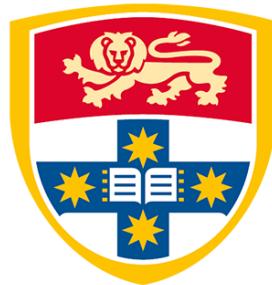


Designing a low-cost, non-toxic method to produce aqueous silk fibroin solutions in the lab

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Statement of Contribution

- *I carried out the literature review to understand and critically analyse the current state of the science in the field and develop a case for my thesis.*
- *I designed the experimental process in this thesis, with reference made and proper citation to the literature from which it was inspired. The idea to compare the degumming ratios of raw silk and cocoons to open the literature was my own.*
- *I carried out all the experiments myself, with inductions and a few suggestions for getting around lab limitations from Joseph Yang.*
- *I carried out the analysis in the discussion of this thesis. The conclusions are my own.*

The above represents an accurate summary of the student's contribution.

Student:







Supervisor:

Date:

22/11/2023

Abstract

This thesis looks to design a low-cost, non-toxic method for producing aqueous silk fibroin solutions in the laboratory with a specific focus on the complex challenge of silk degumming. Despite not fully achieving this primary objective, significant strides were made in standardising and understanding the degumming process. The introduction of Standardised Degumming Ratios (SDR) and Relative Degumming Efficacy (RDE) provides a novel framework for comparing degumming methods across different sericin contents and silk types, addressing a crucial gap in existing literature.

Through a series of experiments, this research evaluated the efficacy of various degumming agents and conditions on both cocoons and raw silk. The findings underscored the delicate balance between complete sericin removal and the preservation of silk fibroin, highlighting the inherent challenges in processing natural biomaterials like silk. The study identified several potential methods that may achieve complete degumming without substantial fibroin damage, though further research is required.

Furthermore, this thesis shed light on the ethical considerations of using animal-derived biomaterials. It emphasised the need for sustainable and humane practices in silk production, and the necessity for comprehensive documentation and standardisation in biomaterial research to address the variability inherent in biological materials.

Overall, this work contributes to the broader understanding of silk degumming and offers a foundation for more systematic, replicable, and ethically responsible research in the field of silk-based biomaterials. It sets a precedent for future studies to build upon, particularly in the development of efficient and sustainable methods for silk fibroin extraction and application in various biomedical contexts.

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As I reflect upon the journey that culminated in this thesis, I am filled with a profound sense of wonder and gratitude. Writing this thesis has been like wandering through an endless labyrinth. It's been huge challenge, one that I've only overcome with a tremendous amount of help. This thesis is not just a reflection of my academic endeavors but a mosaic of the invaluable contributions from the people below. I dedicate this thesis and my thanks to:

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List of Abbreviations

- CDR:** Control Degumming Ratio.
- DR:** Degumming Ratio.
- ECM:** Extracellular Matrix.
- GAGs:** Glycosaminoglycans.
- HTHP:** High Temperature, High Pressure.
- MSC:** Mesenchymal Stem Cell.
- MW:** Molecular weight.
- NSF:** Native Silk Fibroin.
- RDE:** Relative Degumming Efficacy.
- RSF:** Regenerated Silk Fibroin.
- SAEW:** Strongly Alkaline Electrolysed Water.
- SEM:** Scanning Electron Microscope.
- SF:** Silk Fibroin.
- U:** Enzyme Unit.
- US:** Ultrasonication.

Glossary

Bath Ratio: The ratio of the mass of silk to the liquid volume for degumming.

Bave: A silk bave consists of two fibroins (or brins) glued together and encompassed by sericin.

Control Degumming Ratio: The degumming ratio of a control method according to the literature..

Cycles: For testing, cycles are inclusive of the first run. For example, the process for a test with one cycle is performed once. The process for a test with two cycles is performed twice..

Degumming: The removal of sericin from the silk fibroin core of silk fibers.

Degumming Ratio: The percent weight loss of silk after degumming, representing the efficacy of the process.

Enzyme Unit: The amount of an enzyme that catalyzes the conversion of 1 micromole of substrate per minute under specific conditions.

Extracellular Matrix: The network of proteins and other molecules that surround, and support tissues in the body.

Native Silk Fibroin: Silk fibroin extracted directly from the silk glands of a silkworm larvae.

Raw Silk - yarn: Raw silk fibers that have been spun into a yarn, similar to wool yarn.

