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Optimal Condition of Natural Silk 3D Matrix Production by Silkworm Spinning

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Abstract

Silk is appealing materials for many biomedical applications involving tissue engineering and implantable devices, because of its biocompatibility, environmental stability, controlled proteolytic biodegradability and morphologic flexibility. Silk matrix is required for the treatment of a wide wound area, but the present silk matrix is made by the second processing, and thus, the labor and the cost are high. In this work, we investigated the optimal production condition of natural silk 3D matrix using the silkworms and invented Automatic Silk Matrix Making Machine (ASMMM) for natural silk 3D matrix production. As a result, we determined that optimal production condition for making A4 paper size natural silk 3D matrix was used Rough aquarelle paper on surface at 25 ºC and 30 silkworm larvae. These results are expected to provide basic data for the efficient production of the natural silk 3D matrix, and it is suggested that the produced natural silk 3D matrix is useful as a medical biomaterials.

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Introduction

A silkworm cocoon from silkworm *Bombyx mori* is a natural polymer complex. The silkworm uses a substantial proportion of their mass and energy gained in the larva stage for the production of silk for constructing cocoons. The most important function of cocoon in the wild is to protect it from the environment, pathogens and predators. *Bombyx mori* cocoon consists of two main components called fibroin and sericin (Thurber *et al*., 2015). Other minor components include small proteins, lipids, and carbohydrates (Gauthier *et al*., 2004). It is made of silk fibers consisting of two fibroin brins and conglutinated by sericin binder. Fibroin is a natural fibrous protein with a semicrystalline structure, which provides stiffness and strength (Zhou *et al*., 2000). Sericin is an amorphous protein polymer, which acts as an adhesive binder to maintain the structural integrity of the fibers and the cocoon (Hakimi *et al*., 2006).

Silk has been widely used as a high performance and luxurious textile material due to its excellent mechanical and physical properties. Silk is usually produced within specialized glands after biosynthesis in epithelial cells, followed by secretion into the lumen of these glands where the proteins are stored prior to spinning into fibers (Kaplan *et al*., 1998). In recent years, the mechanical properties of silk have made this protein polymer highly attractive for developing progressive applications, which mainly fall within the scope of technical textiles and devices for biomedical uses (Altman *et al*., 2003). Silk has numerous advantages such as excellent blood compatibility (Sakabe *et al*.,

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1989; Um *et al*., 2002), minimal inflammability (Santin *et al*., 1999), excellent biodegradation (Ari *et al*., 2004), antioxidant activity (Bae *et al*., 2016) and excellent cytocompatibility (Minoura *et al*., 1995; Jo *et al*., 2016).

During decades of use, silk is proven to be effective in many medical applications. Silk is now being rediscovered and reconsidered as a potentially useful biomaterial for a range of applications in clinical repairs and *in vitro* as scaffolds for tissue engineering (Minoura *et al*., 1995; Cho *et al*., 2013; Yun and Lee., 2013). Silk matrix is required for the treatment of a wide wound area, but the present silk matrix is made by the second processing, and thus, the labor and the cost are high. Therefore, many researchers have studied for decades aiming to produce natural silk fiber as medical materials (Mandal *et al*., 2010).

The purpose of this study is to produce a natural silk 3D matrix by silkworm spinning on the surface, and to investigate the optimal conditions for its production. The production condition factor was designed temperature, silkworm count and surface material.

Materials and methods

Machine for silk matrix production

We invented the Automatic Silk Matrix Making Machine (ASMMM) for silk matrix production (Fig. 1). This machine can automatically regulate the tilting time interval of surface frame, the range of angle of the surface frame and the tilting speed of surface frame. Using this machine, it is feasible to produce silk matrices of various sizes. In this study, the angle of the surface frame was set to 20° and the tilting time interval was set to 1 hour. The time required to make the natural silk 3D matrix was about 96 hours.

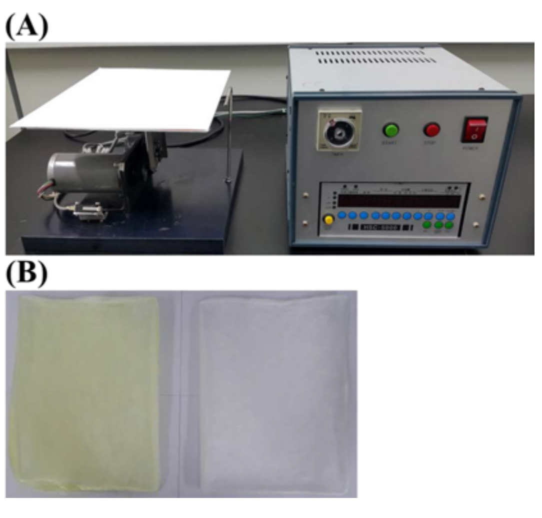

Fig. 1. Invented machine and natural silk 3D matrix (A) Automatic Silk Matrix Making Machine (B) A4 paper size natural silk 3D matrix was produced by ASMMM.

