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Review Article

Silk gland fibroinase: Case study in *Bombyx mori* and *Samia cynthia ricini*

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Abstract - Fibroinase is a cathepsin L-like cysteine proteinase isolated from the silk glands of the silkworm, *Bombyx mori,* and the Eri silkworm, *Samia cynthia ricini,* and characterized by our research group. In *B. mori*, its physiological functions are disclosed as follows. (1) During each molt period in the larva, fibroinase is secreted into the lumen of the silk glands and digests the fibroin and sericin completely to make the lumen empty so that the silk gland cells can start synthesizing fibroin and sericin, and then secrete them into the lumen of the silk glands in the late molt period. (2) In day zero to day one pupa, fibroinase is secreted into the lumen of the silk glands and digests the remaining fibroin and sericin. (3) In the feeding period for each instar of the larva and in the spinning period for the last fifth instar larva, fibroinase functions as a lysosomal proteinase in lysosomes within the silk gland cells for the digestion of obsolete proteins and organelles, such as mitochondria, endoplasmic reticulum, and ribosomal proteins, transported into the lysosomes for regeneration of the highly efficient protein synthesis machinery. In *S. cynthia ricini*, the properties of fiboroinase are different in several points from those of *B. mori*, such as the N-terminal amino acid sequence, developmental profile, and maximum activity. Higher maximum activity, 38 times the maximum activity of *B. mori* per individual insect, is observed at the end of spinning. The silk glands of *S. cynthia ricini* degenerate just after the end of the spinning and the day zero pupa has no silk glands. Accordingly, the physiological functions of fibroinase in *S. cynthia ricini* are the fibroinase functions (1) and (3) in *B. mori*. The purpose of this review article is to encourage researchers of insect science to start studying fibroinase of silk glands in other insect species and to find out the species specificity for a comprehensive view of silk gland fibroinase of a diverse array of insect species. As a guide for the study, we will present the study path in which knowledge of fibroinase in *B. mori,* and *S. cynthia ricini,* was obtained with the logic utilized.

Keywords: Fibroinase, silk gland, *Bombyx mori*, *Samia cynthia ricini*

1. Introduction

This review article is written to encourage researchers of insect science to start studying silk gland fibroinase using a diverse array of insect species to spin cocoons. As a guide of the study, we will present the study path by which knowledge of silk gland fibroinase in the silkworm, *Bombyx mori,* and the Eri silkworm, *Samia cynthia ricini,* was elucidated with the logic utilized. Two review articles on silk gland fibroinase (Sumida, 2001; 2010) published previously will be useful for the researchers to know the early phase of the study. The studies of silk gland fibroinase in the various insect species will provide the knowledge of species specificity of silk gland fibroinase. The knowledge will provide clues that are indispensable for further understanding of the mechanism of evolution of silk proteins and silk gland fibroinase in insects.

2. Why is the question worthy of studying?

Sometime in the late 1980s, MS (Motoyuki Sumida) decided to study the question, do the silk glands of the domesticated silkworm, *B. mori,* make fibroin digestion enzyme or fibroinase? This question came from three main events.

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3. Three events

In 1987, MS recalls an experience in his career that formed the question worthy of studying. There were three events. The first event was a request in 1979 by Prof. Keizo Hayashiya, Kyoto Institute of Technology (KIT), Japan, in which he asked MS to study silk glands in *B. mori*. Prof. Hayashiya thought it important that at least one faculty of the Department of Sericultural Biology, KIT, should study silk glands. Silk glands are a pair of organs in the silkworm, *B. mori*, that synthesize large amounts of silk proteins, fibroin, and sericin in the fifth instar larva at the posterior and middle silk gland, and that secrete the proteins into the lumen where the proteins are stored until the spinning of a silk thread for cocoon production. The reason for the request was that KIT was an institute that had a good tradition for more than 100 years of serving the sericulture industry and science, and KIT needed to strengthen its potential in the study of silkworms. MS obtained a position in KIT in 1979 as an assistant professor and became a candidate to support Prof. Hayashiya's ideals for the future of KIT.

The second event was that MS found joy in studying protease with the support of Prof. Masaharu Eguchi, Applied Genetics Laboratory, KIT, in the study of digestive protease in the midgut of *B. mori*.

The third event was an experience of MS in the USA from 1985 to 1986. MS worked as a research associate in the Laboratory of Prof. John H. Law, University Department of Biochemistry, the University of Arizona. Tobacco hornworm, *Manduca sexta,* was an experimental animal for the molecular mechanism of lipid transport in insects (Prasad *et al*., 1986). *M. sexta* does not make a cocoon when it reaches maturity in late larval life. When the final fifth instar larva of *M. sexta* is fully grown, it digs a hole in the ground and in the hole it transforms into a pupa without making a cocoon. Knowing this, MS decided to study the cocoon proteins of *B. mori* after coming back to Japan.

Thus, there were three items that must be studied, namely the silk glands, protease, and cocoon proteins. For cocoon proteins, there were two proteins, fibroin and sericin. The question related to these items was 'do silk glands make fibroin digestion enzyme or fibroinase?'

4. Fibroinase in *B. mori*

4.1 Why was fibroinase studied first in *B. mori*?

There were two reasons: one reason was that the preparation method of fibroin solution (Katagata *et al*., 1984) and identification method of fibroin subunits by 10% SDS-PAGE (Oyama *et al*., 1984) had been developed in *B. mori* by the research group of Prof. Kensuke Shimura, Tohoku University, Japan. MS could construct an assay system for fibroinase by combining these methods with slight modifications. Later when the preparation method of sericin (Takasu *et al*., 2002) was developed in *B. mori*, the question of if purified fibroinase digests sericin was studied. The answer was yes (Watanabe *et al*., 2007).

Another reason was that cocoonase had been studied in the wild silkmoths, which digests sericin only (Kafatos and Williams, 1964; Kafatos *et al*., 1967a, b; Hruska *et al*., 1969; Berger *et al*., 1971; Felsted *et al*., 1973a, b; Hruska *et al*., 1973; Kramer *et al*., 1973; Law *et al*., 1977; Law, 2015).

4.2 When should silk glands be collected from the silkworm as the source of fibroinase?

MS selected day one pupa to collect silk glands (Sumida *et al*., 1993a). This was because when one dissects pupae from day zero onwards, every day one can find that the remaining silk glands in the pupa become more slender. This suggested that fibroinase is secreted into the lumen of the remaining silk glands in day zero pupa and digests the fibroin and possibly sericin in the lumen of the silk glands from day zero pupa to day one pupa. Therefore, the silk glands in day one pupa must contain fibroinase.

4.3 Assay of fibroinase using the fibroin solution as the natural substrate

Based on the experience of studying digestive protease in the midgut of *B. mori* in KIT, MS had decided that if fibroinase were studied, he would use a natural substrate, fibroin solution. Fortunately, the research group of Prof. Kensuke Shimura, Tohoku University, Japan had established a method of preparation of fibroin solution (Katagata *et al*., 1984). They also developed an identification method for fibroin subunits by 10% SDS-PAGE (Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis) (Oyama *et al*., 1984). The heavy chain (H-chain) and light chain (L-chain) fibroin subunits were clearly separated. If H-chain was digested by fibroinase, fragments of the fibroin polypeptide should be identified on the gel at the position below the band of the H-chain. MS borrowed these methods and, with slight modifications, constructed an assay system for fibroinase. First, make an enzymatic reaction at 30 ºC for 20 min after mixing the silk gland homogenate as the fibroinase source and the fibroin solution as the natural substrate. After stopping the reaction, detect the fibroin fragments produced from the fibroin solution by fibroinase with 10% SDS-PAGE. Unfortunately, things were not going as MS planned. MS had to spend a huge amount of time on trial and error until MS detected fibroinase activity by this system. We will discuss this in 5. Difficulties. For our information, silk fibroin is now known to be secreted from the posterior silk gland cells into the lumen, assembling a high molecular mass elementary unit consisting of H-chain, L-chain, and P25, with a 6:6:1 molar ratio (Inoue *et al*., 2000).

4.4 Fibroinase is likely a cysteine proteinase and it is different from a cocoonase

Once the activity of fibroinase became detectable (Sumida *et al*., 1993a), the next thing to do was clear. To determine what type of proteinase the fibroinase is. Currently, proteases are classified into seven types (Oda, 2012), including the classic four types of 1. Serine proteases, 2. Cysteine proteases, 3. Aspartate proteases, and 4. Metalloproteases. The optimum pH of fibroinase and an effective inhibitor against fibroinase were determined

(Sumida *et al*., 1993a). The pH optimum was an acidic pH, at pH 4.0. The most effective inhibitor was E-64, among other inhibitors. These facts suggested that fibroinase belongs to the cysteine proteinases (Turk *et al*., 2012). This also suggested that fibroinase is different from cocoonase, a famous protease that we learn about in biochemistry textbooks, that digests the silk protein sericin only. Cocoonase is synthesized by pupa in the galeae (Kafatos and Reich, 1968; Kafatos and Feder, 1968; Berger and Kafatos, 1971a, b; Kafatos and Kiortsis, 1971; Kafatos, 1972; Selman and Kafatos, 1975; Law *et al*., 1977), secreted outside, and stored on the surface of the galeae as pure protein crystals until adult emergence. At adult emergence, cocoonase is dissolved by the secretion of the labial glands (Kafatos, 1968; Hakim and Kafatos, 1974) of the adult moth, and the adult moth applies the cocoonase solution to the inside of the cocoon. Cocoonase solution digests sericin only, and it dissolves the outer part of the silk fiber forming cocoons. The adult moth pushes its head to the part where the cocoonase solution was applied and makes a hole, from which the adult moth emerges. Cocoonase is a serine proteinase and functions at a slightly alkaline pH. Based on our results and taking into consideration knowledge of the properties of cocoonase, to continue the study of fibroinase was meaningful. For our information, the reason for naming the enzyme from galeae, cocoonase, is found in Law *et al*. (1977). Cocoonase from *B. mori* was studied by Eguchi and Iwamoto (1975), Yamazaki *et al*. (1992; 1995), Tsuda *et al*. (2005), Yang *et al*. (2009), Rodbumrer *et al*. (2012), Fukumori *et al*. (2014), and Unajak *et al*. (2014). Cocoonase from the Chinese oak silkworm, *Antheraea pernyi,* was studied by Geng *et al*. (2014).

