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Preparation of Water-Insoluble Films Based on Silk Fibroin and Polysaccharide

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Abstract: Silk fiber consists of fibroin (72-81%), sericin (19-28%) and other oils and waxes. Bombyx mori fibers consist of at least 16 amino acid residues, Silk has high molecular weight (200-350 kDa or more), large repetitive modular hydrophobic domains interrupted by small hydrophilic groups. Bombyx mori silk fibroin consists of heavy (H) and light (L) chains (H – and L – fibroin, respectively) connected by disulfide bonds. Ajizawa reagent (calcium chloride:ethanol:water (1:8:2) molar ratio solution) was used to dissolve the isolated fibroin in our further studies . Fibroin was dissolved in ajizawa solution (fibroin: ajizawa solution in a ratio of 1:20) at a temperature of 55 o C for 1 hour under constant stirring. Fibrous materials derived from polysaccharides and proteins affect the body's natural environment and thereby provide optimal conditions for tissue growth and regeneration. We obtained fibroin-polysaccharide based composites in our study as well. For this, fibroin and dextran, purified according to the above methods (dialdehyde dextran) composite material in the form of a film was obtained.

Keywords: Bombyx mori, fibroin, silk, amino acid, structure, cocoon, sodium carbonate, films, fibroin-polysaccharide, dextran, dialdehyde dextran.

Bombyx mori called silk fibroin, it forms the central core of the silk fiber and is covered with a sericin coating. Silk fiber consists of fibroin (72-81%), sericin (19-28%) and other oils and waxes. Fibroin acts as the inner core of the silk fiber and provides mechanical strength. According to the chemical composition, *Bombyx mori* fibers consist of at least 16 amino acid residues, the ratio of which varies between different regions of the fibroin supramolecular structure. The total mole fraction of glycine, alanine, serine and tyrosine residues is 90%; their sequence is represented by a general formula. Figure 1 shows projections of segments of macromolecules forming a-helix and b-sheet structures. The α-helical structure is formed by hydrogen bonds within the molecule, while the hydrophobic fragments move to the periphery. In the β-folded structure, macromolecules are arranged parallel or antiparallel, forming a sheet or layer (β-sheet).

Although sericin is a protein in structure, unlike fibroin, it causes allergies in the body and has side effects. Therefore, fibroin requires purification from sericin before its use for medical purposes.

Silk has high molecular weight (200-350 kDa or more), large repetitive modular hydrophobic domains interrupted by small hydrophilic groups. Silk fibroin, like creatine and collagen, belongs to fibrillar proteins. The structural elements of silk fibers are macrofibrils with a width of up to 105 nm, which in turn consist of spirally wound nanofibrils with a diameter of 90–170 nm. Nanofibrils play an important role in increasing the strength of silk. The length of fibroin macrochain is 150 nm; *macrochain diameter is 0.45 nm* . Silk fibers are mainly composed of two proteins, sericin and fibroin; they also

contain small amounts of other amino acid residues and various impurities: oils, waxes and mineral salts. Depending on the type of cocoon, fibroin is 66.5-73.5%, sericin is 26.5-33.5%.

Bombyx mori silk fibroin consists of heavy (*H*) and light (*L) chains (H* – and *L* – fibroin, respectively) connected by disulfide bonds. In addition, silk contains a 25 kDa glycoprotein called P-25, which is also non-covalently attached to these chains. Hydrophobic domains of heavy chains contain amino acid repeats *(Ala, Ser, Tyr, Val) and can form antiparallel β-layers (β-layer structure). L* – chain (light) is hydrophilic and relatively elastic in nature.

The P-25 protein plays an important role in maintaining the integrity of the silk complex. *H* -fibroin, *L* -fibroin and P-25 are found in silk in a ratio of 6:6:1. Refined and processed silk is purified from light chain *L*- , glycoprotein P-25. Instead, it contains heavy chain homodimers with a molecular weight of ~330 kDa, which are formed by individual proteins (~160 kDa). Hydrophobic domains of silk polymer chains consisting of repeated amino acid sequences are assembled into nanocrystals (β-layers). Hydrophilic bonds between these hydrophobic domains consist of large and polar side chains, forming the amorphous part of the protein's secondary structure.