Silkworm larvae

Silkworm larvae (*Bombyx mori*) were reared by feeding mulberry leaves at the National Institute of Agricultural Science. The silkworm varieties used for the experiment was Baekokjam. Only the mature silkworms were separated from those that mounted up in the cocooning frame. After that, only those that released the urine were selected again and placed on the surface frame of ASMMM.

Environmental condition during silkworm spinning

The materials of the surface frame were used three types of aquarelle paper, which are classified as Rough, Cold pressed and Hot pressed aquarelle paper depending on surface roughness. Size of aquarelle paper was equal A4 paper (297 x 210 mm). In this study, 15, 20, 25 and 30 larvae were used to determine the number of silkworm larvae suitable for natural silk 3D matrix production of A4 paper size, and environmental conditions were 25ºC and 65% humidity in the dark room. The experimental temperature condition was ranged from 15, 20, 25, 30, and 35ºC s at 65% humidity and 30 silkworm larvae were used.

Measurement of produced natural silk 3D matrix

The produced A4 paper size natural silk 3D matrix was immediately cut into nine fragments in the room temparature. Each fragments size is approximately 7 x 10 cm. The weight of each fragment was measured by an electronic scale, and a total of nine fragments were averaged. The thickness of each fragment was measured with a distance of approximately 1.5-2 cm from two sides, and a total of four points were measured using the Digimatic Caliper (Mitutoyo Coperation, Japan). Therefore, average value was obtained by measuring the thickness value of 36 points from nine fragments. The standard deviation was calculated using the measured weight and thickness values.

Results and discussion

Determination of density condition for natural silk 3D matrix production

In order to make various sizes of natural silk 3D matrix and to save labor and production costs, it was essential to develop machines. Mature silkworms have a behavior of ascending to a higher place for construct cocoon. Using this behavior, we considered to construct an environment artificially with a certain slope. Slope condition is to produce a uniform natural silk 3D matrix while causing the silk to move up and down. It is necessary to use a device that tilts the surface frame at an appropriate angle and the surface frame after a certain period of time. Therefore, we invented machine was possible automatically regulate the tilting time interval of surface frame, the range of angle of the surface frame and the tilting speed of surface frame. Machine named Automatic Silk Matrix Making Machine (ASMMM). Because ASMMM automates all controls, it is thought to be very useful in saving labor and production costs for manufacturing natural silk matrices (Fig. 1).

The density condition of silkworm larvae is a necessary study for the production of the most suitable natural silk 3D matrix, which means that it is directly related to high productivity. The density condition of silkworms suitable for the A4 size (297 x 210 mm) natural silk 3D matrix was investigated. We used 15-30 larvae and when density of 30 more over larvae was over half-filled in the surface frame, the silkworms were difficult move up and down. Also, there were many difficulties in fabricating natural silk 3D matrix for reasons such as falling on the floor or spinning on silkworms hitched with each other. As a result of the experiment, the weight of the natural silk 3D matrix was increased as the number of silkworms increased. (Fig. 2A). Thickness was also investigated to be the thickest when 30 larvae were used (Fig. 2B). In addition, the thickness was uniform when 30 larvae were used, which confirms that the density condition is the most suitable for producing A4 size natural silk 3D matrix. Previous reports showed that 1 larva/900 cm^2 and 1 larva/30 cm^2 were used for natural silk 3D matrix production (Garay *et al*., 2014; Jung *et al*., 1999; Nakajima and Shikata., 1983). However, we investigated the best results with a high density of 1 larva/20 cm^2. Based on these results, we performed the following experiments. Especially, the thickness of natural silk 3D matrix from 15 larvae was similar to that from 20 larvae.