Raw Silk - 6A: The highest quality grade of silk, known for its long, uniform fibers.

Raw Silk - crêpe: A textured silk fabric characterised by its thin, crinkly surface created by using highly twisted yarns.

Regenerated Silk Fibroin: Silk fibroin that has undergone degumming to remove sericin.

Relative Degumming Efficacy: The degumming ratio of each Linked Method to the degumming ratio of it's Original Control.

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CHAPTER 1

Introduction

Tissue engineering is an emerging field at the intersection of engineering, biology, and medicine. Researchers seek to augment and enhance the body's own regenerative mechanisms to repair damage and degradation that the body cannot effectively repair itself. Among its areas of research, tissue engineering may provide a treatment to regenerate articular cartilage, the connective tissue that provides an essential lubricating and shock-absorbing interface between articulating bones. Articular cartilage is an avascular and aneural connective tissue consisting of an Extracellular Matrix (ECM) within which lives the primary cartilaginous cell, chondrocytes. This ECM consists of water (65-80% of total mass), proteins, the most abundant of which is collagen, polysaccharides, complexes, and other molecules that support the cells that live within [1]. Due to the lack of direct blood supply or access to the lymphatic system, normal wound healing processes cannot operate in articular cartilage [2]. The ongoing degeneration of articular cartilage is the hallmark of osteoarthritis, a disease that affects more than 30% of Australians over 65 [3]. While there is promising research into cell therapies, including the use of mesenchymal stem cells to slow the progression of osteoarthritis [4], these cells require a physical scaffold for delivery, protection, and for effective integration into the articular cartilage ECM [5].

Silk hydrogels are a promising research area in cartilage tissue engineering for such scaffolds. Hydrogels are substances whose structures consist of cross-linked polymer chains that swell and hold a large amount of water [6]. Silk hydrogels offers high biocompatibility in cartilage, suitable biodegradability, are easily sterilised, and can be fabricated into tunable scaffolds [7]–[9]. To produce these hydrogels, researchers require aqueous silk fibroin solutions from the cocoons of the *Bombyx mori* silkworm. The silk cocoon fibers are primarily comprised

of two different molecules: silk fibroin (70-80%) and glue-like sericin proteins (20-30%) [10]. Unlike silk fibroin, sericin elicits an immune response from the body [11]. To minimise inflammatory responses from silk hydrogels and thus maximise biocompatibility, a process called degumming removes sericin from silk fibroin.

The methods of degumming are broadly categorised into three strategies: biological, chemical, and physical processing [10]. Each of these methods have effects on the silk fibroin molecules and thus the final properties of the hydrogel. Once degummed, the insoluble silk fibroin must undergo further processing to make an aqueous solution, often using toxic chemicals [12]. These toxic chemicals pose a safety challenge to the researchers using them and may also affect the viability of cells implanted within silk hydrogels derived from these silk fibroin solutions.

1.1 Problem Statement

Aqueous silk fibroin solution is expensive and limited in available concentrations. For example, Sigma Aldrich sells 20mL of 50 mg/mL silk fibroin solution for \$557 with no other options in concentration levels [13]. Upon further enquiry, Sigma Aldrich was unable to provide information about the proprietary processing methods or the chemicals used in producing the solution. Significant evidence exist that shows silk fibroin molecules experience damage and property change during all degumming and solubilising methods [10]. This damage can include effects to the β -sheet content of the fibroin molecule [14], which is strongly correlated with changes to the properties of silk hydrogels [15]. Further damage can occur during long-term storage, even in ideal conditions [16].

Designing a method, or series of methods, for producing silk fibroin solution in small batches in a lab environment will provide researchers with greater control over further research. This is important considering the variables involved in the processing of *Bombyx mori* cocoons into silk fibroin solution and the impact of these processes on the physical properties and biocompatibility of the resulting silk hydrogels. With a small scale production methods producing consistent silk fibroin solutions with known, and potentially customisable properties

and concentrations, researchers will be able to perform more precise silk hydrogel research, hopefully facilitating further advancements in the field.

