushing waste). Efforts were made to avoid reeling when multiple strands of cocoon filament were pulled out from the cocoon. For cocoons treated with proteinase K, the following conditions were set: solvent: sterile water, temperature: 40–50 °C, and reeling speed: approx. 60 m/min. For the cocoons boiled by the conventional method, the following conditions were used: solvent: 0.05% $NaHCO_3$ solution, temperature: 55–65 °C, and reeling speed: approx. 60 m/min. Six samples were tested in each group. The numbers of dropping ends (number of cocoon filaments re-fed into the reeling machine) and fiber breaks (number of fiber breaks with no need for refeeding because the filament running in the pulley in the reeling machine had adhered to the pulley during the reeling due to adhesion, remaining at the surface of the cocoon filament, for example) were counted (data not shown). By using the number of dropping ends, reelability [%] in each cocoon was calculated. Reeling was continued until the cocoon could no longer be reeled. The remains, including brushing waste and thin inner shell layer, were collected for weighing after measuring fiber fineness.

6.3. Fiber fineness

Reeled cocoon filaments were immersed in warm water to facilitate wrap reeling. A motor-driven wrap reel, SSD-3 (Daiei Kagaku Seiki MFG, Kyoto, Japan), was used to measure the length of cocoon filaments and the longest unbroken silk length (LUSL) in one reeling was recorded. Cocoon filaments were divided into 100-m sections when the reeled filaments were longer than 100 m, to determination the variation in fiber fineness (data not shown).

Each cocoon filament and the rest of each cocoon were air-dried overnight. After air-drying, each sample was weighed and the total amount of silk and weight of the remaining cocoon shell were also determined. Recovery [%] was calculated as the percentage of total silk weight relative to the cocoon shell weight. Fiber fineness (entire denier [d]) was calculated using the length and weight of total silk.

6.4. Tensile test

Boiling and reeling additionally performed to prepare samples for the fiber tests. The longest unbroken cocoon filaments were prepared from four cocoons in each sample group, namely, those treated with proteinase K and those subjected to the conventional method. Filaments for the tensile test were straightened and fastened on the lattice-formed

frame made of graph paper to fix their length at 20 mm as samples. For fastening the filaments, double-coated adhesive tape was used to adhere both paper frame and filament on different sides. After straightening, another double-coated adhesive tape (of which only one side was used) was pressed on the adhered parts of filaments to tightly fix the filaments and prevent peeling off from the paper frame.

Samples were preserved in a stable environment (RH 65% and 20 °C) at least overnight until use for testing under the same conditions using a tensile testing machine, Tensilon (A&D, Tokyo, Japan). Regarding the conditions of the tensile test, samples were tested under strain of 20 mm/min, using a 50 N load cell. Break load [N] and break strain [mm] were measured 25 times for each sample, with a total of 100 times in each sample group. Cocoon filaments were wrap-reeled for 100 m and their weight was measured to calculate their fiber fineness (denier). By using the results of tensile test and fiber fineness, break load and strain per denier in samples were calculated.

6.5. Observing cross section of fibroin

Wrap-reeled cocoon filaments were also used for observing the cross section of silk fibroin using a digital microscope, VHX-2000 (Keyence, Osaka, Japan). For preparing samples to observe the cross section of silk fibroin, wrap-reeled cocoon filaments were bundled and passed through the hole (diameter: 1 mm) on the board. Filaments were cut off along the surface of the board to observe the cross section of silk fibroins [(a) in **Fig. 5**]. Efforts to cram bundled cocoon filaments into the hole were made to avoid preparing a sample in which filaments remaining in the hole fell out of it due to the space between the filaments and the hole. In cases in which there was difficulty cramming bundled cocoon filaments into the hole caused by a shortage of filament (e.g., part of the filament used as a sample was approximately only 20 m), cocoon filaments from *B. mori*, the cross section of which differs from that of *A. yamamai* cocoon filaments, were mixed in to make up for the shortage of sample filaments (**Fig. 6**). Cross-sectional areas of fibroin were observed and their areas were measured 50 times per sample [(b), (c) in **Fig. 5**], giving a total of 200 times in each sample group; their cross-sectional areas of fibroin [μm^2] were calculated per denier.