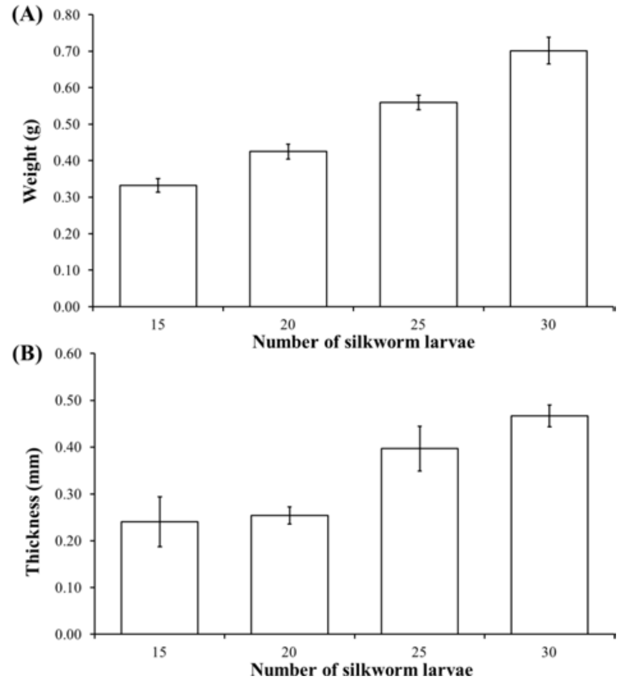

Fig. 2. Comparison of natural silk 3D matrix characteristics by number of silkworm larvae (A) Weight of natural silk 3D matrix fragments (B) Thickness of natural silk 3D matrix fragments. The temperature was set to 25 ºC and the humidity was set to 65 %. Nine fragments of A4 sized natural silk 3D matrix were measured each and averaged. Each bar corresponds to the average of nine fragments; each error bar indicates the standard deviation.

We did not know exact cause, but we estimate that natural silk 3D matrix layer would have formed more tightly when produced from 20 larvae. Recently, the analysis of fiber and structural characteristics of cocoon shows that several layers constitute it (Zhao *et al*., 2007). Once certain structural characteristics of natural silk 3D matrix have been identified, we suggest that they can be used for a variety of biomaterial depending on the production methods.

Optimal condition of environment and surface frame

We investigated the optimal temperature for natural silk 3D matrix production. Considering the other natural silk 3D matrix weight, it was more advantageous at 20, 25 and 30 than 15 and 35ºC (Fig. 3A). Thickness was also demonstrated same result (Fig. 3B). However, these results showed that the standard deviation value was the lowest at 25ºC. A lower standard deviation value means that uniform natural silk 3D

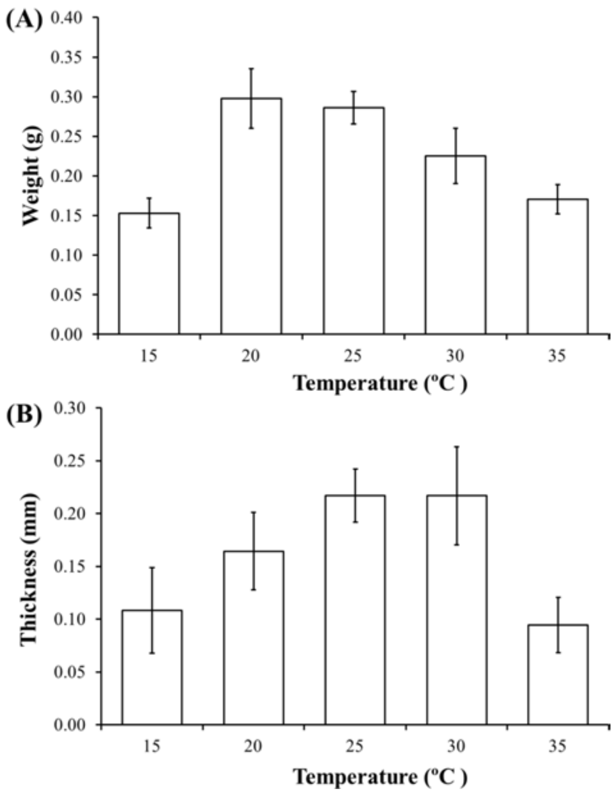

Fig. 3. Environmental effects on natural silk 3D matrix production (A) Weight of natural silk 3D matrix fragments (B) Thickness of natural silk 3D matrix fragments. The humidity was set at 65 % and 30 silkworm larvae were used. Nine fragments of A4 sized natural silk 3D matrix were measured each and averaged. Each bar corresponds to the average of nine fragments; each error bar indicates the standard deviation.

matrix was produced. In additionally, 20 and 30ºC results were contrasted weight and thickness. The reason for this is that the silk was distributed irregularly, resulting in non-uniform natural silk 3D matrix. At 20ºC condition, outside part of natural silk 3D matrix was weighed heavily and the thickness of the inside part of natural silk 3D matrix was thin. The results of the experiment at 30ºC condition were contrast. The effect of temperature on spinning behavior of silkworm was studied (Ramachandra *et al*., 2001; Manisankar *et al*., 2008). Temperature conditions contribute towards the properties of the cocoon as well as through direct effect on silkworm's behavior (Tazima., 1978). Amongst the environmental factors, temperature is a major one. At higher temperatures the rate of spinning is faster than at lower ones (Ramachandra *et al*., 2001); and at very low temperatures silkworms fail altogether to spinning (Sehnal and Akai., 1990). It was also showed that the physical properties of the cocoons change by the influence of temperature (Offord *et al*., 2016). These reports show the same results in our experiment. Therefore, we determined that the optimum temperature for manufacture the natural silk 3D matrix was 25 ºC.