1.2 Thesis Aims

The aims of this project are to:

- (1) Designing a low-cost, non-toxic method to produce aqueous silk fibroin solutions in the lab. The major steps for this project are:
 - (a) Source and prepare *Bombyx mori* silkworm cocoons for degumming.
 - (b) Design a degumming process to isolate, without damage, silk fibroin from the silk fibers.
 - (c) Design a method for an aqueous solution from the silk fibroin

CHAPTER 2

Literature Review

In the past decades, there has been a significant increase in interest in the potential of silk as a biomaterial. From sutures, to hydrogel scaffolds, films, resins, mats, and sponges, tissue engineers have tailored silk's properties to suit a range of uses in a range of tissues like the eyes, skin, bone and cartilage [12], [17]. Processing techniques used to produce silk products from the cocoons of the *Bombyx mori* silkworm can have significant impacts on the final material properties. Given the range of possible permutations through processing and desired properties, it's important to first narrow the scope of investigation of this thesis. The intended use for the final aqueous silk fibroin solution is as hydrogel scaffolds in articular cartilage tissue engineering. Thus it's important to make sure that the processing methods don't affect properties such as the mechanical strength required for a hydrogel scaffold or that any residual chemicals don't affect biocompatibility in articular cartilage. We will begin with a brief overview of the target tissue, articular cartilage.

2.1 Articular Cartilage

Articular cartilage is the avascular, aneural, and alymphatic connective tissue that caps the ends of articulating bones. It acts as a load distributor for and aids in lubrication of joints for low-friction articulation. Unlike most tissues in the body, articular cartilage is sparsely populated (5% v/v) primarily by one cell type, chondrocytes [18]. These are mature cells, differentiated from mesenchymal stem cells, that are responsible for production of the tissue's extracellular matrix and its limited remodelling.

2.1.1 Structure of the Extracellular Matrix

The ECM of articular cartilage is a highly hydrated (65-80% by mass [19]) network of collagen (primarily Type II), glycosaminoglycans (GAGs), proteoglycans, and other non-collagenous proteins. The network contains a significant volume of water (65 - 80% by mass [19]), which acts as a medium for the diffusion of ions (Ca^{2+} , Na^+ , K^+ , Cl^-), and other molecules that play an important part in the structure and function of the tissue [2].

Type II collagen consists of more than 60% of the dry weight of healthy adult cartilage. It's responsible for providing much of the tensile strength and resilience of the tissue. A key factor in articular cartilage degradation is its inability to remodel and repair damage to these structural fibers[20], [21].

GAGs are molecules that provide much of load-bearing and frictional properties of cartilage [22]. They are polysaccharides that consist of repeating disaccharide units of two different six-carbon sugars. Chondroitin sulphate, keratan sulphate, and hyaluronan (hyaluronic acid) are the most prevalent in cartilage. Their carboxyl and sulphate groups give the glycosaminoglycans a strongly negative charge, resulting in a highly hydrophilic molecule [19]. GAGs exist as both free molecules in the ECM and bound to protein cores, forming proteoglycans.

The dominant proteoglycan in the ECM is aggrecan [22]. It consists of a protein core with over a hundred GAG side chains. The strong negative charge of the GAGs cause them to splay out like bristles on a brush (as in figure 2.1). Aggrecan can then bind to a hyaluronan

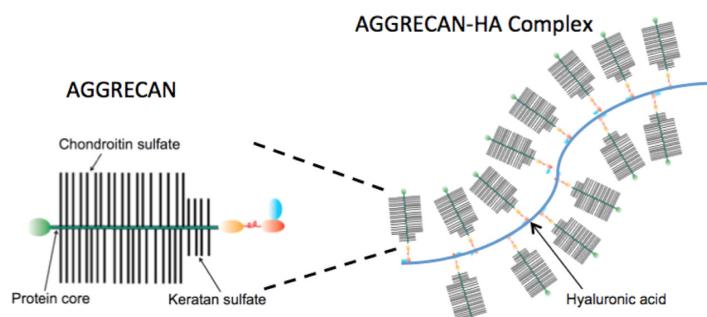


FIGURE 2.1. Drawing of the structure of aggrecan (left) and the aggrecan-HA complex (right). Figure from Horkay, Douglas and Raghavan [22].

core, producing large (>1000 kDa) aggrecan-hyaluronan complexes that weave between the collagen network. This highly hydrophilic complex results in a network capable of holding and controlling water movement throughout the tissue [19]. The incompressible water provides the compressive and stress-shielding properties of articular cartilage.

