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Proteomic Level Solubilisation of Spider Silk by Two Step Heating strategy in an Aqueous Solution

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Abstract –
Spider silk is a proteinaceous fiber with remarkable mechanical properties spun from spider silk proteins (spidroins).
Spider silk is a protein-based fiber with outstanding mechanical properties, composed primarily of spider silk proteins (spidroins). In nature, spidroins are spun directly into fibers, whereas under controlled in vitro conditions they can be processed into a variety of morphologies, such as films, hydrogels, fibers, capsules, and particles. The structural versatility and biochemical properties of silk proteins make silk-derived materials promising candidates for applications in tissue engineering, regenerative medicine, and the controlled delivery of protein drugs and peptide vaccines. In this work, we present a two-step heating solubilization strategy to extract spidroins from the web of *Neoscona mukerjii*, taking advantage of their high thermal stability in concentrated urea solutions. The influence of pH and temperature on the solubilization efficiency was examined, and the optimized conditions were determined as follows: heating at 55 °C-90 °C for upto 60 minutes, pH 9.0–10, and 7-8 M urea in standard biological buffers. The spidroins obtained through this method exhibited the capacity to self-assemble into spherical nanoparticles with smooth morphology. Compared with conventional solubilization approaches, this two-step heating method provides a more effective strategy for dissolving spider silk proteins, which may be valuable for downstream applications. Nevertheless, care should be taken in cases where urea-induced modifications could interfere with the intended application. Furthermore, the method demonstrates adaptability across a wide range of buffer systems, pH levels, and temperature conditions, suggesting its potential applicability for solubilizing other thermally stable proteins.
Keywords: Web Protein, Solubilization, Heating, Degumming, Incubation

Here, we present a mild solubilization strategy—one-step heating method to solubilize spidroins from IBs, with combining spidroins' high thermal stability with low concentration of urea. A 430-aa recombinant protein (designated as NM) derived from the minor ampullate spidroin of Araneus ventricosus was expressed in E. coli, and the recombinant proteins were mainly present in insoluble fraction as IBs. The isolated IBs were solubilized parallelly by both traditional urea-denatured method and one-step heating method, respectively. The solubilization efficiency of NM IBs in Tris-HCl pH 8.0 containing 4 mol/L urea by one-step heating method was already comparable to that of 7 mol/L urea with using traditional urea-denatured method. The effects of buffer, pH and temperature conditions on NM IBs solubilization of one-step heating method were evaluated, respectively, based on which the recommended conditions are: heating temperature 70–90 °C for 20 min, pH 7.0–10, urea concentration 2–4 mol/L in normal biological buffers. The recombinant NM generated via the one-step heating method held the potential functions with self-assembling into sphere nanoparticles with smooth morphology.

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Introduction -

Spider silk proteins are gaining significant attention as potential alternatives to petroleum-derived fibres and materials. The unique combination of strength and toughness in native spider silk makes it an excellent candidate to replace conventional fibers such as nylon. However, unlike silkworms, spiders cannot be farmed on a large scale, necessitating the synthetic production of spider silk proteins through alternative organisms and biotechnological methods. These synthetic systems, however, cannot fully replicate the intricate biological processes that spiders use to produce silk proteins and preserve their solubility until fiber formation. Spiders have developed a highly specialized production and storage system capable of generating very large proteins (over 250 kDa), transporting them to the gland lumen, and maintaining them in a soluble state until they are required for fiber spinning [1–3]. To achieve the remarkable mechanical performance of silk fibers, spiders have evolved protein sequences that are among the most repetitive known in nature [4]. These repetitive motifs within the spidroin sequence are directly responsible for the robust and diverse mechanical properties of spider silk [5–10].

Proteins serve as fundamental building blocks in the formation of many complex hierarchical biological materials [11–15]. The diverse functionality of protein-based materials often arises from their hierarchical organization, beginning at the molecular level and extending across multiple structural scales [14, 16, 17]. Frequently, these systems are composed of relatively weak molecular components that are readily available, capable of large-scale assembly through low-energy processes, or naturally inclined to self-assemble. The emerging field of materiomics [18] seeks to characterize such material systems across different length scales, linking the properties of weak molecular building blocks to the mechanical performance observed at the macroscopic level. A prime example is spider silk, a protein-based fiber in which hierarchical organization—driven largely by weak hydrogen bonds—governs material behavior across scales, ultimately producing exceptional mechanical properties.

Silk represents an outstanding example of nature's design, remarkable for its exceptional properties that even surpass those of high energy-absorbing materials such as Kevlar and carbon fiber, while offering the advantage of being extremely lightweight [19–22].

Spider silk has attracted significant interest in materials science owing to its exceptional tensile strength, unique torsional behavior, and water-induced physical response (23-26). Beyond its mechanical properties, spider silk holds great promise for biomedical applications, as it demonstrates antimicrobial activity, wound-healing capability, biodegradability, low immunogenicity, potential in cancer treatment, and minimal toxicity while supporting cell adhesion and growth (27-31).