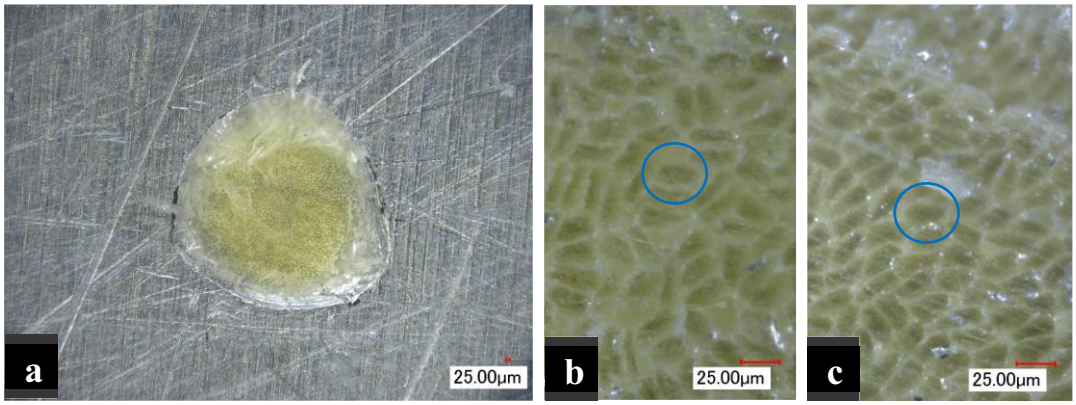

Figure 5. Cross sections of raw silk from *A. yamamai* cocoons observed by a digital microscope: (a) Silk was bundled and passed through the hole (diameter: 1 mm) on the board, followed by cutting to observe the cross section of silk (magnification: ×100). (b) Upon treating with proteinase K (magnification: ×1000), cross sections of fibroin were produced forming a rectangular shape with a dark color, as circled in the image. (c) Upon boiling by the conventional method (magnification: ×1000).

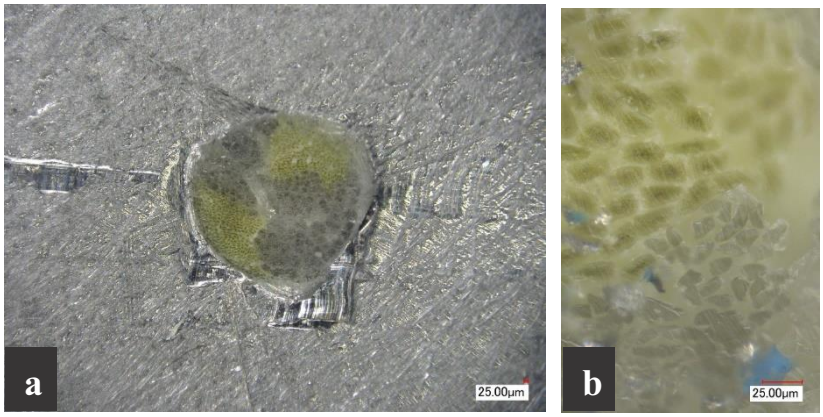

Figure 6. Cross sections of a mixture of raw silk from *A. yamamai* cocoons and *B. mori* cocoons observed by a digital microscope: (a) Parts of filaments from *A. yamamai* or *B. mori* are distinguishable by their coloration (magnification: ×100). (b) Fibroins from *A. yamamai* and *B. mori* are easy to distinguish by their size, color, and cross-sectional shape (upper part of the image: *A. yamamai* filaments, bottom part: *B. mori* filaments, magnification: ×1000).

6.6. Comparing fiber properties in each strain

Cocoon filaments from the cocoons of SUB-11, SUB-52, and $B_{3–7}$ mentioned in **Chapter 2** were also measured and compared in terms of whether their properties were affected by their strains and backcrossing.

The cocoons harvested in 2018 were desiccated. The weight, width, and length of the cocoons were measured before desiccation. After these measurements, cocoons were desiccated using a forced-air-flow oven (WFO-400; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at 115 °C for 1 h and then treated at 60 °C for 8 h. Desiccated cocoons were sealed in a zippered bag and stored in a dark place until use.

Boiling, reeling, and a fiber test were performed on the cocoons of each strain; however, cocoons of SUB-11 and B_n were larger than the others, so 70-ml centrifuge tubes were used for their boiling. Here, 0.1 mg/ml proteinase K solution and only phosphate buffer for boiling were prepared in the ranges of 30–35 ml and 30–50 ml, respectively, in cases with cocoon widths greater than 25 mm. Four cocoons in each strain were used for reeling and their cocoon filaments were measured in terms of their break load, break strain, and cross-sectional area of fibroin. These variables were each calculated per unit fineness (break load: mN/d, break strain: mm/d, and cross-sectional area of fibroin: μm^2/d). These recalculated values of break load, break strain, and cross-sectional area of fibroin per unit fineness were used for statistical analyses by ANOVA (Tukey–Kramer method).

7. Results and discussion

7.1. Reeling

The results for all variables in the reeling results for the conventional method and boiling with proteinase K or only phosphate buffer are shown in **Table 13.** These items were also compared by *t*-test.

Weight of cocoon shells: Upon comparing the weights of cocoon shells between the conventional method (mean ± SD: 506.4 ± 35.6 mg) and boiling with proteinase K (mean ± SD: 548.2 ± 74.3 mg), there was no significant difference ($\rho = 0.24$). This means that cocoon shell weight had little effect on reeling.