4.5 Physiological role of fibroinase in the early pupa

Based on the facts collected so far, fibroinase is secreted into the lumen of the degenerating silk glands in the early pupa and plays a physiological role in the digestion of fibroin and possibly sericin that remained in the lumen of the silk glands in the early pupa, which might enable the smooth apoptosis of the silk glands. Later, fibroinase was shown to digest sericin as well as fibroin (Watanabe *et al*., 2007). Thus, it is now established that the digestion of fibroin and sericin in the remaining silk glands in the early pupa is done by fibroinase. Then, the proteinaceous components of the silk gland cells are likely to be digested by other proteinases (Repnik *et al*., 2012).

4.6 The question, "can MS logically use the fluorescent peptide substrate of cathepsin L for assay of fibroi nase?" led to a paper by Prof. Dr. Akai

When fibroinase was found to be a likely cysteine proteinase, MS thought of using an artificial substrate of cysteine proteinase as an alternative substrate for the fibroinase assay, such as Z-Phe-Arg-MCA, a fluorescent peptide substrate routinely used for the assay of cathepsin L. An assay using a fibroin solution as the substrate and SDS-PAGE as the detection method might be an ideal system for the fibroinase assay, but it required much time. In the early assay system, it took two days for the detection of activity. Then the assay results were qualitative and not quantitative. The assay using Z-Phe-Arg-MCA gave quantitative results and enabled a quick assay. However, MS could not go instantly to an alternative substrate. MS had two concerns. One concern was that although fibroinase was likely a cysteine proteinase, it had never been identified as such. Fibroinase was not yet purified and the characteristics of the purified fibroinase were not determined. Under the situation, could MS adopt an alternative substrate? MS asked this question of himself. The answer was that it was not yet time to go to the alternative substrate. Another concern was that cysteine proteinase usually functions within cells, in lysosomes (Turk *et al*., 2012). The only exception is cathepsin L in osteoclastic cells that is secreted outside the cells and digests bones (Tagami *et al*., 1994). Fibroinase, on the other hand, must be secreted into the lumen of silk glands by day one pupa and digests the remaining fibroin and possibly sericin in the lumen of the silk glands. Accordingly, for fibroinase, secretion might be the key, considering its function in an analogy with cathepsin L in osteoclasitc cells. MS finally answered that if he could find some evidence that fibroinase is secreted outside of silk gland cells, he could use the alternative substrate. MS surveyed the literature on silk glands to see if there is a paper describing the secretion of fibroinase. A paper was found (Akai, 1965). It described the complete digestion of fibroin and sericin occurring in the lumen of silk glands at the middle of the fourth molt period of *B. mori*. Prior to this, vacuoles were enlarged within the silk gland cells and their contents seemed to be discharged into the lumen of the silk glands. MS thought that this was the description to indicate that fibroinase is secreted. For our information, the newest research on bone resorption indicates that cathepsin K is involved in the process (Rachner *et al*., 2011; Turk *et al*., 2012).

4.7 Fibroinase at the fourth molt period in the fourth instar larva became a new study subject

MS had never imagined that he would perform the sampling of silk glands at the fourth molt period in the fourth instar larva as the enzyme source. However, a paper (Akai, 1965) had clearly suggested that fibroinase occurs at this molt period. MS collected silk glands and performed an assay for fibroinase. High activity comparable to the high activity from day one pupa was obtained (Sumida *et al*., 1993b). There was no doubt that fibroinase is present in silk glands at the fourth molt period in the fourth instar larva.

4.8 Second physiological role of fibroinase at the fourth molt period in the fourth instar larva

The fact that fibroinase digests sericin in addition to fibroin was later demonstrated (Watanabe *et al*., 2007). Thus, the fibroinase of the silk glands from the fourth molt period in the fourth instar larva digests the fibroin and sericin in the lumen of the silk glands and makes the lumen of the silk glands empty. As a result, the silk gland cells can start synthesizing fibroin and sericin in the late fourth molt period and secrete them into the lumen of the silk glands as described by Akai (1965).

4.9 Spiracle index for identification of the developmental time of silk glands during the fourth molt period in the fourth instar larva

During the molt period, the silkworm does not eat and does not move. The overall appearance does not change except for the gradual emergence of the next head capsule under the cuticle at the dorsal side near the anterior thorax. How can we precisely identify the developmental time of the silk glands during the molt period? Here we can utilize a spiracle index (Kiguchi and Agui, 1981). We could determine the developmental time of the silk glands by using the spiracle index when the absolute fibroinase activity was determined in the silk glands in the larva-pupal development (Sutthikhum *et al*., 2004a).

4.10 Purification of fibroinase at the fourth molt period in the fourth instar larva

The purification of the fibroinase from the silk glands at the fourth molt period, stage D_2 in the spiracle index, was performed (Watanabe *et al*., 2004a). This confirmed that fibroinase is cysteine proteinase. Why were the silk glands at the fourth molt period chosen first? This was because the silk glands at the fourth molt period seemed to contain less of the other proteinases compared to the silk glands from day one pupa. The silk glands in day one pupa will soon to be degenerated completely by apoptosis, after one and a half days. MS worried about purified fibroinase that might be contaminated by other proteinases present in the silk glands from the day one pupa.

4.11 Both the fibroin solution and the fluorescent peptide substrate must be used for the assay of fibroinase in the purification of fibroinase

For the purification of fibroinase, the assay with the fluorescent peptide substrate, Z-Phe-Arg-MCA, (Rawlings and Barrett, 1994), was handy, especially for assays of fractions from column chromatography and for assays of pooled fractions at each step of purification. It gave quantitative results and a quick assay. At one time in the study of the purification of fibroinase, MS used Z-Phe-Arg-MCA only as a substrate for the assay. As a result, MS could purify an enzyme after several steps of purification procedure. To our surprise, the finally purified enzyme preparation could not digest the fibroin solution at all. It was apparent that the purified enzyme was not fibroinase. We will discuss this in 6. Difficulties, 6.10. Based on this surprising result, MS determined to use both substrates for the assay in the purification of fibroinase. As a result, fibroinase was purified from the silk glands at the forth molt period (Watanabe *et al*., 2004a). It was a two step purification method. First, the supernatant fraction was collected by centrifugation of the silk gland homogenate, followed by acid precipitation. The supernatant after acid precipitation was centrifuged to collect the resultant supernatant, which was dialyzed against the buffer. The dialysate was applied to a SP Sepharose FF column equilibrated with the buffer. The column was washed with the buffer only. Then 0.1 M NaCl in the buffer was applied and active fractions were collected and pooled. The pooled fraction was purified fibroinase. The purity was checked by SDS-PAGE. The purified fibroinase showed characteristics of cysteine proteinase, such as pH optimum and effective proteinase inhibitors (Watanabe *et al*., 2004a). Purified fibroinase hydrolyzed preferentially the peptide bond between Gly and Ala from the fibroin molecule (Watanabe *et al*., 2004a). This produced ladder bands of fragments of fibroin polypeptides on a 10% SDS-PAGE. Each ladder band consisted of fibroin polypeptide, for which the N-terminal amino acid was Ala and the carboxyl terminal amino acid was Gly. This is an interesting fact. This result is brought about by the characteristics of the fibroin protein that has numerous Gly-Ala sequences in it (Zhou *et al*., 2000) and the characteristics of fibroinase that preferentially splits the peptide bond between Gly and Ala (Watanabe *et al*., 2004a). Purified fibroinase hydrolyzed the oxidized β-insulin chain at the peptide bonds, which is characteristic of cathepsin L (Watanabe *et al*., 2004a).

4.12 Purification of fibroinase from day one pupa

A similar purification procedure was applicable to the purification of fibroinase from day one pupa. Fibroinase was successfully purified from silk glands (Watanabe *et al*., 2006a). The purity was checked by SDS-PAGE and no contamination by other proteins was observed. The pH-activity profile was slightly different from that of fibroinase at the fourth molt period. There was sensitivity to pepstatin in the fibroinase from day one pupa while there was no sensitivity in the fibroinase from the fourth molt period. These points will be discussed in 4.19. The other properties were similar to those of fibroinase at the fourth molt period.

4.13 Determination of absolute activity of fibroinase of silk glands using the fibroin solution as the natural substrate and the SDS-PAGE as the detection method in the development from the fourth instar larva through the fifth instar larva until day two pupa

The method of assay of the absolute activity of fibroinase using the fibroin solution as the natural substrate and SDS-PAGE as the detection method was established as described in 7. Innovations, 7.9 before VS (Vallaya Sutthikhum) joined our research group in 2000. We used this assay system to determine the absolute fibroinase activity in the developmental period from the fourth instar larva, through the fifth instar larva until day two pupa (Sutthikhum *et al*., 2004a). Two things became apparent. One thing was that two peaks are clearly observed in this developmental period. One peak is at the fourth molt period at stage D_2 in the spiracle index. The other peak is from day zero pupa. The absolute level of activity for each peak is almost the same. Another thing was that in the feeding period in the larva, the silk glands show fibroinase activity. Further, in the spinning period in the fifth instar larva, the silk glands show considerably higher activity. Thus, the physiological role of fibroinase in the feeding period and in the spinning period had to be considered, which is discussed in 4.15.

4.14 Purification of fibroinase in the spinning period of the last fifth instar larva

In 4.13, we observed clearly that there is fibroinase activity in the silk glands in the feeding period in the fifth instar larva and that considerably higher activity occurs in the spinning period in the fifth instar larva. We decided to purify fibroinase in the spinning period and characterize the purified fibroinase. A similar purification method was applicable as for the purification of fibroinase in the spinning period except that we used a longer column of SP Sepharose FF to remove contaminating proteins, for which the amount increased in this developmental stage (Sutthikhum *et al*., 2004a, b). We noticed that the purified fibroinase in the spinning period shows different properties from those of the other two fibroinases, from the fourth molt period and from day one pupa. The differences were apparent and we considered the reason for it. We will discuss this in 4.19 and 4.20.

4.15 Third physiological role of fibroinase in the feeding period in the larva and in the spinning period in the last fifth instar larva

Fibroinase activity is detectable within the silk gland cells in the feeding period and in the spinning period in the last fifth instar larva. Fibroinase in this developmental period is not secreted into the lumen of the silk glands but stays in the lysosomes within the silk gland cells. The physiological role of fibroinase in this developmental period is to function as a lysosomal enzyme within the lysosomes. Fibroinase digests obsolete proteins and organelles, such as mitochondria, endoplasmic reticulum, and ribosomal proteins, that were transported into the lysosomes for the regeneration of the protein synthesis machinery for the highly active biosynthesis of fibroin and sericin in silk glands.