Additionally, spider silk has been explored for biochemical sensing applications (32). Spidroins, the main silk proteins, can be processed into a wide range of physical and colloidal forms including gels, films, capsules, emulsions, foams, porous matrices, non-woven fabrics, and fibers. This versatility opens vast opportunities to harness the beneficial qualities of spidroins (26). Moreover, advances in biotechnology have enabled the design of spidroin-inspired sequences that may yield silk-like materials with tailored properties (33-34).

Despite this progress, proteomic studies of Indian spider silk remain scarce. The present study therefore focuses on the proteomic solubilization of silk from the spider *Neoscona mukerjii*.

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Material and Methodology –

Collection of Spider Web:

The visual search sampling method described by Sebastian et al. (2005) was adopted in this study to survey spider fauna within randomly selected quadrants of the chosen sites. This approach is particularly advantageous as it enables repeated censusing of spiders without causing disturbance (Lubin, 1978). Random sampling was carried out across all four seasons at the selected sites. All three study locations were inhabited by the same species, *Neoscona mukerjii,* which is predominantly observed from winter through early summer.

Spiders were sampled between late October and early January, approximately 1–2 months after the disturbance event. This interval allowed the immediate effects of disturbance to subside, while still permitting the detection of vegetation differences between disturbed and undisturbed sites. Webs of *N. mukerjii* along with adult spiders were collected using nets from grasslands, trees, abandoned houses, and construction sites across all locations. The collected webs were stored in plastic containers without consideration of their age or developmental stage.

Primary treatment of Web:

The collected spider webs were pre-treated to eliminate dust and unwanted materials. Initially, the webs were washed under running tap water, followed by soaking overnight in distilled water to remove residual debris. Finally, they were dried in a hot-air oven at 60 °C for one hour.

Degumming by using Degummed agent:

The collected spider silk mass was subjected to a degumming process in order to remove the tightly bound shell layer and prepare the fibres for protein extraction. The material was boiled in a sodium carbonate (Na_2CO_3) solution at 100 °C with continuous stirring. The alkaline environment created by sodium carbonate facilitated the breakdown and removal of the non-fibroin components adhering to the silk, particularly the shell-like impurities. Following boiling, the fibres were thoroughly rinsed several times with distilled water to remove any residual sodium carbonate and detached debris. After washing, the fibres were dried in a hot-air oven at 60 °C until complete dryness was achieved. The resulting cleaned and degummed fibres represented the purified silk protein material, which was then subjected to further processing for spidroin extraction. This degumming step was adapted from the method described by Sah & Pramanik (2010), originally developed for the removal of the sericin layer in silkworm fibres, and was modified here to suit spider silk protein preparation.

Two way heating method for Dissolution of Spider web to extract the Spidroin:

The pre-treated spider silk was subjected to a solubilisation process using a concentrated denaturant. Specifically, the silk was mixed with an equal volume of 8.2 M urea solution in a 1:1 (w/v) ratio of silk to urea. The pH of the resulting mixture was carefully adjusted to 9.3, creating an alkaline environment favourable for the unfolding and solubilisation of silk proteins. The mixture was then incubated at 55-60 °C for 2 hours under static conditions (without stirring) to allow the urea to effectively disrupt hydrogen bonds and promote dissolution of the spidroins.

This procedure was adapted with modifications from the protocol described by Tszydel et al.,

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originally designed for the electrolysis of spider silk, in order to improve solubilisation efficiency. To further process the material, a hydrolysis step was employed. Approximately 100 mg of spider silk was accurately weighed and immersed in 10 mL of 0.1 M sodium hydroxide (NaOH) solution. The mixture was then heated to 60-90 °C for 1 hour, during which the alkaline hydrolysis helped break down non-fibrous residues and facilitated the release of spidroins from the silk matrix. After hydrolysis, the solution was allowed to cool to room temperature. Throughout the procedure, the critical parameters—such as incubation temperature, duration, and pH conditions—were maintained in accordance with previously established methods, with minor modifications to optimize protein solubilisation for the present study.

Result and Discussion –

In the present study, we introduce a two-step heating strategy that enables efficient extraction and recovery of spider silk proteins, particularly spidroins, with a high degree of purification. Remarkably, the spidroins obtained through this method exhibit the inherent ability to spontaneously self-assemble into well-structured spherical nanoparticles, a phenomenon consistent with earlier reports describing nanoparticles generated from other types of silk proteins. Furthermore, the two-step heating approach demonstrates broad compatibility across different biological buffers and a wide pH range, thereby making it versatile for various downstream applications.

To determine the optimal heating conditions required for effective solubilization of spider webs, we systematically investigated the influence of temperature and pH. Specifically, heating experiments were performed in 10 mL of 8 M urea, with temperatures ranging from 40 °C to 100 °C and pH values spanning 7.0 to 10.0. The results indicated that at pH 7.0 and temperatures up to 50 °C, no significant solubilization occurred. However, when the pH was progressively increased, solubilization efficiency improved in parallel with elevated heating temperatures, becoming more evident from 55 °C onwards and reaching its maximum effect at approximately 90 °C with pH 9.1. Considering the inherent thermal stability of spidroins, we identified 90 °C at pH 9.1 as the optimal condition for further evaluations.

