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# SDS-PAGE for Silk Fibroin Protein

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**[Abstract]** The method and detailed procedure of SDS-PAGE for silk proteins are exactly the same as for other proteins, but the electrophoresis profile of silk protein is often unsatisfactory. The main reason is that their molecular masses are too large, and the regenerated liquid silk is easily coagulated and denatured, resulting in a significant adverse effect on normal electrophoresis. A satisfactory SDS-PAGE profile for silk protein can be obtained by rapidly loading samples, reducing time and temperature when mixing the sample with the loading dye.

**Keywords:** SDS-PAGE, Silk protein, Fibroin, Regeneration, Coagulation, Denature

**[Background]** SDS-PAGE for protein or polypeptide is one of the most classic, basic and commonly used experimental methods for analyzing the molecular masses of protein subunits (Laemmli, 1970). Therefore, it is generally not difficult to obtain a good SDS-PAGE electrophoretic profile with clear bands for most proteins.

However, for researchers involved in electrophoresis experiments with silk protein, it seems that it is not easy to obtain a good SDS-PAGE profile with clear band of light chain, a subunit of silk fibroin. This is especially true for beginners or students who do not have a long experience with electrophoresis. Where is the problem? The main difficulty is not related to the SDS-PAGE technique itself but is related to the preparation of liquid silk fibroin from silk fiber, as well as the unique properties of the silk fibroin itself.

Silk protein is a general term for silk fibroin and sericin. Two parallel monofilaments spun by matured larvae of silkworm *Bombyx mori* are composed of 65%-75% fibroin, 20%-30% sericin, and 5% wax, pigments, sugars, and other impurities. Silk fibroin is a crystalline polymer-based fiber surrounded by several layers of the gum sericin protein. The outermost layer of sericin is easily solubilized in hot or boiling water. The innermost layer of sericin closest to the fibroin fiber is hardly solubilized in boiling water (Wang and Zhang, 2011). All layered sericins are easily solubilized in alkaline hot or boiling water. Therefore, boiling and degumming (removing sericin) in a 0.1%-0.5% $Na_2CO_3$ solution have been used most frequently in the laboratory. However, it not only causes a large amount of sericin degradation and hydrolysis but also leads to a decrease in its mechanical properties (Wang and Zhang, 2013). These layered sericins can also be solubilized in 8 M urea buffer at 80 °C (Yamada *et al.*, 2001), aqueous neutral soap (Yuksek *et al.*, 2012) and surfactant solution (Wang and Zhang, 2017) under repeated treatments. In general, the process of removing sericin from the surface of silk fibers is called as

degumming. The use of these solvents for degumming treatments hardly results in a decrease of mechanical properties. But the overall degumming efficiency is very low and the repetition of treatments for more than 3 times barely remove all the sericin.

The degummed fibroin fibers are about 10-25 μm in diameter and consist mainly of a 391-kDa heavy chain (H) (Zhou *et al.*, 2000) and a 26-kDa light chain (Yamaguchi *et al.*, 1989), which are present in a 1:1 ratio and linked by a single disulfide bond (Tanaka *et al.*, 1999a). In addition, a 25 kDa glycoprotein, named $P_{25}$, is non-covalently linked to these proteins (Tanaka *et al.*, 1999b). The structure of fibroin is primarily attributed to its composition of only 3 amino acids organized in a repeating 6-residue sequence of (Gly-Ala-Gly-Ala-Gly-Ser)n. The fibroin in natural silk fiber is a semi-crystalline macromolecule in which the polypeptide chains are strongly held together by hydrogen bonds in an anti-parallel arrangement to form β-sheets which result in crystalline regions (Silk II), while the random coils and α-helix chains form the amorphous regions (Silk I).

Silk fibroin fiber is processed into an aqueous silk fibroin solution via a series of processing, degumming, dissolution, purification and concentration steps that are often referred to as silk regeneration. The resulting liquid silk fibroin is often referred to as regenerated liquid silk. The regenerated liquid silk is very unstable in aqueous solution and its molecular structure changes easily from Silk I into Silk II form due to environmental factors including physics and chemistry, such as temperature, pH, UV radiation, organic solvents, ion strength, stress and ultrasonic treatment. Due to the structural transition of silk protein, the regenerated liquid silk can be easily made into various forms of silk biomaterials, such as micro- or nano-particles (Zhang *et al.*, 2007), regenerated fibers (Matsumoto and Uejima,1996), artificial skin(Jin *et al.*, 2005), porous matrix or 3D scaffolds (Mandal and Kundu, 2009), biomimetic nanofibrous scaffolds (Park *et al.*, 2006), and a platform for transistors (Capelli *et al.*, 2011) and various classes of photonic devices (Kim *et al.*, 2013), due to its biocompatibility.