4.16 cDNA cloning of fibroinase

cDNA cloning was carried out to investigate further what type of proteinase the fibroinase is (Watanabe *et al*., 2006d). Purified fibroinase was digested by trypsin and peptides of fibroinase were separated by reversed phase chromatography to collect the major peptide fractions. The amino acid sequence of the peptide fractions was determined. By database searching in the protein database, for the five peptides from the purified fibroinase, a similar sequence was found in the amino acid sequence of a *Bombyx* egg cysteine proteinase (Yamamoto *et al*., 1994). Based on the amino acid sequences, we determined and deduced the nucleotide sequences of the *Bombyx* egg cysteine proteinase, and PCR primers were designed, synthesized, and used for the RT-PCR of fibroinase. As a result, the full-length cDNA of fibroinase was obtained. cDNA cloning was carried out. The cDNA sequence was determined. The cDNA sequence of fibroinase (Watanabe *et al*., 2006d) was found to be similar to that of the *Bombyx* egg cysteine proteinase (Yamamoto *et al*., 1994). The amino acid sequence deduced from the nucleotide sequence indicated clearly that fibroinase is a cysteine proteinase (Watanabe *et al*., 2006d).

4.17 An identical gene to the fibroinase gene is expressed in the developing eggs and the gene product functions within the yolk cells in eggs of *B. mori* and consideration of the natural substrates of cysteine proteinase in insects

The results of 4.16 clearly indicate that an identical gene to the fibroinase gene is expressed in the developing eggs of *B. mori* to produce *Bombyx* egg cysteine proteinase (Yamamoto *et al*., 1994) within the yolk cells, for the digestion of the yolk proteins to provide yolk peptides to the developing embryo. In addition, in *B. mori*, fibroinase, or *Bombyx* cysteine proteinase of the silk gland (Watanabe *et al*., 2006d), is expressed in the silk gland cells in the larva and in the early pupa. In the feeding period and in the spinning period in the larva, fibroinase functions within the lysosomes in silk gland cells for the digestion of obsolete organelles, such as mitochondria, endoplasmic reticulum, and ribosomal proteins, that were transported into lysosomes. In the molt periods in the larva and in the early pupa, fibroinase is secreted into the lumen of the silk glands and functions for the digestion of fibroin and sericin. Thus, the natural substrates for cysteine proteinase in *B. mori* are the yolk proteins in the developing eggs, fibroin and sericin in the lumen of the silk glands, and obsolete mitochondria, endoplasmic reticulum, and ribosomal proteins within the lysosomes in silk gland cells. The natural substrate for the cathepsin L-like proteinases from the midgut of *Tenebrio molitor* is reported to be food proteins (Cristofoletti *et al*., 2005). These are the rare cases in which natural substrates for cysteine proteinase are identified, and all the cases are from insects (Turk *et al*., 2012).

4.18 Immunohistochemical localization of fibroinase in the silk glands

An polyclonal antibody against purified fibroinase was raised in guinea pigs and used for immunohistochemical identification of fibroinase in the silk glands by confocal laser scanning microscopy (Watanabe *et al*., 2004b). The fluorescence signal was found in the silk gland cells in the feeding period and in the spinning period. The fluorescence signal was also found in the lumen of the silk glands during the molt periods and early pupa. Accordingly, it was apparent that fibroinase is synthesized in the silk gland cells and secreted into the lumen at the molt periods and early pupa. For further study, it was found that electron microscopic studies are needed for identification of fibroinase in relation to the ultrastructure of silk gland cells. However, it was noteworthy that the immunohistochemical detection of fibroinase does not always mean that active fibroinase is found. The detection of a signal at stage D_2 in the spiracle index in the lumen of the anterior part of the middle silk gland (Watanabe *et al*., 2004b), for example, is defiantly active fiboinase when the data of absolute activity of fibroinase is referenced (Sutthikhum *et al*., 2004a). However, in some cases, fibroinase is likely to be inactivated but detectable by this method. Thus, it is important that the data (Watanabe *et al*., 2004b) should be compared with the data of the absolute activity of the fibroinase from the silk gland (Sutthikhum *et al*., 2004a). For example, the immunohistochemical method detected

the signal in the lumen of the anterior part of the middle silk gland in the early fifth instar larva (Watanabe *et al*., 2004b). Absolute activity data (Sutthikhum *et al*., 2004a) indicated that there was no activity by fibroinase in the middle silk gland at the late fourth molt period, which indicates that no fibroinase activity was present in the lumen of the silk gland before the silkworm larva reached the early fifth instar larva. Since fiborinase is secreted into the lumen of silk glands in *B. mori* only at the molt period, the fluorescence signal detected in the early fifth instar larva (Watanabe *et al*., 2004b) should indicate the remains of the fibroinase secreted into the lumen of the anterior part of the middle silk gland at the fourth molt period that would be inactivated.

4.19 Differences in the properties of fibroinase collected from the different developmental stages

We noticed that the pH-activity profile of fibroinase was slightly different between the fourth molt period (Watanabe *et al*., 2004a) and the day one pupa (Watanabe *et al*., 2006a). The optimum pH itself was not different appreciably. However, the former showed asymmetry in the pH-activity profile and had a shoulder at an acidic pH. The latter showed a typical symmetry in the profile and had the maximum at the center of the profile. The sensitivity to pepstatin was different. The former fibroinase was insensitive and the latter fibroinase, sensitive. The pH-stability was also different. These properties are summarized in Table 1.

Then we noticed that the properties of fibroinase in the spinning period (Sutthikhum *et al*., 2004b) were different from those of the fibroinase at the fourth molt period (Watanabe *et al*., 2004a). The notable difference was in the K*cat*/K*m* value. The value for the fourth molt period was 15.9 S^{-1} x mM^{-1} and in the spinning period it was 147.8 S^{-1} x mM^{-1}. These are also shown in Table 1.

4.20 Reason for the differences in the properties of fibroinase from the different developmental stages

Fibroinase is secreted into the lumen of silk glands at the fourth molt period (Watanabe *et al*., 2004b) and for day one pupa (Watanabe *et al*., 2004b). On the other hand, fibroinase in the spinning period stays within the lysosomes (Watanabe *et al*., 2004b). Thus, we reasoned that the difference in the properties of the fibroinase was brought about by post-translational modification of the fibroinase protein in the lumen of the silk glands.

4.21 Production of the recombinant fibroinase using *Escherichia coli* and its purification and characterization

If we can produce recombinant fibroinase, it might be that the fibroinase was just synthesized on the endoplasmic reticulum in the silk gland cells. We could compare the properties with those of the fibroinases purified from silk glands. We produced recombinant fibroinase in *E. coli* and the recombinant was purified and characterized. Purification methods used previously were not applicable, and a new purification method was established (Watanabe *et al*., 2006d). The properties of the purified recombinant fibroinase are shown in Table 1. When the properties were compared with those of the fibroinase in the spinning period, the K*cat*/K*m* value was lower in the recombinant. The properties of the pH–stability were better in the recombinant than in the fibroinase from the spinning period.

4.22 cDNA cloning and sequencing of fibroinase from the three developmental stages of *B. mori*

If we can find the presence of a single species of cDNA in the silk glands from the different developmental stages of *B. mori*, we could say that one species of fibroinase protein was produced in the silk glands at the different developmental stages. We could say further that the differences in the properties of the fibroinase are brought about by post-translational modification of the fibroinase protein. RT-PCR was carried out (1) at the fourth molt period, stage D_2, (2) at the spinning period, at day nine in the fifth instar larva, and (3) at day zero pupa. Identical cDNA was obtained from the three developmental stages (unpublished data). Based on the results, one species of fibroinase protein seemed to be expressed in the three developmental stages in *B. mori*. It is likely that the difference in the properties of fibroinase from the different developmental stages is due to post-translational modification of the fibroinase protein.

4.23 Can fibroinase digest sericin?

If purified fibroinase digests sericin, the presence of fibroinase alone in the lumen of silk glands is sufficient for the digestion of both fibroin and sericin at the fourth molt period and in the early pupa. The preparation method of sericin was developed by Takasu *et al*. (2002). We tested if purified fibroinase digests sericin using the sericin preparation by Takasu *et al*. (2002). Purified fibroinase digested sericin (Watanabe *et al*., 2007). The digestion products of sericin were not detectable by 10% SDS-PAGE. We separated the digestion products of sericin by chromatography and the major sericin peptides were collected. Sericin peptides were determined from their mass and amino acid sequence. The agreement of the amino acid sequences of the sericin peptides with the deduced amino acid sequences from the nucleotide sequence from the sericin genes was observed (Watanabe *et al*., 2007). These results clearly indicated that fibroinase digests sericin. Thus, it is conceivable that fibroinase digests both fibroin and sericin in the lumen of silk glands at the fourth molt period and in the early pupa.

4.24 Determination of the absolute activity of fibroinase using the fluorescent quenched peptide substrate in the larval-pupal development from day zero, the first instar larva through the second, third, fourth, and fifth instar larva until day one pupa

We could determine the absolute activity of fibroinase using the fibroin solution as the natural substrate and SDS-PAGE as the detection method in the development from the fourth instar larva through the fifth instar larva until day two pupa, as shown in 4.13. The result showed clearly that at the fourth molt period, stage D_2 in the spiracle index, a clear, sharp peak of fibroinase activity is present. Then in the earlier molt periods, i.e., at the first, second,

Table 1. Comparison of properties of purified fibroinase of the silkworm, *Bombyx mori* from the fourth molt period, stage $4D_2$, day one pupa, spinning period, and recombinant enzyme produced in *E. coli*.