Total silk length: In the case of the conventional method, total silk length results were as follows: min.: 413.0 m, max.: 632.8 m, mean: 491.7 m and SD: 74.1 m. In the case of using proteinase K, the results were min.: 538.1 m, max.: 684.2 m, mean: 631.6 m and SD: 49.6 m. The *t*-test results showed that treating with proteinase K produced longer silk than the conventional method ($\rho = 0.003$).

Total silk weight: In the case of the conventional method, for total silk weight, the

following results were obtained: min.: 265.5 mg, max.: 393.8 mg, mean: 319.3 mg and SD: 48.5 mg. In the case of using proteinase K, the results were as follows: min.: 339.7 mg, max.: 479.2 mg, mean: 396.3 mg and SD: 53.7 mg. The *t*-test showed that the quantity of silk using proteinase K was greater than that by the conventional method ($\rho = 0.026$).

Recovery: In the case of the conventional method, the recovery results were as follows: min.: 53.3%, max.: 76.1%, mean: 63.0% and SD: 7.9%. In the case of using proteinase K, the results were: min.: 68.0%, max.: 78.6%, mean: 72.4% and SD: 3.8%. The *t*-test showed that recovery was significantly improved by using proteinase K ($\rho = 0.026$) compared with the conventional method. Regarding treatment only with phosphate buffer, the equivalent results were as follows: min.: 29.8%, max.: 69.3%, mean: 50.2% and SD: 16.8%. Upon comparison with treatment with proteinase K, it was shown that treatment with only phosphate buffer resulted in lower recovery of silk ($\rho = 0.01$). This was because there were cases in which the outer layer of cocoon shell itself was frequently peeled off as brushing waste upon feeding into the reeling machine. We consider that calcium oxalate crystals were inadequately removed and maintained their adhesiveness to cocoon filaments, which prevented single-strand filaments from being pulled out from the outer layer of cocoon shell.

Percentage of brushing waste: In the case of the conventional method, the results for the percentage of brushing waste were as follows: min.: 18.4%, max.: 32.7%, mean: 26.3% and SD: 6.2%. In the case of using proteinase K, the corresponding results were as follows: min.: 10.3%, max.: 23.3%, mean: 16.0% and SD: 4.4%. The *t*-test showed that the quantity of brushing waste was significantly reduced by using proteinase K ($\rho = 0.008$) compared with that in the conventional method. We consider that cocoon filaments at the outer part of the layer were easily pulled out as a single strand by using proteinase K for reducing calcium oxalate crystals.

Percentage of thin inner shell layer: In the case of the conventional method, the results for the percentage of the thin inner shell layer were as follows: min.: 4.9%, max.: 15.2%, mean: 10.7% and SD: 4.4%. In the case with proteinase K, the corresponding results were: min.: 8.7%, max.: 15.1%, mean: 11.6% and SD: 2.5%. The *t*-test showed that there was no significant difference in the percentage of thin inner shell layer ($\rho = 0.66$).

From the results of total silk length, total silk weight, recovery, percentage of brushing waste and percentage of thin inner shell layer, we can conclude that boiling using proteinase K is superior regarding the quantity of reeled silk compared with the conventional method, which is achieved by reducing the quantity of brushing waste.

Reelability: In the case of the conventional method, the following results for reelability were obtained: min.: 14.3%, max.: 33.3%, mean: 26.6% and SD: 8.2%. In the case of

using proteinase K, the equivalent results were as follows: min.: 16.7%, max.: 50.0%, mean: 26.1% and SD: 12.1%. The *t*-test showed that reelability could not be improved or worsened by using proteinase K ($\rho = 0.94$).

LUSL: In the case of the conventional method, the results for LUSL were as follows: min.: 129.6 m, max.: 382.7 m, mean: 260.5 m and SD: 81.6 m. In the case of using proteinase K, the equivalent results were min.: 273.0 m, max.: 598.1 m, mean: 404.5 m and SD: 138.8 m. Although the mean LUSL for the treated sample with proteinase K was nearly one and half times higher than that for the conventional method, the difference did not quite reach significance ($\rho = 0.053$).

Fiber fineness: As for the entire denier, in the case of the conventional method, the results for fiber fineness were as follows: min.: 5.208 d, max.: 6.713 d, mean: 5.857 d and SD: 0.497 d. In the case of using proteinase K, the corresponding results were: min.: 4.749 d, max.: 6.303 d, mean: 5.653 d and SD: 0.651 d. The *t*-test showed that there was no significant difference in the entire denier ($\rho = 0.55$). This suggests that proteinase K did not markedly degrade silk protein.