The structure and properties of the final forms of silk biomaterials depend evidently on the molecular size of the regenerated liquid silk which is affected by a series of processing, degumming, and dissolution steps. The purification, concentration, and storage conditions easily induce regenerated liquid silk gelling or coagulating and denaturing. Therefore, determination of the molecular mass of the protein by SDS-PAGE is a necessary step before processing silk biomaterials.

## Materials and Reagents

*Notes:*

a. *The purification grade, CAS No., catalog number and manufacturer of the main reagents used in the electrophoretic experiment are listed as below. It should be emphasized here that the reagents used in the experiments need to be of high purity or electrophoresis grade. It is important to note that all water used to prepare the reagents, including water in the running buffer, must be ultrapure water.*
b. *MB, molecular biology; GR, Guaranteed reagent.*

 

1. Eppendorf tube
2. DP$H_2O$ (Double distilled purified water)
3. Tris (CAS No. 77-86-1, MB > 99.9%) (Bio Basic, catalog number: TB0195-500g)
4. SDS (CAS No. 151-21-3, MB > 99%) (Bio Basic, catalog number: SB0485-500g)
5. APS (Ammonium persulfate) (CAS No. 7727-54-0, ACS) (Amresco, catalog number: 0486-100G)
6. TEMED (N,N,N',N'-Tetramethyl ethylenediamine) (CAS No. 110-18-9, GR) (Sigma-Aldrich, catalog number: T9281-50mL)
7. Glycine (CAS No. 56-40-6, MB) (Bio Basic, Sangon Biotech, catalog number: A610235-0500)
8. Acrylamide (CAS No. 79-06-1, MB ≥ 99%) (Sigma-Aldrich, catalog number: A8887-500G)
9. BIS (N,N'-methylenebisacrylamide) (CAS No. 110-26-9, MB ≥ 99.5%) (Sigma-Aldrich, catalog number: M7279-100G)
10. The cocoons of silkworm *Bombyx mori* (provided by Sericultural Institute, Soochow University, Suzhou, P. R. China)
11. $Na_2CO_3$ (AR, General Chemicals)
12. $CaCl_2$ (AR, General Chemicals)
13. Ethanol (AR, General Chemicals)
14. 0.2% $Na_2CO_3$ solution (see Recipes)
15. $CaCl_2$/ethanol/$H_2O$ ternary system (Ajisawa's reagent) (see Recipes)
16. 30% acrylamide monomer solution (Cryl/Bis) (see Recipes)
17. Condensing gel buffer (1 mol/L Tris-HCl, 0.4%SDS, pH 6.8) (see Recipes)
18. Resolving gel buffer (1.5 mol/L Tris-HCl, 0.4% SDS, pH 8.8) (see Recipes)
19. 10% SDS (see Recipes)
20. 10% APS (ammonium persulfate) (see Recipes)
21. 10% TEMED (see Recipes)
22. 5% condensing gel buffer and 10% resolving gel buffer (see Recipes)

## Equipment

1. Beaker
2. Hot plate
3. Triangular bottle
4. Water bath
5. Centrifuge (Beckman Coulter, model: Avanti J-30I, Rotor, catalog number: 363420)
6. Dialysis cassette (Thermo Fisher Scientific, 5,000 MW cutoff, Slide-A-Lyzer™)
7. Mini-PROTEAN® 3 electrophoresis cell (Bio-Rad Laboratories, catalog number: 165-3301)
8. PowerPac 1000 (Bio-Rad Laboratories, catalog number: 165-5054)

## Procedure

1. Degum clean cocoon shells of a silkworm or raw silk twice in a boiling solution of 0.2% $Na_2CO_3$ for 0.5 h in a beaker on a hot plate.
2. Wash the degummed silk fibroin fiber repeatedly in deionized water and then air-dry at 105 °C for 2 h.
3. Dissolve the resulting degummed fiber a ternary system ($CaCl_2$/ethanol/$H_2O$ at 1:2:8 molar ratio) at a stuffed triangular bottle in a shaking water bath (70 °C) for 4 h .
4. Centrifuge the silk fibroin–salt solution at 10 °C, 7,740 *x g* for 10 min. Dialyze the supernatant continuously for 48 h against stirring pure water to remove $CaCl_2$, smaller molecules and some impurities using a Dialysis cassette.
5. Store the dialyzed silk fibroin solution at 4 °C for short time storage (12 h-48 h).
6. Determine the molecular mass range of regenerated silk fibroin by SDS-PAGE with 10% resolving gel and 5% condensing gel according to the method reported by Laemmli (1970). The method and detailed procedure of SDS-PAGE for silk protein are exactly the same as for other common proteins.