Healthy articular cartilage is typically 1-2 mm thick. Dividing this depth into three distinct segments, as seen in Figure 2.2, highlights the importance of the fibre network to the unique biomechanical properties of the tissue [2]. The superficial layer contains fibres running mostly parallel to the joint surface, high-density flatter chondrocytes, and a lower proteoglycan content than that of deeper layers. The middle zone consists of less organised, thicker fibres with more spherical chondrocytes. The deep zone features thick fibres perpendicular to the joint surface, the highest proteoglycan content, the lowest water content and the presence of sub-structures called chondrons. These are large chondrocytes surrounded by a collagen VI and proteoglycan matrix that provides both protection and acts as a transducer of chemical and mechanical signals [23], [24].

Crucially, the physical properties of articular cartilage change throughout its depth [2], [18], [19]. The superficial layer interfaces with the synovial fluid, itself a complex mixture that acts in tandem with cartilage to provide lubrication to the joint. Due to the lower levels of hydrophilic proteoglycans in the superficial layer, here the hydrostatic pressure is lowest,

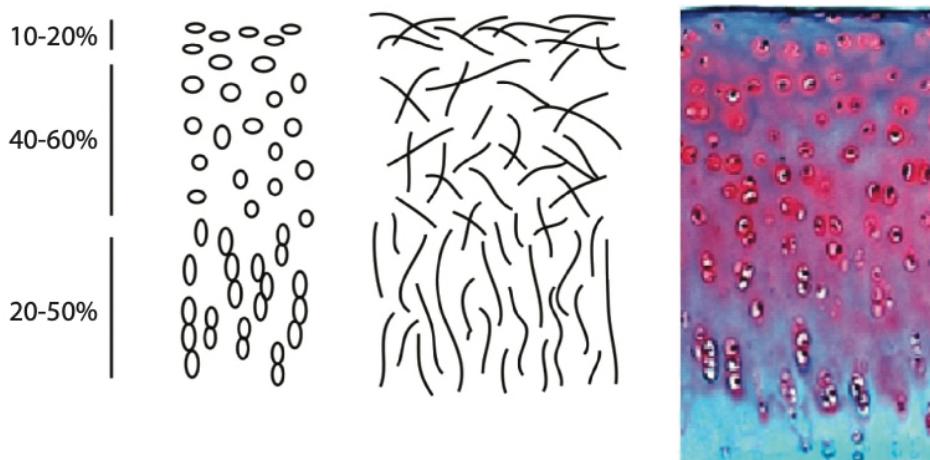


FIGURE 2.2. Organisation of chondrocytes (left) and collagen fibres (middle) alongside the approximate proportion of each of the three zones (superficial, middle, and deep) in articular cartilage (stained, right). Figure from Athanasiou, Darling, DuRaine *et al.* [18].

with the highest fluid flow and the highest resistance to shear stress [2]. Conversely, the higher proteoglycan content in the deeper layers results in much higher hydrostatic pressure and the lowest fluid flow under loading, affording this layer superior compressive strength. This complex structure is able to evenly disperse a load across the joint while providing a low-friction articulating surface for decades of cyclic fatigue.

Chondrocytes are the cells responsible for maintaining the structure and biochemical composition of articular cartilage. They respond to stimuli such as mechanical loads, growth factors, piezoelectric forces, and hydrostatic pressures to produce a variety of molecules, such as inflammatory response proteins, matrix-degrading enzymes, as well as synthesising proteoglycans amongst other ECM molecules [21]. As with zonal changes in the ECM, chondrocytes exhibit different phenotypes based on their depth [25]. Superficially, chondrocytes are smaller, flatter, and release more lubricating proteoglycans while those found in the deeper layers are larger, rounder, and often grouped together in columns, with higher levels of the protein vimentin, resulting in a stiffer cell with greater mechanical stability [26].

2.1.2 Cartilage Damage and Repair

Articular cartilage has limited regenerative potential due to its lack of direct blood supply. The nutrient supply for the cells in articular cartilage is through the slow process of diffusion through the ECM. Upon superficial damage or wear, chondrocytes are capable of limited proliferation and healing, but given the cyclic loading experienced by articular cartilage, small defects often lead to progressive deterioration of the tissue [27]. The ongoing degeneration of articular cartilage is the hallmark of osteoarthritis, a disease that affects more than 30% of Australians over 65 [3]. Though the specific causes of osteoarthritis are more complex than as described here, the result is softening and erosion of cartilage, often leading to full thickness cartilage loss, exposing the subchondral bone. The joint, unable to articulate without significant friction becomes swollen, painful, and often deformed [19]. Tissue engineering techniques may be able to combat the imbalance between the slow deterioration of articular cartilage and the body's capacity for its regeneration that lies at the core of osteoarthritis.