According to the chemical composition, *Bombyx mori* fibers consist of at least 16 amino acid residues, the ratio of which varies between different regions of the fibroin supramolecular structure (Table 1). The total mole fraction of glycine, alanine, serine and tyrosine residues is 90%; their sequence is represented by a general formula.

***Table 1. Bombyx mori* silk fibroin**

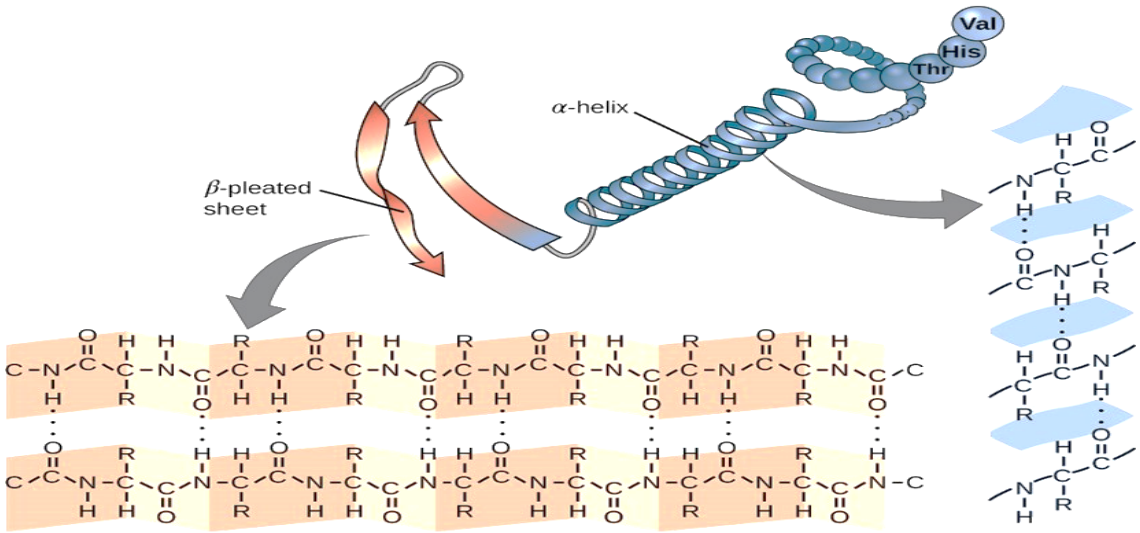
Amino acid	Content, mol.%		
	General	H-fibroin	L-fibroin
Glycine	42.9	49.4	10.0
Alanine	30.0	29.8	16.9
Cool	12.2	11.3	7.9
Tyrosine	4.8	4.6	3.4
Valin	2.5	2.0	7.4
Aspartic acid	1.9	0.65	15.4
Glutamic acid	1.4	0.70	8.4
Threonine	0.92	0.45	2.8
Phenylalanine	0.67	0.39	2.7
Methionine	0.37	-	0.37
Isoleucine	0.64	0.14	7.3
Leucine	0.55	0.09	7.2
Proline	0.45	0.31	3.0
Arginine	0.51	0.18	3.8
Histidine	0.19	0.09	1.6
Lysine	0.38	0.06	1.5

The secondary structure of fibroin is stabilized by various types of interactions. Hydrogen bonds appear between functional groups of peptide macrochains and side parts of macromolecules. Currently, three main "types" of secondary structures of natural silk fibroin are distinguished: in the crystalline regions, there are α-helical and β-folded structures (silk I and silk II, respectively), and in the amorphous regions, there are random globules in an irregular conformation. Liquid silk synthesized by the silkworm gland is an aqueous solution of fibroin with a concentration of 26 vol.%, in which macromolecules are in the form of globules or α-helices. *Bombyx mori* fibroin contains 56±5% of β-folded macromolecules and 13±5% α-helical macromolecules. Thus, the percentage of high-order (crystalline) regions of the polymer reaches 60-70%.