   *Notes:*

   a. *Sample treatment before loading into SDS-page: take out the sample from 4 °C and add the loading buffer. Invert the sample several times in an Eppendorf tube, and then immediately put it in hot or boiling water for a few seconds or a shorter time and take it out immediately otherwise the intact silk fibroin easily gels or denatures in staining buffer.*
   b. *After the last sample is loaded, run the electrophoresis immediately. In our laboratory, we often run at a constant current of 20 mA for several minutes in the beginning, and then adjust to 40 mA to continue running until the dye front reaches the end of gel. Usually, the entire running time for electrophoresis is about 1.5-2.0 h.*
   c. *The loading amount of silk fibroin sample is much higher than that of common protein for electrophoresis. The main reason is that there are a lot degraded products in regenerated silk fibroin samples. They are not a single band but a smeared band on the electropherogram. For example, the loading amount of common protein is generally controlled at 10 μl, while the loading of silk fibroin solution needs 20 μl. A better method is that the loading amount of sample can be kept unchanged, still 10 μl, but the concentration of silk fibroin solution is 2-5 times higher than that of common protein.*

## Notes

1. Silk fiber degumming

   The degumming solvents and the degumming temperature have a big influence on the range of the molecular mass of the regenerated silk fibroin, especially the former. If preparing an intact silk fibroin sample, add the clean silkworm cocoon in 8 M urea buffer (pH 7.0) and incubate at

 

80 °C for 2-5 h (Yamada *et al.*, 2001), or in neutral soap or surfactant aqueous solution, boiling 3-5 times (Wang and Zhang, 2017). Boiling of silkworm cocoon shell in $Na_2CO_3$ solution causes different degrees of peptide cleavage of silk fibroin, while degumming in urea buffer or neutral soap solution has little effect on the peptide chain of silk fibroin.

2. Silk fibroin dissolution

   The solvent and dissolving temperature of silk fibroin fiber also have a significant effect on the range of molecular mass of the regenerated silk fibroin. In general, the $CaCl_2$ ternary solvent has more effect on the peptide chain of silk fibroin than that of the LiBr solvent; The higher the dissolution temperature, the faster the dissolution rate of the silk fibroin fiber is, and the more easily the polypeptide chain of the silk fibroin gets broken.

3. Dialysis of silk fibroin-salt solution and storage of regenerated liquid silk fibroin

   In fact, the above two factors can significantly affect the quality of silk fibroin for SDS-PAGE. So dialysis of the silk fibroin-salt solution and storage of the liquid silk fibroin solution have an impact on the electrophoresis results. It is best to perform dialysis at 4 °C for 24-36 h since a higher environmental temperature will lead to denaturation and coagulation of liquid silk fibroin solution. Moreover, fresh water should be exchanged frequently during dialysis. The dialyzed liquid silk solution should be concentrated to 15%-20% by air-drying or by polyethylene glycol and can be temporarily stored at 4 °C before use.

4. Mixing sample and staining buffer prior to sample-loading

   After the standard marker (protein ladder) or other sample solutions have been loaded, the final step is that the silk fibroin solution is mixed with the staining buffer for reaction.

## Data analysis

Figure 1 shows an SDS-PAGE map of three silk fibroin samples prepared in our laboratory. The first sample is a liquid silk fibroin taken from the posterior silk gland of the $5^{th}$ instar silkworm, called as the natural silk fibroin (NSF), and the second sample is a degummed in 8 M urea buffer (pH 7.0) according to the method described by Yamada *et al.* (2001). After shakily degumming at 80 °C for 5 h, the resulting silk fibroin fiber is dissolved in 9.3 M LiBr solution at 40 °C. This sample is called as regenerated silk fibroin (RSF). The third sample called as degraded silk fibroin (DSF) is degummed silk fibroin fiber in 0.2% $Na_2CO_3$ aqueous solution. The degummed silk fibroin is dissolved in $CaCl_2$ ternary solvent at 70 °C. The three fibroin-salt solutions are all dialyzed continuously for 2 days against purified water to remove salts, smaller molecules and some impurities using a Dialysis Cassette (cutoff 5 kDa), and finally sample solution of silk fibroin is concentrated to about 10% for electrophoresis.

