Road to Development of New Materials from "Silk" by Elucidation of Mysteries in "Spinning Mechanism" and "Unique Structure" of Silk

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Composition by Hiroyoshi Otsuki

Silk fibers possess both beauty and high strength. Such fibers are skillfully spun by silkworms and are characterized by a structure unique to silk. Silk fiber is stronger than steel of the same diameter. In what state is silk when it is inside the silkworm? What mechanism produces silk fibers in small silkworms? How are unique structural characteristics of silk and silk fibers useful for humans?

Dr. Tetsuo Asakura is a professor at the Department of Biotechnology of Tokyo University of Agriculture and Technology. He is working on the development of "New Materials from "Silk" and addresses the above questions:

As you know, silk is queen of fibers. There are much efforts to develop new synthetic silk fibers continuously. Such synthetic fibers are only mimicking the appearance of silk fibers, such as non-uniformly shaped fibers with small diameters. Much remains unknown regarding the mechanism of skillful spinning of silk proteins by silkworms and the detailed molecular structures of silk fibers. Silk threads have been used for a long time for surgical sutures because they have many excellent properties and functions for use in humans.

In recent years, remarkable advance of analytical techniques for determination of molecular structures has been attained. In particular, there are remarkable advance in nuclear magnetic resonance (NMR). The mysteries in the spinning mechanism and unique structures of silk both before and after spinning will be clarified using this advanced NMR spectroscopy.

Professor Tetsuo Asakura, Department of Biotechnology, Tokyo University of Agriculture and Technology

Dr. Asakura was born in Kanagawa Prefecture in 1949. In 1972, he graduated from the Department of Chemistry at Tokyo University of Science. In 1977, he completed his Ph.D. degree from the Department of Chemical Engineering at Tokyo Institute of Technology. In 1980, he began working as an assistant professor at the Department of Dental Materials of Nihon University School of Dentistry at Matsudo. He began working as an associate professor in 1981 and as a professor in 1993 at the Faculty of Technology of Tokyo University of Agriculture and Technology. In 1990, he was an invited professor of the Department of Chemistry at Florida State University. In April 2012, he became the president of the Nuclear Magnetic Resonance Society of Japan.

Mystery of Silk I Structure

Silk proteins produced by silkworms consist of two proteins: silk fibroin and silk sericin. Silk fibroin molecules produced in the posterior silk gland move to middle silk gland and store in there. Silk sericin is produced at the middle silk gland and coats the fibroin stored in the gland. Then the silk proteins move through the anterior silk gland and exit the spinnerette as silk fibers. Thus, a silk fiber has a unique structure in which silk fibroin is coated by silk

sericin.

There is a long history about the structural study of silk fibroin because of its potentiality as mentioned above. In 1941, Dr. Masanori Shimizu of the Sericultural Experiment Station proposed two kinds of the crystalline forms of silk fibroin: pre-spun silk (silk I) and post-spun silk (silk II). In 1955, the Nobel Prize winner Linus Pauling and his research team proposed the basic structure of silk II (post-spun).

Dr. Asakura explained:

The silk fibroin consists of roughly a peptide structure with a repeated alternating sequence of glycine and alanine. The former is the simplest amino acid and the latter is the secondary simplest amino acid. Inter-molecular hydrogen bonds are formed among adjacent molecules. Pauling et al. reported that these bonds bind peptide molecular chains together to form a stable and strong planar structure which are stacked on top of each other. They called such protein structure as anti-parallel "beta (β) sheet." A beta sheet structure of silk fibers is formed after a silkworm expels silk from the silk gland through the orifice of the spinnerette. Therefore, it is clear that silk fibroin stored in silkworm is not in such a beta-sheet form.

Then silk proteins in silkworm form what type of structure?

Dr. Asakura answered:

How do silkworms have a mechanism to produce the fiber with comparable strength of steel with the same diameter from silk proteins dissolved in water? This is a very important and interesting problem. Therefore many research groups had long competed to elucidate the specific structure of silk I but they were not successful.