In comparison, the conventional single-step heating method this two-step incubation of spider webs in 8 M urea at 55 °C and pH 9.1 achieve partial solubilization is more effective. In contrast, our optimized two-step heating protocol involves an initial mild solubilization under these conditions, followed by treatment of the semi-dissolved material with 0.1 M NaOH at 90 °C for 30 minutes, which results in complete dissolution of the spider web.

The findings further revealed that solubilization efficiency is significantly influenced by the working pH. While a marked improvement was observed when the pH increased from 5.0 to 7.0, differences among pH values 7.0, 8.0, 9.0, and 10.0 were minimal. This suggests that the two-step heating method is capable of operating effectively under a broad pH spectrum, thereby enhancing its robustness.

Finally, before the recovered proteins can be processed into desired formats, extensive dialysis is necessary to eliminate excess denaturants such as urea or NaOH. This step, however, inevitably results in additional sample dilution and partial precipitation of proteins, which must be carefully managed during downstream applications.

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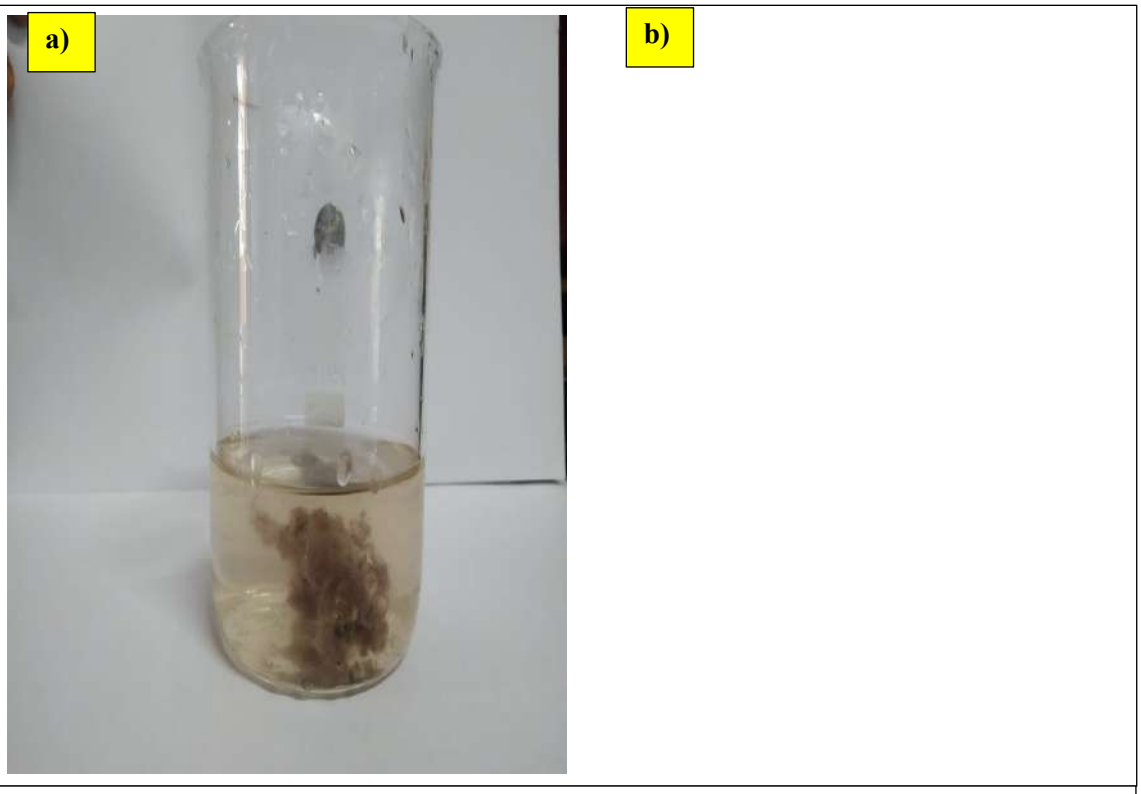

Fig. 1 a) Degummed *N. mukerjii* spider web b) Spidroin protein from *N. mukerjii*

Conclusion –

This project successfully demonstrated the solubilization of *Neoscona mukerjii* spider web silk at the proteomic level using a two-step heating strategy in an aqueous solution. The optimized protocol developed in this study provides a valuable tool for the solubilization and analysis of spider silk proteins, which can be used for various applications in biomaterials, biomedical research, and biotechnology. The results of this study contribute to our understanding of the structure and properties of spider silk proteins and provide insights into the development of novel biomaterials. The solubilization of spider silk proteins using a two-step heating strategy in an aqueous solution has significant implications for the development of novel biomaterials and biomedical applications. The findings of this study can be used to design and develop new biomaterials with improved properties, such as biocompatibility, biodegradability, and mechanical strength. Overall, this project contributes to the advancement of our understanding of spider silk proteins and their potential applications, and provides a valuable foundation for future research in this field.

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