Property	Fibroinase			
	Forth molt period, stage $4D_2$ [1)]	Day one pupa [2)]	Spinning period [3)]	Recombinant enzyme produced in *E. coli* [4)]
Molecular mass (SDS-PAGE)	34.9 kDa	34.2 kDa	32.5 kDa	33.0 kDa
pH optimum (Liquid fibroin)	pH 3.71	pH 3.71	pH 3.87-5.85	pH 4.0*
pH optimum (Z-Phe-Arg-MCA)	pH 4.8-5.0	pH 5.0-5.4	pH 5.0	pH 5.0
pH-activity profile	Not symmetrical	Almost symmetrical	Symmetrical	Symmetrical
Detectable activity at low pH	From pH 2.3	From pH 3.2	From pH 3.2	From pH 4.0
pH-stability remaining activity at 30ºC for 6 hr	54 % at pH 5.0 53 % at pH 4.0	20 % at pH 5.0 10 % at pH 4.0	21 % at pH 5.0 12 % at pH 4.0	60 % at pH 5.0 29 % at pH 4.0
K*cat*/K*m* (s^{-1} x mM^{-1}) (Z-Phe-Arg-MCA)	15.9	18.8	147.8	34.8
Effective proteinase inhibitors, 50% inhibition, from lower to higher conc.	1. Leupeptine 2. E-64 3. Z-Phe-Phe-CHN_2 4. Antipain	1. E-64 2. Leupeptine 3. Z-Phe-Phe-CHN_2 4. Antipain	1. E-64 2. Leupeptine 3. Chymostatin 4. Antipain	1. Leupeptine 2. E-64 3. Antipain 4. Chymostatin
Pepstatin	Insensitive	Sensitive	Insensitive	Sensitive
Localization in silk glands	In the lumen**	In the lumen**	In the cells only	—

[1)], Watanabe *et al.* (2004a)
[2)], Watanabe *et al.* (2006a)
[3)], Sutthikhum *et al.* (2004b)
[4)], Watanabe *et al.* (2006d)
*, At pH 4.0, high activity of digestion of liquid fibroin was observed.
**, Almost all activity is found in the lumen of silk glands and some activity in the cells.

and third molt periods, can a similar peak of activity be observed? This was an important question for considering the physiological role of fibroinase. However, the experiment to answer this question was not easy to do because to obtain sufficient activity on a 7% SDS-PAGE, an extremely large number of silk glands was needed. This was practically impossible. We selected a different approach. We designed and synthesized fluorescent quenched peptide substrates for the assay of fibroinase based on the substrate specificity of fibroinase towards fibroin. Since the fibroinase preferentially splits a peptide bond between the Gly and Ala of the fibroin molecule (Watanabe *et al*., 2004a), we designed and synthesized a peptide substrate, which included the Gly-Ala sequence in the peptide. The peptide substrate also contained a fluorescent quenching compound. As a result, two artificial substrates were designed. They worked as the substrates for the fibroinase assay. Using the fluorescnet quenched peptide substrate, we could determine the absolute fibroinase activity from day zero, the first instar larva through the second, third, fourth, and fifth instar larva until day one pupa (Watanabe *et al*., 2006b). A clear peak of activity was observed at the second, third, and fourth molt periods. At the first molt period in the first instar larva, a clear peak was not observed. We do not know the reason. In the feeding period, in the spinning period, and in the early pupa, the activity of fibroinase was detectable by this substrate.

4.25 Significance of using the natural substrate for assay for fibroinase

It was noteworthy that although the artificial substrates we designed and synthesized for the experiment in 4.24 worked for the purpose, a clear result, such as the one that we obtained with fibroin solution as the natural substrate and 7% SDS-PAGE as the detection method were used (Sutthikhum *et al*., 2004a), was not obtained with the fluorescent quenched peptide substrate. This may be because of the difference in the assay principle of each assay, although the splitting of a peptide bond between the Gly and Ala was common. In the assay using the fluorescent quenched peptide substrate, the mass of the substrate is considerably smaller than that of the H-chain of the fibroin of 350 kDa. Fibroinase is cysteine proteinase and endoproteinase, which might need considerable mass for the preferable substrate (Turk *et al*., 2012). When this condition is fulfilled, the 'correct' activity or full activity will be obtained. In a separate study, we used several artificial peptide substrates, including Z-Phe-Arg-MCA, for the assay of fibroinase during the development of *B. mori* (Watanabe *et al*., 2004c). Different results were obtained from those by Sutthikhum *et al*. (2004a), which is now believed to be the best profile we can obtain on the absolute activity of fibroinase from *B. mori*. Throughout our study of fibroinase of *B. mori*, the proper selection of the substrate for the assay was a key issue.

4.26 One more word on the assay of fibroinase

Fibroinase preferentially digests larger mass substrates, which was observed in the first detection of fibroinase activity on 10% SDS-PAGE (Sumida *et al*., 1993a). Based on this result, we evaluated the meaning of our assay of the absolute activity of fibroinase using fibroin solution as a natural substrate and 7% SDS-PAGE as the detection method, and the determination of the dilution factor of the fibroinase preparation to observe the phenomenon of the accumulation of a polypeptide of 100 kDa from the H-chain of fibroin until 120 min of incubation, followed by its digestion thereafter. By this assay, we observed the initial stage of the digestion of the H-chain of fibroin. That is, the intact H-chain of fibroin of 350 kDa is digested into several fragments of smaller molecular mass. Among the fragments, there is a fragment of the H-chain of fibroin of 100kDa, which is formed during the incubation and accumulated until 120 min of incubation. After 120 min of incubation, the fragment of the H-chain of 100 kDa started to be digested. Thereafter, fragments of the H-chain of fibroin became the next substrates for fibroinase. The fragments are digested to produce further fragments of polypeptides and peptides of smaller molecular masses. This process is repeated. Finally, fibroin peptides of four or eight amino acids, Ala-Gly-Tyr-Gly or Ala-Gly-Ala-Gly-Aal-Gly-Tyr-Gly, are produced (Watanabe *et al*., 2006b). This mode of digestion of fibroin was possible only because the H-chain of fibroin has numerous Gly-Ala sequences in it (Zhou *et al*., 2000) and fibroinase preferentially splits the peptide bond between Gly and Ala (Watanabe *et al*., 2004a).

Among the cascade in the digestion of the H-chain of fibroin, the step of the digestion of the intact H-chain of fibroin of 350 kDa into a fragment of the H-chain of 100 kDa is located at the upper most part of the cascade. This means that the accumulation and then digestion of the fragment of 100 kDa could be used as an index of absolute fibroinase activity. This index enabled the measurement of the initial rate of digestion of the intact H-chain of fibroin. Here was the reason why we could obtain fibroinase activity as close as possible to the correct activity (Sutthikhum *et al*., 2004a). Another condition for the ideal assay of proteinase activity was to use, as the substrate, a high molecular mass protein, which also fulfilled our assay system by using a fibroin solution as the natural substrate.

4.27 Our answer to the question by MS

To the question asked by MS, do silk glands of *B. mori* make fibroinase? We answer, yes. Silk glands of *B. mori* make fibroinase. We can say further that fibroinase is a cathepsin L-like cysteine proteinase and that it performs the following three physiological roles for silk glands: (1) digestion of fibroin and sericin in the lumen of silk glands at each molt period to make the lumen empty so that silk gland cells can start synthesizing fibroin and sericin at the late molt period and secrete them into the lumen of the silk glands. (2) Digestion of fibroin and sericin in the lumen of degenerating silk glands in the early pupa for smooth apoptosis of the silk glands by day three of the pupa. (3) Digestion of obsolete proteins and organelles, such as mitochondria, endoplasmic reticulum, and ribosomal proteins, that were transported into the lysosomes in the

silk gland cells in the feeding period and in the spinning period in the last fifth instar larva for regeneration of highly efficient, protein synthesis machinery for the biosynthesis of fibroin and sericin in the silk glands.

Our study provides the rationale to utilize fibroinase, or more broadly, cysteine proteinase in the digestion of fibroin and sericin for the preparation of products beneficial for human health (Sumida and Sutthikhum, 2015), because silkworms synthesize cysteine proteinase in the silk glands and utilize it for the digestion of fibroin and sericin in the lumenal contents of the silk glands. This is analogous to the situation in which cocoonase, or serine proteinase or trypsin, is utilized for the degumming of raw silk in the silk industry, because the silkworm synthesizes trypsin in the galeae and utilizes it for the digestion of sericin in the silk thread to escape from the cocoon.

5. Fibroinase in the wild silkmoth, Eri silkworm, *S. cynthia ricini*

5.1 Suggestion by Prof. Dr. Akai

When fibroinase in *B. mori* emerged as a clear figure, Prof. Dr. Akai suggested to MS to study fibroinase in the wild silkmoth, such as the Eri silkworm, *S. cynthia ricini*. Prof. Dr. Akai kindly requested Dr. Osamu Shimizu from the Gumma Prefectural Sericulture Experiment Station to provide Eri silkworm larvae to MS. MS collected castor leaves in the University Farm of KIT in Saga, Kyoto Japan in the early mornings and brought them by bus and subway to the laboratory, Faculty of Textile Science, KIT, Matsugasaki, Kyoto, Japan, and reared the Eri silkworm larvae.

5.2 Like *B. mori*, digestion of fibroin and sericin in the lumen of the silk glands of the Eri silkworm occurs at the fourth molt period in the fourth instar larva reported by Prof. Dr. Akai (1971)

At the fourth molt period of the Eri silkworm larvae, a similar phenomenon to that of *B. mori* was observed by Prof. Dr. Akai (1971), in that the complete digestion of fibroin and sericin occurs in the lumen of silk glands in *S. cynthia ricini*. The fibroinase of the Eri silkworm was supposed to be secreted into the lumen of the silk glands at the fourth molt period like in *B. mori*.

5.3 Preparation of the fibroin solution of Eri silkworm

Solid fibroin of the Eri silkworm was successfully prepared by the method used for *B. mori* (Katagata *et al*., 1984). However, the fibroin solution could not be prepared. The reason might be that we hastened too much. MS was told later in 2005 that the fibroin solution from Eri silkworms can be prepared after one day in 60% LiSCN instead of after 1 hr like in *B. mori* (I. Kobayashi, personal communication). At that time we had no choice. We decided to use the fibroin solution of *B. mori* for the assay of the fibroinase of the Eri silkworm. Another substrate used for the assay was Z-Phe-Arg-MCA.

5.4 Fibroin solution of *B. mori* was used as the substrate for assay of fibroinase of *S. cynthia ricini*

The preliminary experiment showed that the fibroin solution of *B. mori* could be used as a substrate for the assay of fibroinase from the Eri silkworm. We could use it for the assay in the purification of the fibroinase from the Eri silkworm (Watanabe *et al*., 2006c; Watanabe and Sumida, 2006) and for the assay of the absolute fibroinase activity of Eri silkworms during the development from the fourth instar larva through the fifth instar larva until the end of spinning in the fifth instar larva (Watanabe *et al*., 2006c), which was one day before day zero pupa. The result of the absolute fibroinase activity of the Eri silkworm that we obtained can be compared directly with that of *B. mori* because the same substrate and the same assay method were used in both insect species.