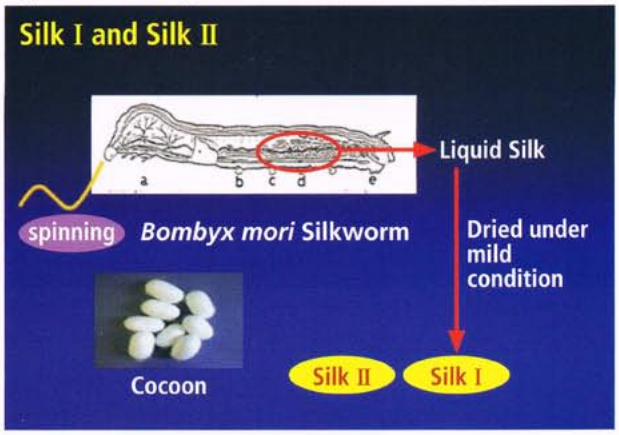
Why was it so difficult to elucidate the structure of silk I present in the silkworm?

Dr. Asakura explained:

It took me 20 years since I began my research to elucidate the silk I structure. For example, suppose one tried to obtain the precise structure of silk I using well-developed structural analyses such as x-ray and electron diffraction methods 20 years ago. To obtain high-quality analytical data, one had to stretch or heat the pre-spun silk samples so that the orientation of silk I molecules was as uniform as possible.

However, this crystallization procedure causes the silk I structure to easily change to the silk II structure. In other words, if some type of external force is applied, silk fibroin proteins with silk I form easily change to a state of silk fiber (silk II). Later, it is noticed that this remarkable change in the structure

Silk fibroin protein is produced in the silk glands in the silkworm. The protein is in a water-dissolved state (silk I structure) in the silkworm. The external forces such as spinning, pressure at press part and dehydration by silkworm cause the silk fibroin molecules to be organized and change into silk fibers (silk II structure).



under weak external force is a favorable characteristic of silk protein when we use this protein as regenerated biomaterials. However, it makes difficult to elucidate the silk I structure. We had to develop an analytical method to determine solid state structure of silk I without any application of external force.

Determination of Silk I Structure attained by Solid-state NMR

Dr. Asakura focused on "solid-state NMR (nuclear magnetic resonance)" as a tool to analyze silk I structure. An NMR spectroscopy uses the phenomenon in which certain nuclei resonate with electromagnetic waves when they are placed in a strong magnetic field. Then NMR is used to elucidate the structure of the substance and its movements. Today NMR spectroscopy has become the most important tool in the determination of structures of unknown substances in solution. However, the substance needs to be in solution for solution NMR study. Thus, it cannot be used to study the solid state structure.

In addition, if the unknown substance is in a single crystal, x-ray diffraction analysis is undoubtedly the most powerful technique for the determination of the structure. However, the silk fibroin with silk Iform is not in a single crystal and therefore this technique is also not suitable. Therefore, the only usable technique was solid-state NMR spectroscopy.

Dr. Asakura described:

Twenty years ago, expensive solid-state NMR could not be installed at our institutiondue to budgetary constraints. Fortunately, a research topic of our laboratory, the "elucidation of silk protein struc-

ture and properties," was selected for a 5-year large-scale project of the Bio-oriented Technology Research Advancement Institution, an extra-governmental organization of the Ministry of Agriculture, Forestry and Fisheries in Japan. Thus, we were able to obtain research funds enough to purchase a solid-state NMR machine, enabling a comprehensive structural study of silk I. Thereafter we have been actively engaged in collaborative researches with many NMR researchers including overseas researchers.

When Dr. Asakura started his research work on silk at Tokyo University of Agriculture and Technology as an NMR expert more than 30 years ago, he had a dream to directly observe silkworms producing silk via solution NMR. Thus his first NMR experiment at this university is study of silk synthesis and the solution structure in live silkworm. Just before spinning of the 5th larval stage of silkworm,a live silkworm was placed as is in an NMR tube (liquid), and the process of silk synthesis and the solution structure was studied directly in a live silkworm. The silk I structure in the solid state appear when the silk fibroin stored in the silkgland was extracted and dried under mild conditions. Therefore the solution structure of silk fibroin stored in the middle silkgland is important, but it was difficult to determine the detailed structure in at the atomic level.

The silk I study progressed remarkably with the purchase of a solid-state NMR machine, a more powerful structural analysis tool. As a result of using this tool and working to develop the analytical method, Dr. Asakura was able to succeed in the "elucidation of silk I structure." He stated that the most distinctive characteristic of the pre-spun silk I structure was that it was formed by alternating "intramolecular hydrogen bonds" and "intermolecular hydrogen bonds."

As mentioned previously, the basic structure of silk proteins is a peptide chain complex formed by linking simple amino acid molecules. The structure is stabilized by the numerous hydrogen bonds. A characteristic of silk I structure is that there are two patterns of hydrogen bonds.