2.1.3 Cartilage Tissue Engineering

Cartilage tissue engineering research centres around three pillars: scaffolds, cells, and bioactive molecules. In the body, cells grow within the extracellular matrix, within which they receive stimuli, nutrients, and physical support. Scaffolds are 3-D polymeric structures that mimic this function. When used for wound repair, they provide physical support to the damaged tissue site, promote integration of the implanted cells into the target tissue, and then degrade within the body as regeneration occurs [4], [18], [28]. Scaffolds must then be porous to allow diffusion of nutrients and bioactive molecules, mechanically suitable to support damaged tissue, and biocompatible as a scaffold and throughout its degradation, ideally with a degradation rate that matches the rate of tissue regeneration [29]. Hydrogels are three-dimensional, cross-linked polymer networks that can absorb and hold large amounts of water in much the same way as the extracellular matrix. Silk hydrogels are a promising research area in cartilage tissue engineering for such scaffolds.

2.2 Silk

Silk, the long and robust fiber derived from the cocoon of the *Bombyx mori* silkworm, has been used for more than 3,000 years in the textile industry [30]. The first documented mention of silk as a ‘medical device’ comes from Aulus Cornelius Celsus (25 BC - 50 CE), who used silk threads in the reconstruction of a fractured jaw and fixation of moving teeth. Further mentions from history include its use as a suture material for sewing the severed tendons of gladiators [12]. In 1869, Joseph Lister introduced sterility to silk sutures as the gold standard in clinical practice. The historical significance of silk in the field of medicine can be attributed to its remarkable biocompatibility and exceptional tensile strength. The enduring recognition of these notable properties underscores the continued use of silk as a biomaterial [31].

It wasn’t until the 1990’s that silk saw use in modern medicine, first as an FDA approved suture material, and then in the emerging field of tissue engineering [12]. Some of the earliest work in 2001 was the use of a silk fibroin sponge as a bone scaffold by Sofia, McCarthy,

Gronowicz *et al.* [32]. They too noted the strong biocompatibility with osteoblasts and impressive mechanical properties under both tensile and compressive loads.

Silk fibers consist primarily of two distinct phases: silk fibroin (70-75% by mass), and the successive sticky sericin layers (25-30%) that glues the fibers into a cocoon [33], [34]. The sericin layers are primarily sericin proteins with a further 1-5% consisting of pigments, wax, carbohydrates, and other impurities. Silk fibroin's unique structure results in processing versatility, biocompatibility, ease of sterilisation, thermal stability, and controllable degradation features. This makes it a promising biomaterial for the regeneration of diverse tissues as shown in Figure 2.3 [10], [17], [29]–[31], [35]–[37].

Silk fibroin solution can be extracted directly from the silk glands of the *Bombyx mori* silkworm larvae, known as native silk fibroin (NSF) solution. More commonly, silk cocoons can undergo two processing steps to produce regenerated silk fibroin (RSF) solution [37]. Degumming removes the glue-like sericin and other impurities to produce insoluble silk fibroin, which is then processed further to produce an aqueous solution.