5.5 Absolute activity of fibroinase of Eri silkworm using the fibroin solution of *B. mori* as the substrate and the 7% SDS-PAGE as the detection method in the development from the fourth instar larva until the end of spinning in the fifth instar larva

In this study, we realized that the Eri silkworm is different from *B. mori* in the development of the silk glands, although they both make cocoons. In the Eri silkworm, the silk glands are found in the fifth instar larva until the end of spinning while being absent on the next day, at a day zero pupa. The silk glands of the Eri silkworm are completely degenerated by apoptosis in the day zero pupa. In *B. mori*, the silk glands are found in day zero pupa, in day one pupa, and in day two pupa. We cannot find silk glands in *B. mori* in day three pupa. The silk glands of *B. mori* are completely degenerated by apoptosis in day 2.5 pupa. Another noteworthy difference was the absolute activity of fibroinase. In the Eri silkworm, extremely high activity of fibroinase was observed towards the end of spinning. Approximately a 38-fold higher activity was observed at the end of spinning in the Eri silkworm (Watanabe *et al*., 2006c) compared to the maximum activity in *B. mori* at the fourth molt period, stage D_2 in the spircle index, or in a day zero pupa. This knowledge could be utilized for practical applications in sericulture in which the silk glands of the Eri silkworm at the end of spinning are used as a source of degumming enzyme. Freddi *et al*. (2003) showed the usefulness of cysteine proteinase for degumming and Prasad *et al*. (2012) proposed *Antheraea mylitta* cocoonase for cocoons cooking.

5.6 Significance to study the kinetics of digestion of the fibroin solution of Eri silkworm by fibroinase of Eri silkworm

It was fortunate that the fibroin solution of *B. mori* could be used for the assay of the absolute activity of fibroinase of Eri silkworms during the development. We obtained data for the Eri silkworm (Watanabe *et al*., 2006c) that was exactly comparable with that of *B. mori* (Sutthikhum *et al*., 2004a). On the other hand, it is well known that fibroin of the Eri silkworm contains many repetitive sequences of polyalanine (Craig and Riekel, 2002; Fedic *et al*., 2002; Sezutsu and Yukuhiro, 2014), which are not found in the sequence of the gene for the H-chain of fibroin from *B. mori* (Zhou *et al*., 2000). Thus, for studying the physiological

role of fibroinase in the Eri silkworm, a fibroin solution from the Eri silkworm should be used as the natural substrate. The immediate study subject is as follows. In the digestion of the fibroin of the Eri silkworm, how does the fibroinase of the Eri silkworm split the fibroin molecule of the Eri silkworm? Does it split at the middle of the poly-alanine sequence? Or is there another mode of digestion? This is an interesting and fundamental question related to the substrate specificity of fibroinase towards its natural substrate of fibroin.

5.7 Purification of fibroinase from Eri silkworm at the fourth molt period in the fourth instar larva

The purification method for the fibroinase of *B. mori* at the fourth molt period (Watanabe *et al*., 2004a) was applicable to the purification of the fibroinase of the Eri silkworm at the fourth molt period (Watanabe *et al*., 2006c).

5.8 Purification of fibroinase from Eri silkworm at the end of spinning in the last fifth instar larva

The purification method for fibroinase from *B. mori* at the fourth molt period (Sutthikhum *et al*., 2004b) was not applicable to the purification of the fibroinase of the Eri silkworm at the end of spinning. The fibroinase of the Eri silkworm at the end of spinning was bound to the column of SP Sepharose FF more firmly than the *B. mori* fibroinase. We increased the concentration of the buffer used for the chromatography to strengthen the ionic charge of the buffer and tried to weaken the interaction of the fibroinase with the SP Sepharose FF. Additional separation methods were further needed to remove the contaminating proteins. For this, experience from the purification of the recombinant fibroinase, as described in 4.21 Production of recombinant fibroinase using *E. coli* and its purification and characterization, was utilized to find the effective measures for the purification of fibroinase from Eri silkworms at the end of spinning (Watanabe and Sumida, 2006).

5.9 Characterization of fibroinase from the Eri silkworm

Some of the properties of the purified fibroinase of the Eri silkworm from the fourth molt period and at the end of the spinning are summarized in Table 2. Not so many differences were observed between the two fibroinases of the Eri silkworm unlike in *B. mori*. The K*cat*/K*m* value showed higher values in the Eri silkworm than *B. mori* and was similar between the fourth molt period and at the end of spinning in *S. cynthia ricini*. It should be noted that the fibroinase from the Eri silkworm at the fourth molt period is secreted into the lumen of the silk glands (Akai, 1971), as in *B. mori* (Akai, 1965), and functions in the lumen of the silk glands and digests the fibroin and sericin there. It was noteworthy that the pH-stability relationship was different between the Eri silkworm and *B. mori* at the fourth molt period. The remaining activity was 8% at pH 4.95 in the Eri silkworm but 54% at pH 5.0 in *B. mori*. The remaining activity was 0% at pH 3.95 in the Eri silkworm but 53% at pH 4.0 in *B. mori*. The reason for the difference is not clear. The fibroinase from the Eri silkworm at the end of spinning is secreted into the lumen of the silk gland in the manner that it is included in the lysosomes (Akai, 2005), which is different from *B. mori* (Akai, 2007). Thus, the purified fibroinase from the Eri silkworm at the end of spinning was actually purified from the lysosomes even if the fibroinase was in the lumen of the silk glands. Accordingly, the properties of the fibroinase from the Eri silkworm at the end of spinning may reflect the properties of the fibroinase in the lysosomes. In this sense, the properties of the fibroinase from the Eri silkworm at the end of spinning (Watanabe and Sumida, 2006) should be compared with that of the fibroinae of *B. mori* in the spinning period (Sutthikhum *et al*., 2004b). The K*cat*/K*m* value was similar between the two, both with a three digit value.

5.10 N-terminal amino acid sequence of fibroinase of the Eri silkworm

The N-terminal amino acid sequence of the fibroinase from the Eri silkworm at the fourth molt period was X-Pro-Asp-Gln-Val- (Watanabe *et al*., 2006c), and that at the end of spinning was Tyr-Pro-Asp-Gln-Val-Asp-X-Arg-Lys-Lys-Gly-X-Val- (Watanabe and Sumida, 2006). X was an unidentified amino acid. The underlined amino acids were the amino acids that were different from the amino acids at the corresponding position in the N-terminal amino acid sequence of the fibroinase from *B. mori* at the fourth molt period (Watanabe *et al*., 2004a), which was Leu-Pro-Glu-Gln-Val-Asp-Trp-Arg-Lys-His-Gly-Ala-. These results suggest that the gene for the fibroinase from the Eri silkworm is different from that of *B. mori*.

5.11 Silk filament of the Eri silkworm has numerous fine pores

Numerous fine pores were found in the silk filaments of *Antheraea yamamai* and *Antheraea pernyi* (Akai *et al*., 1989). Soon after, a similar phenomenon was observed in the insects of Saturniidae (Akai, 2005). In *B. mori*, no pores were found in the silk filament. The reason is explained as follows: in *B. mori*, the inner membrane in the posterior silk gland is well developed to form a barrier to the lumen and lysosomes in the cells of the posterior silk gland cannot be secreted (Akai, 2007), while in Saturniidae, the inner membrane is not well developed, which is considered to be evolutionarily more advanced, and the secretion of lysosomes into the lumen of the posterior silk gland is carried out (Akai, 2005). In Saturniidae, the lysosomes are secreted into the lumen of the posterior silk gland and then elongated in the anterior direction to form fine tubes in the lumen when more lysosomes and fibroin molecules are secreted into the lumen because the distal end of the posterior silk gland is closed. These fine tubes are maintained in the silk filament until the spinning of the filament and thereafter. As a result, we can observe numerous fine pores when we cut sections from the silk filament of Saturniidae (Akai, 2005).

Table 2. Comparison of properties of purified fibroinase of Eri silkworm, *Samia cynthia ricini* from the fourth molt period, stage $4D_2$, and from end of spinning.

Property	Fibroinase	
	Forth molt period, stage $4D_2$[1)]	End of spinning[2)]
Molecular mass (SDS-PAGE)	28.5 kDa	30.0 kDa
pH optimum (Liquid fibroin of *B. mori*)	pH 4.69 (Activity between pH's 3.51-6.47)	pH 5.62 (Activity between pH's 3.71-5.72)
pH optimum (Z-Phe-Arg-MCA)	pH 5.7	pH 5.7
pH-activity profile	Symmetrical	Almost symmetrical
Detectable activity at low pH	From pH 3.4	From pH 3.4
pH-stability remaining activity at 30 ºC for 6 hr	8 % at pH 4.95 7 % at pH 5.95 0 % at pH 3.95	7.6 % at pH 7.74 0 % at pH 4.95 0 % at pH 3.95
K*cat*/K*m* (s^{-1} x mM^{-1}) (Z-Phe-Arg-MCA)	134	183
Effective proteinase inhibitors, 50% inhibition, from lower to higher conc.	1. Leupeptine 2. Chymostatin 3. E-64 4. Antipain	1. Leupeptine 2. Chymostatin 3. Antipain 4. E-64
Pepstatin	Sensitive	Sensitive
Localization in silk glands	Almost all in the lumen	Almost all in lysosomes in the lumen

[1)], Watanabe *et al*. (2006c)
[2)], Watanabe and Sumida (2006)

6. Difficulties in study

We record the difficulties encountered in this study for reference for those who come after us.

6.1 Silk glands from day one pupa do not show fibroin ase activity

The SDS-PAGE gels after staining and destaining of the proteins did not show any single bands because the entire gel was deeply stained. It was not possible to identify a band for the intact H-chain of the fibroin nor the bands of the fragments of the H-chain of the fibroin produced as a result of the fibroinase action on the intact H-chain of fibroin. The reason for this was found to be that the proteins coexisting in the enzyme source are too abundant and interfered with the identification of both the H-chain of fibroin and the fragments of the H-chain of fibroin. The first enzyme source used was the homogenate of the silk glands. The resultant gel was densely stained and not usable for the assay. MS centrifuged the homogenate of the silk glands to obtain a supernatant fraction and a precipitate fraction. The supernatant fraction was used as the enzyme source. This time, the gel was densely stained and it was not usable for the assay. Then a precipitate fraction was used as the enzyme source. This time, again, the gel was densely stained and it was not usable for the assay. Still it was impossible to assay fibroinase activity. Looking at these results, however, MS firmly believed that fiboroinase occurred in the silk glands at day one pupa because there is the fact that the remaining silk glands become slender from day zero pupa to day one pupa. MS could not abandon the study of fibroinase.