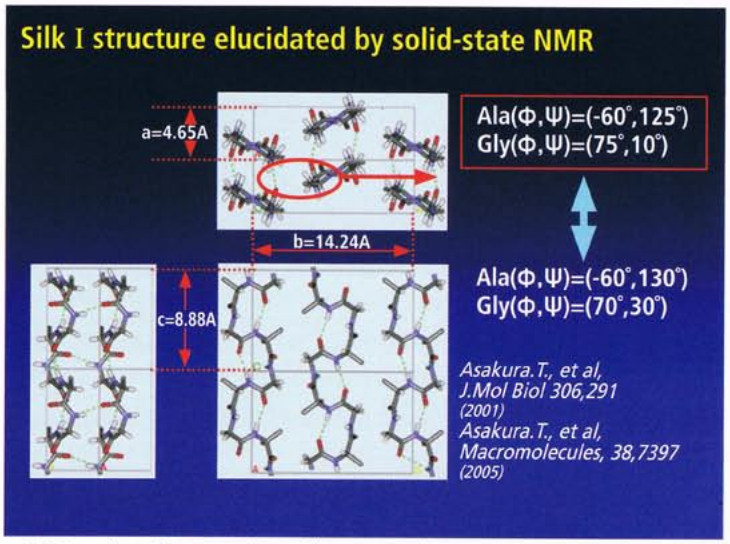
Dr. Asakura stated:

In silk I structure, we found that there is an alternating pattern of hydrogen bonds formed within a molecule (intramolecular hydrogen bonds) and hydrogen bonds formed between adjacent molecules (intermolecular hydrogen bonds). When the silkworm spins silk, between the two types of hydrogen bonds, mainly intramolecular hydrogen bonds are broken. At the same time, the broken bonds form new bonds, intermolecular hydrogen bonds, with adjacent molecules. Thus, a network of intermolecular bonds forms instantaneously, which strengthen the silk fibroin fiber.

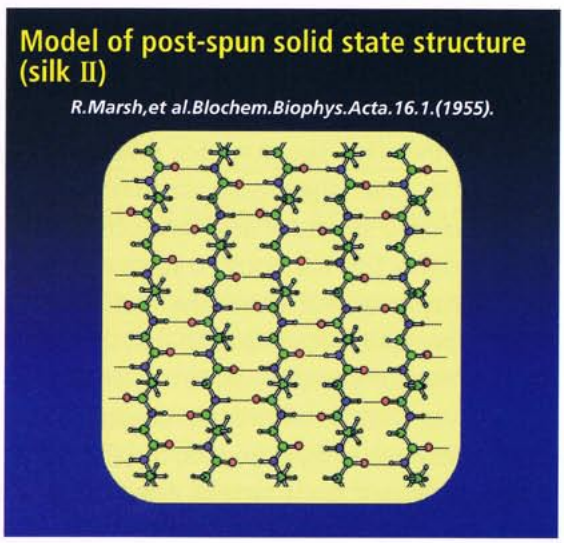
Structural Transition from Silk I to Silk II

Dr. Asakura continued:

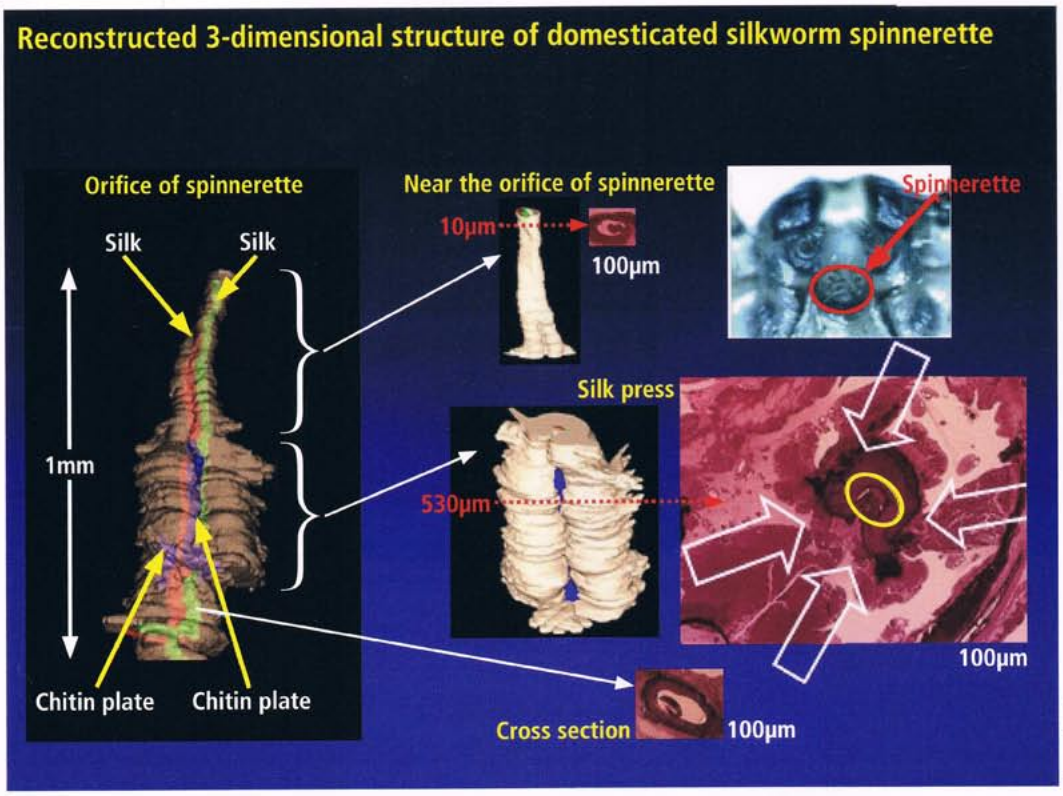
To study the detailed mechanism of silk spinning, namely structural transition from silk I to silk II, we used an optical microscope to observe the cross sections of 1000 slices of the spinnerette, cut approximately 1 mm length from the orifice. The data were used to reconstruct the spinnerette on the comput-



Silk I consists of many amino acid sequences forming repeating and alternating "intramolecular hydrogen bonds" and "intramolecular hydrogen bonds." These bonds enable silk I to be stable in aqueous solution.



"Intramolecular hydrogen bonds" are broken by the force of spinning silk and "intermolecular bonds" are formed, resulting in bonding between silk molecular chains and strong silk fibers.



A force called a "shear force" is applied to liquid silk inside the spinnerette. "Spinning" is added and the structure is transformed.

er. In its center, there is an area called the "silk press" and we were able to elucidate its detailed structure. Spinning of silk involves several complex factors, including external forces applied by the unique structure of "silk press," movement of the silkworm head like the number "8" which applied to silk fiber by stretching force, and dehydration of water surrounding pre-spun silk accompanying spinning.

The silk chains with silk I structure change into silk II structure when these external forces are applied. The mechanism of the structural transition, namely fiber formation mechanism was finally elucidated using molecular dynamics calculations on the computer.

It was also found that silk II structure is not a uniform beta sheet structure which has been proposed by Pauling et al. Thus we must revise the silk II structure proposed by Pauling et al. This is reasonable because Pauling et al. proposed very simple model from their limited X ray diffraction data more than half century ago. Recent advance in the spectroscopic methods will change previously proposed structure after applying the recent methods to clarify more confidential structure. This is the advance in science and such a study is very important to develop new materials from silk according to Dr. Asakura.

Dr. Asakura explained:

In silk II structure, 70% of fibroin proteins are in a stretched anti-parallel beta sheet structure, but mixture of two different intermolecular structures although the conformations are the same. On the other hand, a "distorted beta turn structure (remains of pre-spun silk I structure)" accounts for 30% overall. Namely, we found that the anti-parallel beta sheet structure itself is composed of two types of intermolecular arrangements. That is, it can be said that the key to characteristics of fibroin structure affecting the silk fiber properties is the non-uniform state of mixed beta sheet and beta turn structures.

The use of such an NMR method enabled the examination of the silk production process in the silkworm. It helped to elucidate the spinning mechanism and the pre-spun and spun structures of silk proteins. Dr. Asakura stated that the next step is the application to fields such as regenerated biomaterials.

Dr. Asakura explained:

Silk fibers are dissolved in a solvent to produce a solution. Subsequently, various methods can be used to solidify silk and many useful materials can be made. For example, if the aqueous silk solution is made into a thin transparent film, it becomes a highly transparent membrane that is extremely permeable to oxygen and water vapor. Such a membrane can be a scaffold for regeneration of corneal epithelium of the eye. A method called "electrospinning" can be used where a high voltage is applied to the tip of a nozzle to allow a silk solution to be expelled.