Table 13. Comparison of reeling results for boiling using proteinase K, phosphate buffer, and conventional method [a)]

	Method of cocoon boiling		
	Proteinase K	Phosphate buffer (ρ-value [g)])	Conventional method (ρ-value [h)])
Cocoon shell weight [mg]	548.2 ± 74.3	503.7 ± 59.1 (0.28)	506.4 ± 35.6 (0.24)
Total silk length [m]	631.6 ± 49.6	406.5 ± 100.7 (0.0006)	491.7 ± 74.1 (0.003)
Total silk weight [mg]	396.3 ± 53.7	247.4 ± 67.3 (0.002)	319.3 ± 48.5 (0.026)
Recovery [b)] [%]	72.4 ± 3.8	50.2 ± 16.8 (0.01)	63.0 ± 7.9 (0.026)
Percentage of brushing waste [c)] [%]	16.0 ± 4.4	36.2 ± 17.1 (0.02)	26.3 ± 6.2 (0.008)
Percentage of thin inner shell layer [d)] [%]	11.6 ± 2.5	13.6 ± 4.7 (0.38)	10.7 ± 4.4 (0.66)
Reelability [e)] [%]	26.1 ± 12.1	34.7 ± 13.4 (0.27)	26.6 ± 8.2 (0.94)
The longest unbroken silk length (LUSL) [m]	404.5 ± 138.8	228.0 ± 67.2 (0.02)	260.5 ± 81.6 (0.053)
Entire denier [f)] [d]	5.653 ± 0.651	5.448 ± 0.282 (0.50)	5.857 ± 0.497 (0.55)

a) Each box apart from ρ-value shows the mean (left) and standard deviation (right)

b) Recovery = (Total silk weight) × 100 / (Cocoon shell weight) [%]

c) Percentage of brushing waste = (Weight of brushing waste) × 100 / (Cocoon shell weight) [%]

d) Percentage of thin inner shell layer = (Weight of thin inner shell layer) × 100 / (Cocoon shell weight) [%]

e) Reelability = (number of reeled cocoons) × 100 / {(number of reeled cocoons) + (Dropping ends)} [%]

f) Entire denier = (Total silk weight) × 9 / (Total silk length) [g/9000 m] = [d]

g) ρ-value upon comparing proteinase K and phosphate buffer by *t*-test

h) ρ-value upon comparing proteinase K and conventional method by *t*-test

7.2. Tensile test and cross-sectional area of fibroin

All variables of mechanical properties and the cross-sectional area of fibroin per denier were compared by *t*-test (**Table 14**).

Properties of silk per denier: In the case of the conventional method, the results showed that break load was 44.92 ± 4.37 mN/d and break strain was 1.015 ± 0.289 mm. In the case of using proteinase K, break load was 46.14 ± 4.61 mN/d and break strain was 1.056 ± 0.197 mm. The *t*-test showed that there was no significant difference in both break load ($\rho = 0.71$) and strain ($\rho = 0.82$) between these two cases.

Cross-sectional area of silk fibroin per denier: Cross-sectional area of silk fibroin reeled by the conventional method was 38.71 ± 1.33 $\mu m^2/d$ and that in the case of using proteinase K was 40.17 ± 2.09 $\mu m^2/d$. The *t*-test showed that the cross-sectional area of fibroin was not significantly degraded by proteinase K ($\rho = 0.28$). From these results, we can conclude that proteinase K causes no serious damage to the mechanical properties nor degrades silk fibroin.

Table 14. Comparing mechanical properties and cross-sectional area of fibroin per denier between boiling with proteinase K and the conventional method [a)]

	Method of cocoon boiling		
	Proteinase K	Conventional	ρ-value
Denier [d]	6.253 ± 0.876	6.892 ± 0.742	0.31
Break load [mN/d]	46.14 ± 4.61	44.92 ± 4.37	0.71
Break strain [mm/d]	1.056 ± 0.197	1.015 ± 0.289	0.82
Cross-sectional area of fibroin [$\mu m^2/d$]	40.17 ± 2.09	38.71 ± 1.33	0.28

a): Each box apart from ρ-value shows the mean (left) and standard deviation (right)

According to ANOVA, for the properties in **Table 15**, the results were almost the same among SUB-52, B_n, and SUB-11 (most ρ-values of nearly 1.0: **Table 16**). We thus consider that the properties of fibroin were not affected by the strains and backcrossing.