Silk II is the post-spinning fiber structure of the silkworm, which is mainly anti-parallel β-layer. Physico-mechanical properties of reconstituted silk fibroin depend on the conformation of molecular

chain and crystal structure. Figure 1 shows projections of segments of macromolecules forming a-helix and b-sheet structures. The α-helical structure is formed by hydrogen bonds within the molecule, while the hydrophobic fragments move to the periphery. In a β-folded structure, macromolecules are arranged parallel or antiparallel, forming a folded sheet or sheet (β-sheet).

Figure 1



Antiparallel silk fibroin β-sheets are packed face-to-back: double-layered glycine residues (*interplanar distance 3.5 Å*), double-layered *alanine/serine* residues (*interplanar distance 5.7 Å*), double-layered glycine residues, etc. This structure is the most energetically favorable for hydrophobic fragments of macromolecules.

The chain conformation in the amorphous blocks is a kind of "random coil" that gives silk its flexibility. Important factors that determine the mechanical properties of any silk are precise control over the size, amount, distribution, orientation, and spatial arrangement of crystalline and non-crystalline domains at the nanometer scale. Nanocrystals contribute to the excellent mechanical properties of silk despite microstructural defects in the form of microvoids. In addition to the secondary structure, silk fibers also have a "hierarchical" supramolecular part. Spider and silkworm silk consists of bundles of microfilaments (0.5–2 μm), each of which consists of nanocrystals and/or semicrystalline domains.

There are several ways to extract fibroin from silk. In the textile industry, in order to clean silk fiber from sericin, it is carried out in boiling water or using detergents . In laboratory conditions, it is done using sodium carbonate solution. Sericin-free fibroin fiber or thread is not considered a universal material for obtaining medical devices. It is necessary to obtain a fibroin solution when obtaining bases (frames) for various medical devices, biomaterials in the form of films, as well as when using them for other medical purposes. Dehydrated calcium chloride or lithium bromide solutions are often used for obtaining fibroin solutions. There are some toxicity concerns when using lithium bromide . Lithium is an essential element for the human body and has been used in the treatment of some mental disorders. But due to the high side effects on the body, its use in these treatment methods has been stopped.

***Bombyx mori* silk fibroin**

Figure 2

If these salts are used, after obtaining the fibroin solution, it is necessary to clean the solution from these salts. The dialysis method is used to remove salts from the fibroin solution.

In this study, we studied the effects of boiling water, detergent, and sodium carbonate solution on the efficiency of fibroin extraction from silk fiber and its properties, as well as the effect of dialysis duration on the process of removing fibroin solutions from rapids.

Bombyx mori silkworm filaments in our research . Initially, the cocoons were crushed to increase the surface area. The ground cocoons were freed of sericin in three different ways. In all methods, the silk:solution was taken at 1:100 modulus and heated at 80 $^{\circ}$ C for 1 hour. In the first method, distilled water was used as a solvent. The second method was carried out with the addition of a neutral detergent to distilled water (3:4 ratio of cocoon fragments: detergent). The third method was carried out by adding sodium carbonate (cocoon pieces:Na_2CO_3 10:1 ratio) to deionized water. In all three methods, after the process was stopped, the silk was washed several times with distilled water and dried in the open air.

The characteristics of fibroin obtained by the above three methods were studied and its structure was studied using the methods of physicochemical analysis. As a result of the research, it was shown that a small amount of sericin was preserved in the fibroin extracted by the first and second methods, i.e. with distilled water and detergent, and there was no sericin in the fibroin purified using sodium carbonate.

Ajizawa reagent (calcium chloride:ethanol:water (1:8:2) molar ratio solution) was used to dissolve the isolated fibroin in our further studies . Fibroin was dissolved *in ajizawa solution* (fibroin: *ajizawa solution* in a ratio of 1:20) at a temperature of 55 ^{o} C for 1 hour under constant stirring.