6.2 The day when fibroinase activity was detectable

At last MS could detect a band of H-chain from the fibroin and the bands of the fragments of the H-chain of fibroin on 10% gel of SDS-PAGE on 23rd April, 1991. It was three years and 23 days since MS started the study on 1st April, 1988. As the enzyme source, an extract of the precipitate fraction with Triton X-100 was used. This was because MS thought that the extract should contain far less proteins than the precipitate fraction. The extraction method MS used was the one generally used to extract enzymes from lysosomes (Wattiaux and de Duve, 1956). Why did MS adopt Triton X-100 for the extraction? This was based on the experience of MS who had studied the isolation of the membrane-bound trehalase in the midgut of *B. mori* in

Nagoya University during his Ph.D. study, although MS could not finish the study because MS moved to KIT before the enzyme was isolated from the membrane. In retrospect, it was fortunate for the study of fibroinase that fibroinase was extracted with Triton X-100 from the precipitate fraction of a homogenate of silk gland cells. It suggested that fibroinase was localized in the lysosomes, and further that the fibroinase is a lysosomal proteinase. Indeed this was shown to be true in our later studies. For our information, does fibroinase digest the L-chain of the fibroin in addition to the digestion of the H-chain of fibroin? The answer is yes. The L-chain of the fibroin was digested after 20 min of incubation, and after 180 min of incubation, an appreciable amount of the L-chain of the fibroin was digested (Sumida *et al*., 1993a). We believe that fibroinase digests P25, another subunit of the fibroin molecule (Inoue *et al*., 2000).

6.3 Why was fibroinase not found in the supernatant fraction from day one pupa in the initial studies?

Based on the observation that the silk glands become more slender from day zero pupa to day one pupa, the fibroin and probably the sericin in the lumen of the silk glands were digested by fibroinase in the day one pupa. The fibroinase should have been secreted into the lumen of the silk glands and the fibroinase should be recovered in the supernatant fraction of the homogenate of the silk glands from the day one pupa. However, the fibroinase seemed not to be recovered in the supernatant fraction. This was a mystery for a long time. The mystery was suddenly solved when MS tried an acid precipitation step with the supernatant fraction from the silk gland homogenate, thinking of the purification of the fibroinase. This trial was based on MS's experience that the application of an acid precipitation step to the supernatant fraction of the tissue homogenate is a powerful and efficient step to remove coexisting proteins, which MS experienced in the study of the purification of soluble trehalase in the midgut of *B. mori* (Sumida and Yamashita, 1983). In the case of fibroinase, an acid precipitation step was applicable. A large amount of coexisting proteins were removed after the centrifugation and the resultant supernatant fraction was usable as an enzyme source. The resultant supernatant fraction showed high activity of fibroinase in the assay using the fibroin solution as the substrate and 10% SDS-PAGE as the detection method. Actually, fibroinase had been recovered in the supernatant fraction in the initial experiments in 1988, but the activity detection was not possible by the assay used at that time.

6.4 Early method of assay gave the qualitative results

An assay method for fibroinase was at any rate established that used the fibroin solution as the natural substrate and 10% SDS-PAGE as the detection method and the supernatant fraction after acid precipitation of the original supernatant fraction as the enzyme source. The method gave qualitative results. We could determine that there was activity or there was no activity, but we could not obtain quantitative results. We needed a quantitative result, especially to determine the developmental changes in the fibroinase activity in the silk glands. We could speculate that the activity was high or low, but had no means to express the results quantitatively.

6.5 Method of assay to give the quantitative results

We identified the urgent necessity of a quantitative assay method and decided to establish it, but we did not know how we could obtain it. The chance to establish the method was suddenly brought about by one of our students. We were studying, as a separate study subject, β-fructofuranosidase in the midgut of *B. mori* using a 7% SDS-PAGE, and MS's student accidentally used 7% SDS-PAGE for the assay of fibroinase. To our surprise, on the 7% gel, high molecular mass fragments of the H-chain of the fibroin were more clearly separated than on the 10% gel. We thought that the 7% gel could be used as a better assay for fibroinase. We started a study to establish a quantitative method for a fibroinase assay using a 7% SDS-PAGE. As a result, and as described in 7. Innovatons 7.8, we found out that a 100 kDa polypeptide was derived from the H-chain of the fibroin of 350 kDa, and it accumulates until 120 min of incubation and then it is digested thereafter under our standard assay conditions. Based on this result, we defined a unit of activity of fibroinase to make a measurement of the absolute activity of the fibroinase. The 7% gel could separate the larger mass fragments of the H-chain of the fibroin more than the 10% gel. However, it was not easy to change the assay system from a 10% gel to a 7% gel in the everyday experiment. MS's student pushed us to adopt a 7% gel instead of a 10% gel to establish the quantitative assay method for fibroinase.

6.6 Method of assay to be performed in less than two days

The assay in our first system required the following times for each step: for enzymatic reaction, 3 hr; for electrophoresis, 6 hr; for staining of the gel, 20 hr; and for destaining of the gel electrophoretically, 2 hr. To finally identify the fragments of the H-chain for the fibroin, in total 31 hr was required. This means almost two days. How to develop a method of assay to finish in a shorter time was the next subject.

6.7 Method for preparation of homogenate of silk gland cells from the silk glands containing large amount of fibroin and sericin

To study the fibroinase activity in the development of *B. mori*, homogenization of the silk glands was the first step. Then by centrifugation of the silk gland homogenate, the supernatant fraction was collected. Then, to this supernatant fraction, the acid precipitation treatment was applied. In the first step, to make the homogenate of the silk glands from the fourth molt period was easy and no problem. To make the homogenate of the silk glands from day one pupa was easy and no problem. To make the homogenate of silk glands from day one, day two, and day three of the fifth instar larva was easy and no problem. However, to make the homogenate of silk glands at days four, five, six, and seven gave problems. The silk glands in these developmental

period contained large amounts of fibroin and sericin. When these silk glands were homogenized using a Potter-Elvehjem glass homogenizer with a Teflon pestle in an ice cold buffer, the silk proteins clotted and adhered firmly to the glass tube, and further homogenization became impossible. We could not obtain a homogenate of the silk gland cells from the silk glands containing large amounts of fibroin and sericin. The solution was brought about again by MS's student and described in 7. Innovations 7.12.

6.8 Fluorescent peptide substrates usable for the assay of fibroinase

If fluorescent peptide substrates usable for the assay of fibroinase were found, a quick assay becomes possible. Quantitative results will also be given. It was found that Z-Phe-Arg-MCA was a substrate usable for the assay of fibroinase (Watanabe *et al*., 2004a).

6.9 Method of purification for fibroinase from the silk glands of *B. mori*

This was needed to determine the type of proteinase that fibroinase is. The path was opened first by the introduction of an acid precipitation step to the supernatant fraction of the homogenate of the silk glands. The resultant supernatant fraction was collected by centrifugation, and this was applied after dialysis against the buffer to the cationic ion exchange column of the SP Sepharose FF, and the column was washed with the buffer only. Finally, 0.1 M NaCl in the buffer was applied to the column and active fractions from the column were pooled. The pooled fraction was purified fibroinase. This purification method was usable at the fourth molt period and for day one pupa.

6.10 Pitfalls of using Z-Phe-Arg-MCA only as the substrate for assay of fibroinase

The assay with Z-Phe-Arg-MCA as the substrate is quick and it gives quantitative results. One time for the study of the purification of the fibroinase, MS used this substrate only for the assay. As a result, purified enzyme was obtained. However, this purified enzyme did not digest the fibroin solution at all. Thus, the purified enzyme was not fibroinase. From this incident, MS realized that for the purification of fibroinase, both substrates, the fibroin solution and Z-Phe-Arg-MCA, should be used. Later, when MS was preparing this article, MS found the brochure of the Peptide Research Laboratory Inc. in which the manufacturer of Z-Phe-Arg-MCA, presented an article on the precautions to take when using synthetic peptide substrater, such as Z-Phe-Arg-MCA, for an enzyme assay (Iwanaga, 1978). MS translated the article from Japanese into English.

'Synthetic peptide substrates are usually designed and synthesized in reference to amino acid sequence of split site of natural substrate by target proteinase. The product of synthetic peptide substrate is not always specific to target proteinase. Synthetic peptide substrates that are said specific to certain type of proteinases can be digested to some extent by other type of proteinases. Thus in order to assay target proteinase selectively in the presence of multiple proteinases, ample precautions should be made and special reaction conditions should be set up for target proteinase. This is essential as long as synthetic peptide substrate is used. For evaluation of assay with synthetic peptide substrate, recommendation is that both methods should be simultaneously performed in assay, method using natural substrate that has been done so far and method using synthetic peptide substrate.'

6.11 Why are the properties of the purified fibroinase different from the different developmental stages?

This fact was not expected to be observed before we characterize the purified fibroinase. We reasoned as follows, but further studies are needed to prove that the change in localization of the fibroinase from the lysosomes in the silk gland cells into the lumen of the silk glands at each molt period in the larva brings about a post-translational modification of the fibroinase protein in the lumen, which produces different properties in the fibroinase.

6.12 Purification method of the recombinant fibroinase

The method of purification of the fibroinase from the silk glands from the fourth molt period and from day one pupa could not be applied for the purification of the recombinant fibroinase produced in *E. coli*. We developed a new method (Watanabe *et al*., 2006d).

6.13 Purification method for fibroinase of the silk glands from Eri silkworm at the end of spinning

This was another case in which the method of purification of fibroinase from the silk glands from the fourth molt period and from day one pupa in *B. mori* could not be applied. We developed a new method (Watanabe and Sumida, 2006) utilizing our knowledge to develop a new purification method for recombinant fibroinase (Watanabe *et al*., 2006d).

7. Innovations

7.1 First enzyme source that could be successfully used for the assay by 10% SDS-PAGE was prepared by extraction with Triton X-100 of the precipitate fraction from silk gland homogenate

As described in 6. Difficulties 6.2, the first detection of the fibroinase activity was possible when using an extract of the precipitate fraction with Triton X-100 as the enzyme source, the fibroin solution as the natural substrate, and 10% SDS-PAGE as the detection method (Sumida *et al*., 1993a). The key to the success was the extraction of the fibroinase with Triton X-100 into the resultant supernatant fraction, and this enabled the removal of the proteins coexisting in the precipitate fraction and visualization of a band of the intact H-chain of the fibroin in the control lane and the gradual digestion of the intact H-chain of the fibroin in the test lanes. The concomitant appearance of the fragments of the H-chain of the fibroin was clearly observed with an increasing number of smaller mass molecules over the time course. Fibroinase activity was demonstrated for the first time.