Table 15. Mechanical properties and cross-sectional area of fibroin per denier in each strain [a)]

Strain	Break load [mN/d]	Break strain [mm/d]	Cross-sectional area of fibroin [μm^2/d]
SUB-52	46.93 ± 4.11	1.587 ± 0.215	41.26 ± 4.01
B_3	45.27 ± 4.70	1.351 ± 0.202	43.42 ± 5.10
B_4	45.97 ± 2.10	1.263 ± 0.188	42.70 ± 0.50
B_5	47.28 ± 1.53	1.417 ± 0.377	43.27 ± 2.40
B_6	47.29 ± 6.78	1.366 ± 0.127	42.38 ± 1.36
B_7	47.16 ± 0.69	1.521 ± 0.131	44.46 ± 1.95
SUB-11	47.73 ± 5.96	1.364 ± 0.177	43.80 ± 2.11

a) Numbers of samples were unified into four samples in each strain for analyzing mechanical properties and cross-sectional area of fibroin. Each box shows the mean (left) and standard deviation (right).

Table 16. Significant differences determined by ANOVA (Tukey–Kramer method) in mechanical properties and cross-sectional area of fibroin per denier in each strain [a)]

Combination		Break load	Break strain	Cross-sectional area of fibroin
Strain 1	Strain 2			
SUB-52	B_3	1.0	0.72	0.93
SUB-52	B_4	1.0	0.38	0.99
SUB-52	B_5	1.0	0.92	0.95
SUB-52	B_6	1.0	0.78	1.0
SUB-52	B_7	1.0	1.0	0.70
SUB-52	SUB-11	1.0	0.77	0.87
B_3	B_4	1.0	1.0	1.0
B_3	B_5	0.99	1.0	1.0
B_3	B_6	0.99	1.0	1.0
B_3	B_7	1.0	0.92	1.0
B_3	SUB-11	0.98	1.0	1.0
B_4	B_5	1.0	0.95	1.0
B_4	B_6	1.0	0.99	1.0
B_4	B_7	1.0	0.63	0.97
B_4	SUB-11	1.0	0.99	1.0
B_5	B_6	1.0	1.0	1.0
B_5	B_7	1.0	0.99	1.0
B_5	SUB-11	1.0	1.0	1.0
B_6	B_7	1.0	0.94	0.94
B_6	SUB-11	1.0	1.0	0.99
B_7	SUB-11	1.0	0.94	1.0

a) Each value shows the ρ value for differences between the strains.

Chapter 4. Conclusive Summary

1. Backcross breeding

In the group of B_n, we concluded that B_6 is superior in terms of both the mass of silk produced and the efficiency of producing silk per unit weight. From the results for B_7, this might be much closer to SUB-11, so we could consider that the productivity of silk is no longer improved by backcrossing. We also conclude that effectual backcrossing should be limited until breeding in the sixth generation.

As for the breeding, for SUB-11, it is difficult to collect their eggs, whereas it is easy to collect eggs for SUB-52. The purpose of backcrossing is to introduce SUB-52's characteristics to SUB-11, to facilitate breeding. As future research, my colleagues and I will improve the fertilized female rate of backcross strains by hybridization.

In this research, we acquired the B_6 group, which contained male individuals showing outlying silk productivity. We should also use this group for hybridization to ensure maintenance of the *A. yamamai* strain, which shows high productivity of silk filaments.

2. Reeling efficiency and fiber properties by using proteinase for cocoon boiling

We conclude that the use of proteinase K enables more silk to be obtained from *A. yamamai* cocoons without serious damage. As for increasing reeling recovery, it can also contribute to reducing the brushing waste and the number of cocoons for reeling when producing the same weight of silk yarn as reeled by the conventional method. We expect that this approach can also be applied to other wild silkmoth cocoons for reeling. Moreover, we expect that this research can contribute to promote sericulture and the use of silk fabrics.

As for the mechanical properties of *A. yamamai* silk in each strain, the results show that these might not be changed by their particular habitat, at least in Japan; thus, breeding also has little effect on the fiber properties, neither improving nor worsening them.

3. Future prospects

The major goal of backcrossing is to breed backcrossed strains that exhibit a high fertilized female rate, high cocoon weight, and high productivity of silk to obtain more silk protein. Achieving this can lead to the following applications:

1) Identifying the genes that confer high fertilized female rate and growth by comparing the genome sequences among SUB-11, SUB-52, and the established backcrossed strains.

2) Application of this approach to other saturniid silkmoths for predicting the optimal number of backcrosses or for identifying genomic regions of interest when breeding for improved silk production.

3) Investigate the case of backcrossing by hybridize with female SUB-52 as recurrent parent.

As for cocoon boiling with proteinase to improve the recovery of cocoon filament, 0.1 mg/ml proteinase K solution was used in this study. Goals regarding cocoon boiling are as follows:

1) Investigate the minimum concentration with the same effect as 0.1 mg/ml proteinase K solution and how many times it can be applied in succession for cocoon boiling.

2) Use other wild silkmoths such as *A. pernyi*, which has a different content of calcium oxalate monohydrate from *A. yamamai*, for boiling using proteinase. By examining and determining the appropriate conditions for boiling, this information can be applied to other wild silkmoths to predict the optimal boiling conditions with proteinase.