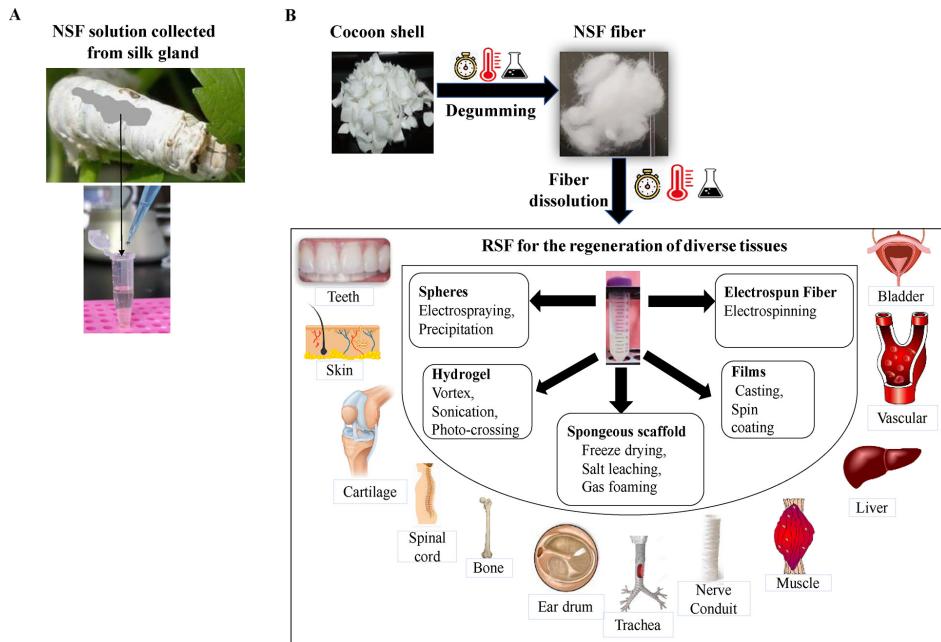


FIGURE 2.3. (A) Native silk fibroin (NSF) sourced from the silk glands of silkworm larvae. (B) Regenerated silk fibroin (RSF) solution derived from silkworm cocoons through a two-step process is the basis for tissue engineering interventions in a wide variety of tissues. Figure from Umuhuza, Yang, Long *et al.* [37].

Researchers have found significant morphological differences between the properties of NSF and RSF [38]. While there is evidence that NSF has superior mechanical and biochemical properties to RSF, it's important to note that the methods of degumming and dissolution can have significant impacts on the properties of silk fibroin [14], [16], [39], [40]. For example, Koebley, Thorpe, Pang *et al.* [41] found that RSF has “significantly diminished” self-assembly capacity compared to NSF under shear. Self-assembly refers to the spontaneous, undirected organisation of the molecule into ordered structures that, for silk, confer its superior mechanical properties [42]. Changes to the silk fibroin molecule, such as cleaving of its heavy chains during degumming will significantly change its capacity for self-assembly [14], [16], [41], [43], [44]. Thus it may be the case that the processing methods to produce RSF contribute a large amount to the differences, and that less damaging degumming methods may result in improved RSF properties. This thesis will focus on RSF (referred to as silk fibroin (SF)) due to its significantly lower price, a key aim for this thesis.

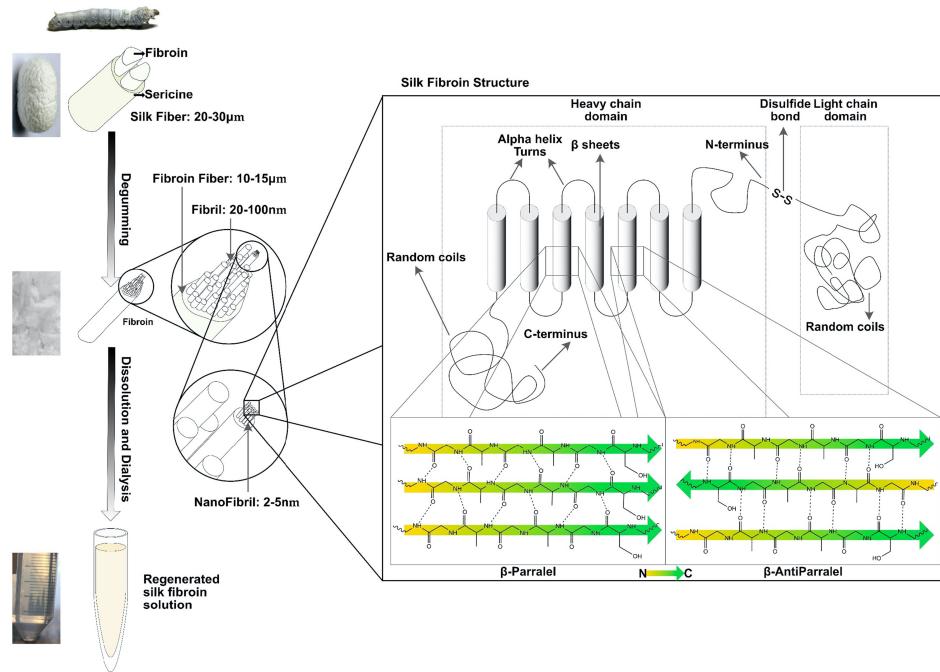


FIGURE 2.4. The structure of silk fibroin, showing the features of the heavy and light chains linked via disulphide bonds at the N-terminus. Below that is a diagram of the orientation of parallel and anti-parallel β -sheets. Figure from Bucciarelli and Motta [12].