7.2 Acid precipitation step to the supernatant fraction of silk gland homogenate opened ways for preparation of enzyme source for the fibroinase assay and for the preparation of starting material for the purification of fibroinase

As described in 6. Difficulties 6.3, the introduction of the acid precipitation step to the supernatant fraction of the silk gland homogenate resulted in the removal of a large amount of proteins that coexisisted in the supernatant fraction. This enabled the resultant supernatant fraction to be used as an enzyme source for the assay of the fibroinase by SDS-PAGE and as the starting material for the purification of fibroinase. The good chance of innovation came when the adoption of the acid precipitation step was tried to establish a step for the purification of fibroinase.

7.3 Choice of the mini gel system of 10% SDS-PAGE in place of the standard sized gel system

The standard sized gel system of 10% SDS-PAGE requires 6 hr for completion of electrophoresis. To decrease the time for the electrophoresis, a mini gel system was adopted. The time for the electrophoresis was shortened to 4 hr by the application of a constant voltage of 80V, which was recommended by the manufacturer of the electrophoresis chamber (ATTO Co. Ltd., Japan).

7.4 Electrophoresis of 10% SDS-PAGE at a constant voltage of 200 V in place of 80 V

To shorten the time for the electrophoresis, an increase in the voltage applied to the apparatus was tried. First the applied voltage increased to 100 V, then 120 V, 140 V, 150 V, 160 V, and finally 200 V. As a result, heat was generated by applying a higher voltage but the resultant curvature of the protein lanes was permissible. At an application of a constant voltage of 200 V, the adverse effects were not observed. We decided to adopt a constant voltage of 200 V. The time of electrophoresis was shortened to 1 hr.

7.5 Silver staining method in place of Coomassie brilliant blue staining method

The staining of the proteins with Coomassie brilliant blue required a total 22 hr; for the staining 20 hr and for destaining electrophoretically for 2 hr. We decided to adopt a silver staining method. This required 1 hr to finally visualize the proteins. The silver staining method was more sensitive than the Coomassie brilliant blue for the detection of proteins and it was possible to use smaller samples of fibroinase for the electrophoresis.

7.6 An assay system with 10% SDS-PAGE that can be performed within 2 hr excluding the incubation time for enzymatic reaction of 140 min by combination of 7.2, 7.3, 7.4 and 7.5

The mini gel system, application of a constant voltage of 200 V for 1 hr, and silver staining method of protein staining for 1 hr enabled the visualization of the proteins on the 10% SDS-PAGE gel after 2 hr from the start of the electrophoresis. For the assay for fibroinase, the enzymatic reaction required 140 min. Thus, now, within 4 hr and 20 min, an assay of fibroinase using a SDS-PAGE became possible.

7.7 The 7% SDS-PAGE revealed far clear profiles of fragments of H-chain of fibroin of higher molecular mass

One day, a student of MS performed accidentally a 7% SDS-PAGE in assay of fibroinase instead of a 10% SDS-PAGE and the gel was stained with silver staining. The profiles of the protein bands were far clear and had better separation, especially for the bands of higher molecular mass. The study of the use of a 7% SDS-PAGE for the fibroinase assay began in this way.

7.8 Discovery that a fibroin polypeptide of 100 kDa accumulates until 120 min of incubation when using the fibroin solution as the substrate and the 7% SDS-PAGE as the detection method

We started to test the usability of a 7% SDS-PAGE in place of the 10% SDS-PAGE for the assay of fibroinase. We confirmed that the 7% SDS-PAGE shows far clear bands for the fragments of the fibroin polypeptides in the upper part of the gel while the 10% gel shows accumulated bands at the same part of the gel. Using 7% SDS-PAGE, our research group observed a time course of the profiles of the fragments of the fibroin polypeptides that were produced by the fibroinase from the H-chain in the fibroin solution. We soon found that a fibroin polypeptide of 100 kDa from the H-chain accumulated until the incubation time of 120 min and then started to be digested thereafter. MS thought that this could be used as an index for a definition of the absolute activity of the fibroinase. One unit of fibroinase activity was defined as the amount of fibroinase to produce and accumulate a fibroin polypeptide of 100 kDa until 120 min of incubation that then began to be digested under the standard assay conditions. In the actual assay, the dilution factor of the fibroinase solution is determined and used for the calculation of the absolute fibroinase activity.

7.9 An assay system of the absolute activity of fibroinase using the fibroin solution as the substrate and the 7% SDS-PAGE as the detection method by combination of 7.6, 7.7 and 7.8

The system to assay the absolute activity of the fibroinase using the fibroin solution as the substrate and the 7% SDS-PAGE as the detection method was established.

7.10 Z-Phe-Arg-MCA as the alternative substrate for fibroinase assay

Z-Phe-Arg-MCA is a fluorescent peptide substrate for the assay of cathepsin L. Purified fibroinase hydrolyzed with this substrate was the most efficient among several fluorescent peptide substrates (Watanabe *et al*., 2004a). This substrate was handy to obtain quantitative results. However, depending on this substrate only is dangerous, especially in the purification of the enzyme, because it is likely to lead us to an enzyme that hydrolyzes this substrate only.

7.11 Establishment of a two step purification method of fibroinase from the silk glands from the fourth molt period in the fourth instar larva and from day one pupa

Our research group established a two step purification method for the fibroinase from the silk glands from the fourth molt period in the fourth instar larva and from day one pupa. The method consisted of the acid precipitation step with the supernatant fraction from the homogenate of the silk glands and then column chromatography of the SP Sepharose FF. The idea for the purification by MS was as follows. MS wanted to make it clear through the efforts to establish a purification method for fiboroinase that fibroinase is a single enzyme and does not consist of multiple species of isozymes. Actually, later it was found that fibroinase is a single enzyme. Anyhow, MS selected ion exchange chromatography to prove that fibroinase is a single enzyme. First, the fibroinase was recovered from the supernatant fraction after the acid precipitation and dialyzed against the buffer, and the condition determined that all the fibroinase is adsorbed on the column. After coexisting proteins are washed out from the column with the buffer only, a shallow gradient of NaCl is applied to the column to gradually elute the fibroinase from the column. If the fibroinase consisted of multiple species of isozymes, we could collect multiple peaks of fibroinase. We could know from the profile of the elution if the fibroinase is a single protein or not. MS used first DEAE-cellulose as the separation media and tried a neutral pH with a low ionic strength of the buffer. This was to make the fibroinase be adsorbed on the column. As a result, a part of the fibroinase was recovered in the flow-through fractions. The condition was found to be bad for all the fibroinase to be adsorbed on the column. Then MS tried CM-cellulose at a neutral pH and then at an acidic pH with low ionic strength of the buffer. The results were not good. Finally, MS tried SP Sepharose FF. This time, the condition was set at an acidic pH of 4.0 and at a considerably higher ionic strength for the buffer at 100 mM. This was for lowering the interaction between the fibroinase and the separation media for the easy elution of the fibroinase from the column after the fibroinase was adsorbed on the column. As a result, all the fibrionase was adsorbed on the column. The column was washed with the buffer only. Then the elution was tried first with 0.1 M NaCl in the buffer, followed by 0.2, 0.3, 0.4, and 1.0 M NaCl in the buffer. Most of the fibroinase was eluted from the column by the 0.1 M NaCl. The profile of the elution indicated that fibroinase is a single protein. The pooled fraction was purified fibroinase. The ion exchange chromatography gave two things. One is the answer to the question by MS as to whether fibroinase is a single enzyme. The other is a useful step for the purification of fibroinase. The two step purification method of fibroinase was handy and quick for the purification. This method contributed to the progress of the study of fibroinase (Watanabe *et al*., 2004a).

7.12 Homogenization method for preparation of homogenate of silk gland cells from the silk glands containing large amounts of fibroin and sericin

A student of MS established the method for the homogenization of the silk glands containing large amounts of fibroin and sericin. The method is as follows. First, store the silk glands in the freezer at -20 ºC for one day, then homogenize the frozen silk glands directly in a Potter-Elvehjem glass homogenizer with a Teflon pestle in ice cold buffer without thawing the frozen silk glands. This is all we must do. Then we can homogenize the silk glands that contained large amounts of fibroin and sericin. Silk proteins, fibroin and sericin, in the frozen silk glands aggregate after homogenization to form solid material. The solid silk material formed was removed with tweezers and cut with scissors and homogenized with a small amount of the buffer again to extract the silk gland tissues included with silk protein clot. The combined supernatants were used as the homogenate of the silk gland cells. This method enabled us to study fibroinase in the feeding period and in the spinning period in the fifth instar larva (Sutthikhum *et al*., 2004a,b). There was an episode for this method as well. A student of MS did experiments to establish this method when MS attended the XXI International Congress of Entomology in Iguasu Falls, Brazil, 20-26 August, 2000. When MS came back to Japan, the student told MS that he established the method. MS was very glad and asked the student to demonstrate the method. However, the student could not reproduce the method. MS firmly believed that once the method was established, then the method could be reproduced. MS encouraged the student to make the experiment again step by step with MS. Finally, two key points were found out. One was to freeze the silk glands at -20 ºC for at least one day and the other was to homogenize the frozen silk glands in the homogenizer without thawing the frozen silk glands.

For our reference, a similar method for the homogenization of silk glands to prepare a tissue homogenate of the silk gland cells has been reported by Takei *et al*. (1987). MS found this fact when writing this case study on 11th January, 2016. They described in the Materials and Methods, the Gel Electrophoresis of Fibroin as follows:

'To prepare tissue (or cellular) proteins from the posterior silk gland, the posterior silk gland was placed at -10 ºC for 12 h to denature fibroin secreted into the lumen, then homogenized in the SDS buffer. The homogenate was filtered through four layers of gauze to remove the coagulated fibroin.'

In our study, we collected, by dissection of the silkworm larvae, whole silk glands containing large amounts of fibroin and sericin, which included the posterior silk gland, middle silk gland, and anterior silk gland. However, both methods were very similar. We did not know of the description in the paper (Takei *et al*., 1987) when we were having difficulty in the preparation of the homogenate of the silk gland cells from the silk glands containing large amounts of fibroin and sericin. It is interesting to note that similar methods have been used in separate research groups about silk proteins.

7.13 Determination of the absolute fibroinase activity from the fourth instar larva through the fifth instar larva until day two pupa by combination of 7.2 and 7.9

This was a case in which we identified the potential of combining two innovations to develop a new method.