3) If there is any proteinase that is cheaper or more effective at degrading protein than proteinase K, it can be used for boiling and determining the appropriate conditions for boiling.

To achieve these goals, the rearing and method of reeling wild silk in this study could be more practically and commercially used. We also expect that the combination of breeding wild silkmoths and improving the methods of cocoon boiling and reeling may

enhance the silk market.

References

1) The International Silkworm Genome Consortium (2008): The genome of a lepidopteran model insect, the silkworm *Bombyx mori*. *Insect Biochemistry and Molecular Biology* **38**, 1036–1045.

2) Y. Banno, T. Shimada, Z. Kajiura, H. Sezutsu (2010): The silkworm: An attractive bioresource supplied by Japan. *Animal Bioresources in Japan* **59**, (2), 139–146.

3) A. Ohnuma (2005): Establishment of the practical male-rearing technology by a balanced sex-linked lethal. *The Journal of Silk Sericultural Science of Japan* **74**, (2+3), 81–87.

4) A. Ohnuma (2006): The history of the development of male-rearing silkworm strains, *Bombyx mori*. *Journal of Dainippon Silk Foundation* **54**, 1–10.

5) A. Ohnuma (2007): Development of special silkworm strain named Platina Boy for male rearing and its attractive points. *Sen'i Gakkaishi* **63**, (9), 270–274.

6) A. Matsumoto, H.J. Kim, I.Y. Tsai, X. Wang, P. Cebe, D.L. Kaplan (2006): Silk, Handbook of Fibre Chemistry. Taylor & Francis Group, LLC, USA.

7) R.M. Robson (1998): Handbook of Fibre Chemistry. Marcel Dekker, New York, 415.

8) S. Zarkoob, D.H. Reneker, D. Ertly, R.K. Edy, S.D. Hudson (2000): U.S. Patent 6, 110, 590.

9) Silk report No.57: http://silk-teikei.jp//pdf/silk57.pdf, *Sannshi Kenngyou teikei-sienn center*, (2018/10/10)

10) Y. Wang, H.J. Kima, G. Vunjak-Novakovicb, D.L. Kaplan (2006): Stem cell-based tissue engineering with silk biomaterials. *Biomaterials* **27**, 6064–6082

11) C.Y. Wu, B.Z. Tian, D. Zhu, X.M. Yan, W. Chen, G.Y. Xu (1996): Properties and application of wound protective membrane made from fibroin. International Silk Congress, Suzou Institute of Silk Technology.

12) Y. Tamada (2014): Utilization of silk as biomaterials. *The Society of Rubber Science and Technology, Japan* **87**, (10), 428–433.

13) D. Porter, F. Vollrath (2009): Silk as a biomimetic ideal for structural polymers. *Advanced Materials* **21**, 487.

14) H. Wen, X. Lan, Y. Zhang, T. Zhao, Y. Wang, Z. Kajiura, M. Nakagaki (2010): Transgenic silkworms (*Bombyx mori*) produce recombinant spider dragline silk in cocoons. *Molecular Biology Reports* **37**, 1815–1821.

15) X. Bai, M. Sakaguchi, Y. Yamaguchi, S. Ishihara, M. Tsukada, K. Hirabayashi, K. Ohkawa, T. Nomura, R. Arai (2015): Molecular cloning, gene expression analysis, and recombinant protein expression of novel silk proteins from larvae of a retreat-maker caddisfly, *Stenopsyche marmorata*. *Biochemical and Biophysical Research Communications* **464**, 3, 814–819.

16) J.H. Zhang, M. Pandey, J.F. Kahler, A. Loshakov, B. Harris, P.K. Dagur, Y. Mo, W.F. Simonds (2015): Improving the specificity and efficacy of CRISPR/CAS9 and gRNA through target specific DNA reporter. *Journal of Biotechnology.*

17) T. Daimon, M. Uchibori, H. Nakao, H. Sezutsu, T. Shinoda (2015): Knockout silkworms reveal a dispensable role for juvenile hormones in holometabolous life cycle. *Proceedings of the National Academy of Sciences of the United States of America* **112**, (31), E4226–E4235.

18) T. Iizuka (2016): Development of commercial transgenic silkworms producing high-performance silks. *Breeding Research* **18** (4), 158–164.

19) W. Chen, F. Wang, C. Tian, Y. Wang, S. Xu, R. Wang, K. Hou, P. Zhao, L. Yu, Z. Lu, Q. Xia (2018): Transgenic Silkworm-Based Silk Gland Bioreactor for Large Scale Production of Bioactive Human Platelet-Derived Growth Factor (PDGF-BB) in Silk Cocoons. *International Journal of Molecure Sciences* **19** (9), 2–16

20) A. Teshome, S.K. Raina, F. Vollrath (2014): Structure and properties of silk from the African wild silkmoth *Gonometa postica* reared indoors. *Journal of Insect Science* **14**, (36), 1–9.