The fibroin solution was dialyzed to remove salts. The dialysis process was carried out for 1-7 hours in distilled water using dialysis bags with a pore diameter of 3 kDa . Complete purification of fibroin from salt ions was determined by qualitative reaction with chloride ion $AgNO_3$, and by measuring electrical conductivity of solutions.

***Table 2. Bombyx mori* silk fibroin purification process**

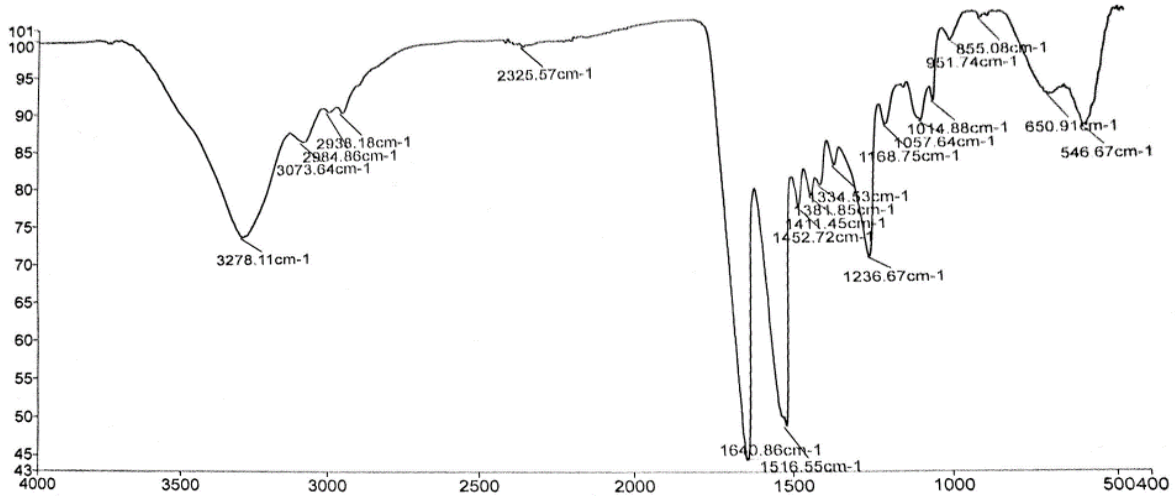
No	Cleaning process	Solutions used	Temperature	Time
1.	Cleaning process	Na_2CO_3 with 0.1M	80 ^{0}C	1 hour
2.	Melting process	(calcium chloride:ethanol: (1:8:2)	55 ^{0}C	1- hour
3.	Dialysis process	Dis-water	At room temperature	5-7 days

Studies have shown that the concentration of salt ions decreases as a result of constant exchange of the dialysis water of the fibroin solution, and the solution is significantly cleaned of salts within 5 days. However, for medical purposes, fibroin should be in a state completely purified from salts. Obtaining such fibroin was achieved by carrying out the dialysis process for up to 7 days.

Thus, after evaluating the three separation methods, it was found that the sodium carbonate cleaning method could separate sericin and fibroin more easily and efficiently than the distilled water and detergent treatment.

The effectiveness of the dialysis method in cleaning the solution of fibroin in the ajizawa reagent from salts was demonstrated. In particular, it was determined that dialysis should be performed for 5 days to significantly remove salts from the solution, and 7 days for complete cleaning.

After that, the structure of the fibroin obtained in the parashock state was studied using IR-spectroscopy and the following conclusions were drawn. In the region of 3278 cm $^{-1}$ -NH and in the region of 1638 - 1640 cm $^{-1}$ - absorptions characteristic of vibrations of COOH groups, in the region of 1516 cm $^{-1}$ - absorptions characteristic of CO-NH- groups, in the region of 2938 cm $^{-1}$ - CH_2, in the region of 2984 cm $^{-1}$ absorbances specific to the tertiary -CH- group, as well as specific absorptions for Amide I, II, and III in the 1638, 1516, and 1236 regions were determined.