7.14 Purification method of fibroinase from the silk glands of spinning larva that contain large amounts of fibroin and sericin by combination of 7.11 and 7.12

This was another case in which we identified the potential of combining two innovations to develop a new method.

8. Further study subjects

In 2016, the study of silk gland fibroinase is assured to produce academically significant results. This was impossible to think of when we were studying silk gland fibroinase 15 years ago. We did not know exactly what direction we should go. Now it is different completely. As long as you use insects to spin cocoons as the study material, you can study silk gland fibroinase. We will show you several examples of further study subjects as follows. We believe that you will surely find new facts about each species of insects.

You are assured to produce important results by studying fibroinase, because you can make significant contributions to insect science. As a result, you will be able to find your own subjects in fibroinase study and you might make fibroinase study your life's work. Or you can simultaneously challenge your separate subjects while studying fibroinase. Even if you could not obtain fruitful results for some time in a separate subject, you need not worry about it because you can contribute to insect science by studying fibroinase. In the end, you might discover unexpected facts by challenging your subject. This is the merit for you and another reason why we recommend you to start studying fibroinase using various kinds of insect species.

8.1 Gene cloning of fibroinase of *B. mori* and sequencing of the gene

This has already been done by genome studies of *B. mori*. Gene information is available in KAIKObase Version 3.1.0 (KAIKObase, 2010). By keyword search, we obtain two results. One is a gene of cathepsin L-like proteinase (public ID, BMgn004622; local ID, Gene015360) and the other is a cathepsin L like protein (public ID, BMgn006893; local ID, Gene05798). The number of exons and introns are apparent.

8.2 Sequencing of 5' flanking region of the fibroinase gene of *B. mori*

The regulation of fibroinase activity in silk glands is highly likely to be under the control of ecdysone. The spiracle index of D_2 corresponds to the period just after the peak concentration of ecdysone was observed in the haemolymph of *B. mori* (Kiguchi and Agui, 1981) and also when the maximum activity of fibroinase was observed (Sutthikhum *et al*., 2004a). If the ecdysone response element (Cherbas *et al*., 1991; Shirai *et al*., 2012) is found in the 5' flanking region of the fibroinase gene, it indicates that its expression is under the control of ecdysone. There is a good paper (Chaitanya *et al*., 2011) that identifies the ecdysone response element in the 5' upstream region of the H-chain of the fibroin gene from the larval salivary glands of the stored grain pest, *Corcyra cephalonica,* and analyzed its functionality.

8.3 Secretion mechanism of fibroinase of *B. mori* at the molt period from lysosomes in the silk gland cells into the lumen of silk glands

The regulation of fibroinase secretion into the lumen of the silk gland at the fourth molt period in *B. mori* is likely under the control of ecdysone. A similar approach might be possible as in 8.2. Knowledge on the secretion mechanism of cathepsin L from the osteoclasts in the process of bone resorption, might be helpful (Tagami *et al*., 1994). The ultrastructural study of the posterior silk gland of *B. mori* was reported (Tashiro *et al*., 1968; Matsuura *et al*., 1968; Morimoto *et al*., 1968), and they will also be good starting points for further studies.

8.4 Re-absorption mechanism of digestion products of fibroin and sericin from the lumen of the silk glands in *B. mori* at the molt period

No study has been done for this subject. New facts will be found that are significant from the cellular and molecular aspects of the development of the silk glands.

8.5 Polyploidy of the silk glands

There is a well-known fact that the nuclei of silk gland cells consist of polytene chromosomes (D'Amato, 1989). Further study will elucidate interesting mechanisms that might be related to fibroinase gene expression.

From the following subjects, you will obtain merit by using the specific insect species for fibroinase study.

8.6 cDNA cloning of fibroinase of the Eri silkworm, *S. cynthia ricini,* and sequencing of the cDNA

We can study this subject in the same way as we did in *B. mori*. The N-terminal amino acid sequence of *S. cynthia ricini* is known to be slightly different from that of *B. mori* (Watanabe *et al*., 2006c, Watanabe and Sumida, 2006). The purification method of the fibroinase from the Eri silkworm has been established (Watanabe *et al*., 2006c; Watanabe and Sumida, 2006). It is likely that the cDNA is slightly different from that of *B. mori*.

Researchers in Thailand, Vietnam, Cambodia, the People's Republic of China, Assam, India, and Japan can enjoy studying *S. cynthia ricini*.

8.7 Gene cloning of fibroinase in the Eri silkworm, *S. cynthia ricini,* and sequencing of the gene

Researchers in Thailand, Vietnam, Cambodia, the People's Republic of China, Assam, India, and Japan can enjoy studying *S. cynthia ricini*.

8.8 Research subjects as follows in *Antheraea mylitta*

1. Preparation of the fibroin solution from each insect species.

2. Method of assay for fibroinase using the fibroin solution as the substrate and 7% SDS-PAGE as the

detection method.

3. Method of assay for fibroinase using Z-Phe-Arg-MCA as the substrate.

4. Observation of the development of silk glands.

5. Profile of activity of fibroinase during the development of insect.

6. Purification and characterization of fibroinase.

7. cDNA cloning and sequencing.

8. Gene cloning and sequencing.

9. Histological and cellular study of silk glands related to fibroinase.

Researchers in India can enjoy studying the Tussar silkworm, *A. mylitta*.

8.9 Similar research subjects in *Antheraea assama* as in 8.8

Researchers in India, especially in Assam, can enjoy studying the Muga silkworm, *A. assama*.

8.10 Similar research subjects in *Attacus atlas* as in 8.8

Researchers in Thailand, in ASEAN countries, and in India can enjoy studying *A. atlas*.

8.11 Similar research subjects in *Antheraea yamamai* as in 8.8

Researchers in Japan can enjoy studying *A. yamamai*.

8.12 Similar research subjects in *Antheraea pernyi* as in 8.8

Researchers in the People's Republic of China can enjoy studying the Chinese oak silkworm, *A. pernyi*.

8.13 Similar research subjects in *Cricula trifenestrata* as in 8.8

Researchers in Thailand, Cambodia, Vietnam, and particularly Indonesia, Assam, India, and other areas in India can enjoy studying *C. trifenestrata*.

8.14 Similar research subjects in the tropical, polyvoltine strains of *B. mori* as in 8.8

We are not sure if we can find new facts by this study, but the study will surely elucidate the physiological characteristics of the strains of *B. mori* from the aspect of fibroinase. Researchers in Thailand, Laos, Cambodia, Vietnam, Myanmar, Malaysia, Indonesia, Assam, India, and other areas in India can enjoy studying the fibroinase of tropical, polyvoltine strains of *B. mori*.

8.15 Similar research subjects in other insect species that spin cocoons as in 8.8

If you can find insect species that spin cocoons, you have an ample chance to study the silk gland fibroinase using that species.

9. Things to be remembered

9.1 Naming of enzyme

At first, MS called the enzyme a long name, fibroin hydrolyzing enzyme. Looking at the poster presentation on fibroin hydrolyzing enzyme in 1989 on the occasion of the International Conference of Invertebrate Reproduction held in Nagoya, Japan, 23rd-28th July, Prof. John H. Law, an invited speaker and boss of MS in the USA from 1985 to 1986, proposed the name fibroinase. MS accepted it. MS is thankful to him. He is all the more thankful because Prof. John H. Law was the main researcher in the study of cocoonase with Dr. Fotis Kafatos in Harvard University from the 1960s to 70s. Later in our study, it became apparent that fibroinase digests sericin (Watanabe *et al*., 2007). In addition, fibroinase was found out to be a cysteine proteinase and similar in nucleotide sequence to the *Bombyx* egg cysteine proteinase (Yamamoto *et al*., 1994). Thus, we renamed fibroinase as *Bombyx* cysteine proteinase of the silk gland, abbreviated as BCPSG (Watanabe *et al*., 2006e). In this case study, we called the enzyme fibroinase and it is BCPSG.

9.2 Significance of study of different insect species

According to the suggestion by Prof. Dr. Akai, when MS started studying fibroinase of the Eri silkworm, MS supposed that he would find similar facts in the Eri silkworm as in *B. mori*. MS was totally wrong. Actually, considerably different facts were obtained from the Eri silkworm. These facts gave the next questions. We believe that different insect species have slightly different fibroinases. This is the reason why we think the study of the silk gland fibroinase in different insect species is important. The obtained facts might be discussed related to the phenomenon of the co-evolution of the enzyme and its substrate in the long history of insect development and differentiation.

9.3 Two studies in 1941 and 1942 on degenerating silk glands in the pupa in *B. mori*

Akao (1941) studied the accumulated silk proteins in the remaining silk glands in the pupa of *B. mori* that were produced by the enforced flat spinning of the matured larva. He observed haemolymph components after the apoptosis of the silk glands in the pupa. Ono (1942) studied the pupa of *B. mori* after enforced flat spinning of the matured larva. He observed physiological changes in the pupa and changes in the silk proteins in the remaining silk glands. Our studies shed light on the observed phenomena by Akao (1941) and Ono (1942) from the silk gland fibroinase. The silk gland fibroinase must have functioned in the digestion of the fibroin and sericin remaining in the lumen of the silk glands in the pupa produced by the enforced flat spinning of the matured larva.

9.4 Two early papers on the fibroinase published in comparative biochemistry and physiology

MS sent two manuscripts on fibroinase in 1992 by registered air mail to the office of Pergamon Press in the UK for submission to Comparative Biochemistry and Physiology. To MS's surprise, the reply letters by the Editor-in-chief, Prof. Kerkut, came unexpectedly fast, within one month of MS sending the manuscripts to the UK. Usually MS received the reply letter after three months. The letters informed him that the manuscripts were

accepted. MS supposed that Prof. Kerkut recognized the significance of the information about the silk gland fibroinase in the manuscripts. They were published in 1993 (Sumida *et al*. 1993a, b).

9.5 Open access journal

All the papers on silk gland fibroinase were published in two journals: the Journal of Insect Biotechnology and Sericology and the International Journal of Wild Silkmoth & Silk, except for the early two papers that were published in Comparative Biochemistry and Physiology. This was because Prof. Koichi Suzuki, Iwate University, Japan asked MS to submit good manuscripts to the first Journal to improve the Journal. Unfortunately, the two Journals do not have good circulation around the world. However, the first Journal is now open access on the internet to anyone who wants to read the contents, at the website, J-Stage, a platform for open access journals published in Japan. This is handy for research.

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