21) Y. Dong, F. Dai, Y. Ren, H. Liu, L. Chen, P. Yang, Y. Liu, X. Li, W. Wang, H. Xiang (2015): Comparative transcriptome analyses on silk glands of six silkmoths imply the genetic basis of silk structure and coloration. *BMC Genomics* **16**, (203), 1–14.

22) S.C. Kundu, B. Kundu, S. Talukdar, S. Bano, S. Nayak, J. Kundu, B.B. Mandal, N. Bhardwaj, M. Botlagunta, B.C. Dash, C. Acharya, A.K. Ghosh (2012): Invited review: Nonmulberry silk biopolymers. *Biopolymers* **97** (6), 455–467.

23) K. I. Devi, K. M. Ponnuvel, L. S. Singh, K. C. Singh, K. Dutta (2012): Genetic Diversity among Indian Oak Tasar Silkworm, *Antheraea proylei* J. Revealed by ISSR Markers. *International Journal of Industrial Entomology* **24** (1), 57–61.

24) R. M. Reddy, N. Suryanarayana, M. K. Sinha, N. S. Gahlot (2009): Silk Filament Progression with Backcross Breeding Generations in Tropical Tasar Silkworm, Antheraea mylitta D. *International Journal of Industrial Entomology* **19** (1), 187–192.

25) W. Li, Z. Zhang, L. Lin, O. Terenius (2017): Antheraea pernyi (Lepidoptera: Saturniidae) and Its Importance in Sericulture, Food Consumption, and Traditional Chinese Medicine. *Journal of Economic Entomology* **0** (0), 1-8.

26) MULBERRY AND VANYA RAW SILK PRODUCTION STATISTICS: http://texmin.nic.in/sites/default/files/Raw%20Silk%20Production%20Statitics.pdf, Central Silk Board, (2019/2/19).

27) K. Kawahara, M. Muguruma, T. Hashimoto, K. Terasawa, A. Miyaji, T. Nakazawa: Identification of Silk Protein Excavated from the Ruin of the Makimuku Site in 3-4 AD Japan by Mass Spectormetly. http://www.jssscp.org/files/abstract/30_oral.pdf, 30-31, (2019/2/21).

28) Z. Kajiura (2013): Tennsann shiiku-no mecca -Azuminosi tennsann center-no rekishi-to konngo-no tennbou - (Mecca of rearing Japanese oak silkworm - History and future prospects of Japanese oak silkworm center in Azumino city -). *Sanshi-Konchu Biotec* **82** (2), 91–95

29) Silk report No.17: http://silk-teikei.jp/pdf/silk17.pdf, *Sannshi Kenngyou teikei-sienn center*, (2019/2/19)

30) H. Kato, T. Hata, M. Tsukada (2004): Potentialities of natural dyestuffs as antifeedants against varied carpet beetle, *Anthrenus verbasci*. *Japan Agricultural Research Quarterly* **38**, 4, 241–251.

31) M. Yang, Y. Shuai, C. Zhang, Y. Chen, L. Zhu, C. Mao, H. Ouyang (2014): Biomimetic nucleation of hydroxyapatite crystals mediated by *Antheraea pernyi* silk sericin promotes osteogenic differentiation of human bone marrow derived mesenchymal stem cells. *Biomacromolecules* **15**, 1185–1193.

32) K. Gellynck, P. Verdonk, R, Forsyth, K.F. Almqvist, E.V. Nimmen, T. Gheysens, J. Mertens, L.V. Langenhove, P. Kiekens, G. Verbruggen (2008): Biocompatibility and biodegradability of spider egg sac silk. *Journal of Materials Science: Materials in Medicine* **19**, 2963–2970.

33) K. Numata, R. Sato, K. Yazawa, T. Hikima, H. Masunaga (2015): Crystal structure and physical properties of *Antheraea yamamai* silk fibers: Long poly(alanine) sequences are partially in the crystalline region. *Polymer* **77**, 87–94.

34) H. Saito (2001): Blue biliprotein as an effective factor for cryptic colouration in *Rhodinia fugax* larvae. *Journal of Insect Physiology* **47**, 205–212.

35) H. Yamada, Y. Kato (2004): Green colouration of cocoons in *Antheraea yamamai* (Lepidoptera: Saturniidae): light-induced production of blue bilin in the larval haemolymph. *Journal of Insect Physiology* **50**, 393–401.

36) A. Yoshido, F. Marec, K. Sahara (2005): Resolution of sex chromosome constitution by genomic in situ hybridization and fluorescence in situ hybridization with (TTAGG)n telomeric probe in some species of Lepidoptera. *Chromosoma* **114**, 193–202.