Natural silk matrices were manufactured by varying the types of surface frames as conditions for silkworm spinning. First, we used acrylic panel, rubber plate, styrofoam plate and three type aquarelle papers. However, acrylic panel, rubber plate and styrofoam plate showed low absorption and a poor contact surface for the silk fiber to be spun. Also, it was observed that the larvae escaped around the edges and fell in the floor. Therefore, these surface frame materials were not distributed in this paper. When aquarelle paper was used, it was found to be the best in all conditions such as adsorption, cleanliness and separation from surface frame. Thickness and weight of the produced natural silk 3D matrix using the three type aquarelle papers were similar, but the standard deviation value of Rough paper was found to be the lowest

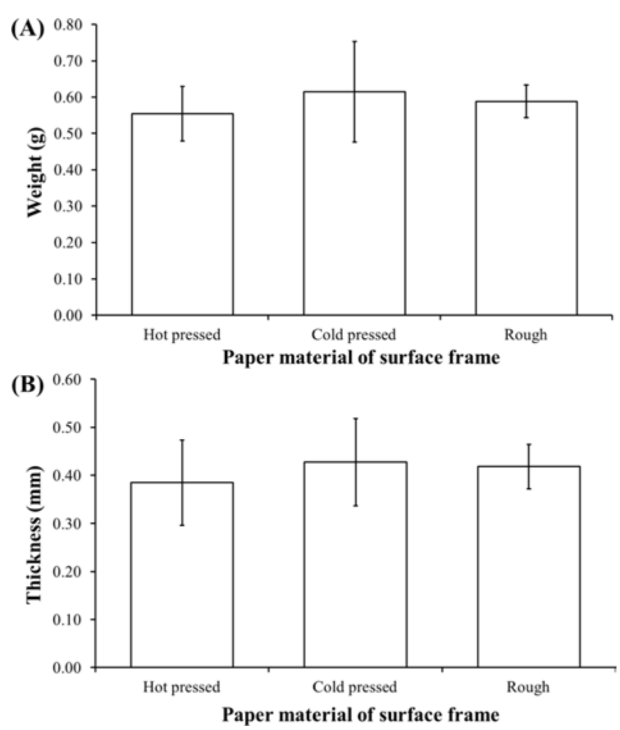

Fig. 4. Effect of surface frame on natural silk 3D matrix production (A) Weight of natural silk 3D matrix fragments (B) Thickness of natural silk 3D matrix fragments. The environmental conditions were set at 65 % humidity and 25 ºC, and 30 silkworm larvae were used. Nine fragments of A4 sized natural silk 3D matrix were measured each and averaged. Each bar corresponds to the average of nine fragments; each error bar indicates the standard deviation.

(Fig 4). Aquarelle paper is the squeezed cotton that is thicker than conventional paper, and has a high water absorption capacity. Depending on the roughness, it is divided into Hot pressed, Cold pressed and Rough paper, and the Rough paper is the roughest. When mould made aquarelle paper is dried it is either pressed using a press or a roller is rolled on it. Depending on the texture of this press or roller the paper gets its texture. The paper with maximum surface roughness or texture is called 'Rough'. This is generally rolled or pressed between a felt cloth from where the texture of the paper is derived. The paper with smooth surface finish is called 'Hot Pressed'. Paper is pressed between smooth plates and sometimes this plate is hot. Hence the name Hot pressed. And the one having roughness somewhere in between these two is called 'Cold Pressed'. This is pressed using a mechanical press with finer grains or rolled with a roller covered with felt cloth of finer texture than what is used to dry Rough paper. We estimated that the Rough paper with the highest roughness will be related to the adsorption and spinning behavior of the silkworm. More research on this is expected to be added in the future. Garay *et al* reported that natural silk 3D matrix was manufactured using glass, steel, zinc and burlap bag, and it was best when burlap bag was used.

In conclusion, we were able to manufacture very uniform, thick, and wide natural silk 3D matrixes. We have developed a new machine for making natural silk 3D matrix and suggested using Rough aquarelle paper at 25ºC using 30 silkworms a condition for making A4 size paper natural silk 3D matrix. The produced natural silk matrices with natural spinning process without any chemical processing could be used in the biomaterial engineering application. In the future, we will study the physical and chemical properties and biocompatibility of the natural silk 3D matrix for biomaterials application.

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