Compositions can be obtained by combining fibroin protein isolated from silkworm cocoons with various polysaccharides. Films of different thickness with variable parameters and characteristics are obtained from aqueous solutions of silk fibroin. Thus, for example, nano-sized fibroin films can be obtained from its aqueous solutions using the "layering" technique. This technique makes it possible to obtain biopolymer coatings of a certain size and thickness. Nano-sized films perfectly support the adhesion and proliferation of mesenchymal stem cells.

Fibroblast growth and proliferation on fibroin films have been shown to be identical to collagen films. Other mammalian and insect cells have also been shown to bind better to fibroin coatings compared to collagen films. Chemically modified fibroin films are used to improve cell attachment and bone formation. To study the proliferative activity of cells, it is necessary to take into account the morphofunctional state of the surface of the product based on silk fibroin (depending on the material - fiber, matrix or hydrogel). Scanning electron microscopy, atomic force microscopy, and interference

microscopy are often used as microscopy methods. One of the most universal parameters describing surface and cell adhesion is the value of the surface energy of the material. Compared to other fibrillar proteins, an important feature of silk fibroin as a biomaterial is the versatility of sterilization options.

Fibrous materials derived from polysaccharides and proteins affect the body's natural environment and thereby provide optimal conditions for tissue growth and regeneration. We obtained fibroin-polysaccharide based composites in our study as well. For this, fibroin and dextran, purified according to the above methods (dialdehyde dextran) composite material in the form of a film was obtained.

For this purpose, a periodate oxidation reaction with dextrane was carried out and dialdehyde derivatives containing 10-90% oxidized units were obtained. It was found that by changing the reaction conditions, it is possible to change the amount of aldehyde groups and the molecular parameters of dialdehyde dextran. The resulting dialdehyde derivatives react with primary amines to form easily hydrolyzable azomethine compounds. This makes it possible to use them as polymer carriers in obtaining macromolecular medicinal systems with prolonged action time.

To carry out the periodate oxidation reaction with dextran, 6 g of dextran was withdrawn. 200 ml buffer solution of pH 4.24 was added to the dextran-containing polyglucin solution, followed by stirring for 30 min. 222 mg of $NaJO_4$ was added. The reaction mixture was stirred for 2 h at 25 °C. The reaction products were precipitated with acetone. The precipitate was washed with 75% aqueous alcohol solution until a negative reaction for $JO_{4\ ions.}$ Dextran is a polysaccharide obtained as a result of microbiological synthesis, its main molecular chain consists of anhydro-D-glucopyranose, the units connected to α-1,6 are considered to be glycosidic bonds.

The film formation process was carried out as follows. An aqueous solution was prepared based on the fibroin and dialdehyde dextran extracted using the above methods. Their ratio was 1/1,1/2,1/3,1/4,1/5, and the concentration of the solution was 30 mg / ml. The solutions prepared in these proportions were poured into petri dishes and left to dry at room temperature for 3 days. The prepared films were washed 2 times in ethyl alcohol and separated from the petri dish surface using a scalpel and dried.

Films obtained from preliminary studies are strong, flexible and elastic, and now their structural and physicochemical properties are being studied.

Thus, as a result of research, *Bombyx mori* silkworm cocoons in three ways: using distilled water as a solvent; if neutral detergent is added to distilled water; by adding sodium carbonate to deionized water, fibroin was isolated in a pure state and its composition and structure were studied.

In order to obtain polymer compositions based on fibroin and dextran, functionalization of dextran, that is, aldehyde groups were introduced into the macromolecule. For this purpose, periodate oxidation reaction with dextran was carried out and polyaldehyde derivatives containing different amounts of aldehyde groups were obtained. Films with high elasticity were obtained based on polyaldehyde destran and fibroin. Currently, the structure and properties of the obtained films depending on their composition are being studied.

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