37) T. Yamada, S. Samoto (1975): Variation of population components caused by the difference of parents in the backcross method. *The Hokuriku Crop Science Society* **10**, 5–9.

38) M. Kobayashi, M. Yonekawa, M.N. Yokoyama, M. Nakagaki, K. Tanaka (1992): Ultramorphological characterization of spermatogenesis of interspecific hybrid (Fl) of *Antheraea yamamai* and *Antheraea pernyi*. *Japanese Journal of Applied Entomology and Zoology* **36** (4), 231–237.

39) K. Komatsu (1984): Structure and properties of the silk filaments of the Japanese oak silkworm. *Technical Bulletin of Sericultural Experiment Station, Ministry of Agriculture, Forestry and Fisheries* **123**, 217–235.

40) Iwate Agricultural Research Center: Method for reeling yarn from cocoon of the wild silkmoth, *Antheraea yamamai* (Tennsann mayu no soushi houhou), www2.pref.iwate.jp/~hp2088/seika/h10/b28.pdf, *The National Agriculture and Food Research Organization*, (2017/7/17)

41) C. Takabayasi (1984): Structure of cocoon layers and characteristic of cocoon cooking in the Japanese oak silkworm. *Technical Bulletin of Sericultural Experiment Station, Ministry of Agriculture, Forestry and Fisheries* **123**, 159–184.

42) T. Gheysens, A. Collins, S. Raina, F. Vollrath, D.P. Knight (2011): Demineralization enables reeling of wild silkmoth cocoons. *Biomacromolecules* **12**, 2257–2266.

43) M. Boulet-Audet, F. Vollrath, C. Holland (2015): Identification and classification of silks using infrared spectroscopy. *Journal of Experimental Biology* **218**, 3138–3149.

44) Y. Kawahara, H. Tamura (1999): Degumming of cocoon shell of Japanese oak silkworm, *Antheraea yamamai*, using hot pressurized water. *The Journal of Silk Sericultural Science of Japan* **68** (5), 433–435.

45) J. Koh, S.-M. Kang, S.-J. Kim, M.-K. Cha, Y.-J. Kwon (2006): Effect of pineapple protease on the characteristics of protein fibers. *Fibers and Polymers* **7** (2), 180–185.

46) G. Freddi, R. Mossotti, R. Innocenti (2003): Degumming of silk fabric with several proteases. *Journal of Biotechnology* **106**, 101–112.

47) Y.R. Devi, L.R. Singh, S.K. Devi (2012): Indigenous knowledge of oak tasar silk cocoon cooking and a method of its improvement using pineapple extract. *Indian Journal of Traditional Knowledge* **11** (4), 724–726.

48) L.R. Singh, Y.R. Devi, S.K. Devi (2003): Enzymological characterization of pineapple extract for potential application in oak tasar (*Antheraea proylei* J.) silk cocoon cooking and reeling. *Electronic Journal of Biotechnology* **6** (3), 198–207.

49) S.V. Naik, T.H. Somashekar (2005): Studies on bivoltine cocoon reeling Part I: Effect of cocoon cooking condition and reeling speed on reeling performance of Indian bivoltine hybrid cocoons. *The Journal of Silk Sericultural Science of Japan*, **14**, 61–65.

Publications

H. Ishikawa, Z. Kajiura (2019): Reeling and fiber property of Japanese oak silkmoth cocoon treated with proteinase. *The Journal of Silk Science and Technology of Japan* **27**, 5-14.

H. Ishikawa, T. Hirano, C. Kiriyama, T. Okuno, M. Jounai, S. Takeuchi, Y. Sobue, M. Jitsukawa, K. Sakurai, Z. Kajiura (2019): Study of breeding large *Antheraea yamamai* for greater silk productivity by backcross. *The Journal of Silk Science and Technology of Japan* **27**, 15-22.

Conference presentations (oral presentations)

石川 浩章, 梶浦 善太(2016); タンパク質分解酵素による野蚕の繭影響を調査. 日本蚕糸学会中部支部第 72 回・東海支部第 68 回研究発表会

H. Ishikawa, Z. Kajiura (2018); Reeling and fiber property of Japanese oak silkmoth cocoon treated with proteinase. 8th International Conference on Wild Silkmoths

H. Ishikawa, Z. Kajiura (2018); Reeling and fiber property of Japanese oak silkmoth cocoon treated with proteinase. Textile Summit 2018

石川 浩章, 平野 智洋, 桐山 知久, 奥野 隆夫, 城内 光治, 竹内 傑, 祖父江 悠大, 實川 未果, 櫻井 聖子, 梶浦 善太 (2018); 戻し交雑による天蚕糸生産に優れた大型ヤママユの育種. 日本蚕糸学会中部支部第 74 回・東海支部第 70 回研究発表会研究

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