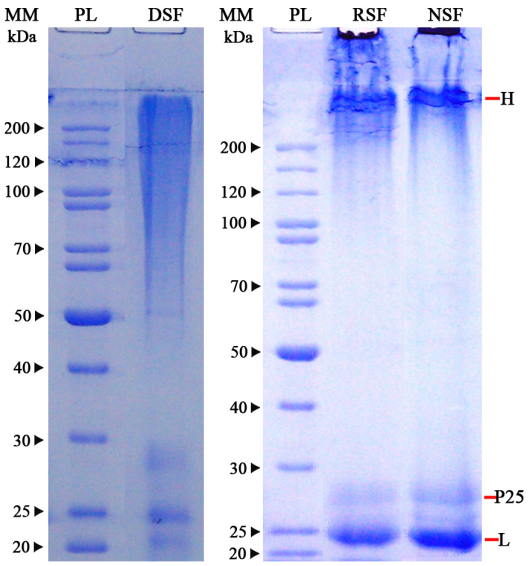


**Figure 1. SDS-PAGE of native (NSF), regenerated (RSF), degraded silk fibroin (DSF) by 10% Gel.** MM: molecular mass; PL: protein ladder (standard marker, 10 μl); NSF: native silk fibroin *in vivo* (2 mg). RSF: regenerated silk fibroin *in vitro* (2 mg); DSF: degraded silk fibroin *in vitro* (2 mg). H, heavy chain; L, light chain; P25, P25 protein.

From the electrophoretic profile, it can be found that the band of the standard protein ladder is very clear, which fully demonstrates that the electrophoresis experiment is very successful. First, the bands of the heavy and light chains of the natural silk fibroin samples (NSF) are clear, and even the band of P25 protein is visible; Similarly, the silk fibroin sample (RSF) degummed from the urea buffer has clear heavy and light chains of silk fibroin. These bands are also considerable clear, almost similar to the natural silk fibroin (NSF), indicating the method degummed in the urea buffer hardly causes significant breakage of the silk fibroin polypeptides. However, for the silk fibroin sample (DSF) degummed in $Na_2CO_3$ solution, both the heavy and the light chain are not clear, indicating that boiling degumming in $Na_2CO_3$ solution causes severe breakage of the silk fibroin polypeptides.

## Recipes

1. 0.2% $Na_2CO_3$ solution
   Dissolve 21.198 g $Na_2CO_3$ to 500 ml $H_2O$, and bring the final volume of the solution up to 1,000 ml with $H_2O$
2. $CaCl_2$/ethanol/$H_2O$ ternary system (Ajisawa's reagent)
   a. Dissolve 55.5 g $CaCl_2$ in 72 ml $H_2O$
   b. After the mixture solution cool to room temperature, add 57.5 ml ethanol to the mixture
   c. The total volume of the ternary solvent is about 165 ml
3. 30% Acrylamide monomer solution (Cryl/Bis)
   a. Add 14.55 g acrylamide and 0.45 g N'N'-bis-methylene-acrylamide to 40 ml $DPH_2O$. Stir

 

until the solution is transparent.

b. Adjust the volume to 50 ml with $DPH_2O$.

c. Filter the solution through filter paper, store the acrylamide/Bis solution in a brown bottle at 4 °C for subsequent use.

*Note: The two monomers and the solution all are toxic to Central nerve system and should be handled with protection.*

4. Condensing gel buffer (1 mol/L Tris-HCl, 0.4% SDS, pH 6.8)
   a. Dissolve 6.06 g Tris-base in 40 ml $DPH_2O$
   b. Adjust to pH 6.8 with 4 mol/L HCl
   c. Adjust the volume to 50 ml with $DPH_2O$, and finally store at 4°C
5. Resolving gel buffer (1.5 mol/L Tris-HCl, 0.4% SDS, pH 8.8)
   a. Dissolve 9.08 g Tris-base in 40 ml $DPH_2O$,
   b. Adjust to pH 8.8 with 4 mol/L HCl.
   c. Bring the final volume up to 50 ml with $DPH_2O$, and finally store at 4°C
6. 10% SDS
   Dissolve 25 g SDS in 250 ml $DPH_2O$, store at room temperature
7. 10% APS (ammonium persulfate)
   Dissolve 0.1 g APS in 1 ml $DPH_2O$, prepare prior to use
   *Note: It is preferable to aliquot 0.1 g APS/tube, and pre-store in a 4 °C refrigerator.*
8. 10% TEMED
   Add 0.1 ml N,N,N',N'-tetramethylethylenediamine to 0.9 ml $DPH_2O$, prepare prior to use
9. 5% condensing gel and 10% resolving gel (Table 1)

**Table 1. Recipes of 5% concentration gel and 10% resolving gel**

| Gel sorts | Condensing gel (ml) | Resolving gel (ml) |
|---|---|---|
| Gel concentration | 5% | 10% |
| 1) $DPH_2O$ | 0.90 | 2.64 |
| 2) 30% Cryl/Bis | 0.43 | 2.67 |
| 3) Gel buffer (condensing/resolving) | 1.0 (pH 6.8) | 2.5 (pH 8.8) |
| 4) 10% SDS | 0.1 | 0.1 |
| 5) 10% ammonium persulfate | 0.05 | 0.05 |
| 6) 10% TEMED | 0.02 | 0.04 |
| 7) Total volume | 2.5 | 8.0 |

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