2.2.1 The Structure of Silk Fibroin

Silk is a complex hierarchical material as shown in Figure 2.4. The fibers spun by the *Bombyx mori* silkworm have a diameter of 20-30 μm , consisting of two fibroins (or brins) glued together and encompassed by sericin, forming a bave. Each fibroin consists of bundles of fibrils, which are in turn comprised of nanofibrils. The primary structure of silk fibroin consists of chain complexes of ~390 kDa heavy chains bonded at the N-terminus to a disulfide bond to ~26 kDa light chains in 1:1 ratio. This chain complex is then bonded via hydrophobic interactions to the ~30 kDa glycoprotein P25 in a 6:1 ratio [36], [38], [41].

Silk fibroin can adopt three distinct structural configurations: silk I, II, and III. The water-soluble NSF within the silkworm gland has the Silk I configuration, characterised by random coils and helices. When the silkworm excretes fibroin, it spontaneously assembles into Silk II, which is characterised by its water-insoluble nature and the presence of parallel and anti-parallel β -sheets. The silk III structure is helical and manifests at the air-water interface [46].

These β -sheets arise from repetitive amino acid sequences (GAGAGS and GAGAGY) on the heavy chain. These sequences align as in Figure 2.4, forming hydrogen bonds between their amine and carboxyl groups to create a strong, rigid structure. Interspersed between these sheets are α -helix turns with ends of the heavy chain characterised by random coils with no discernible order [31], [47].

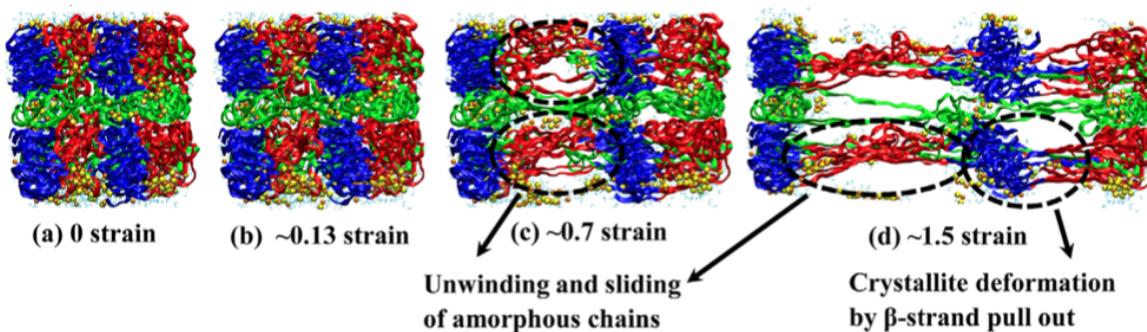


FIGURE 2.5. Showing the deformation of silk fibroin under strain. The red and green amorphous regions offer elasticity while the blue rigid β -sheets provide load transfer between the fibroins. Figure from Patel, Dubey and Singh [45].

The light chain is hydrophilic with no regular repeating units. It similarly forms random coils that entwine with random coils and α -helix turns to form an amorphous matrix. This matrix provide elasticity to silk, allowing significant strain before permanent damage as in Figure 2.5 [36]. Together, the β -sheets create strong interlocking regions that transfer tensile load between the amorphous chains, producing a strong, yet elastic fiber as shown in Figure 2.5 [47]. Silk fibroin is also thermally resistant, able to withstand temperatures of 100°C without damage [48].

2.2.2 Sericin

Sericin is the globular protein that envelops and glues together the fibroins in successive layers to ensure the cohesion of the cocoon (see Figure 2.4. Its molecular weight ranges from 10 to 200 kDa with a large difference in amino acid composition between the weights [49]–[51]. While this range represents a spectrum of different compositions, the delineations established by Wang and Zhag [50] relate to the solubility of each group which will be useful for this investigation of the degumming process.

Wang and Zhag [50] found the outer layer (S1) to be most soluble, with 50% of the sericin removed by boiling in 100°C water at 1 atm for 1 hour. The silk was then placed into a high-pressure boiler and maintained in 120°C water at 2 atm for 1 hour, removing a further 35%. The remaining 15% was less soluble again, undergoing degumming in a 0.5% Na₂CO₃ solution at 100°C for a further hour.

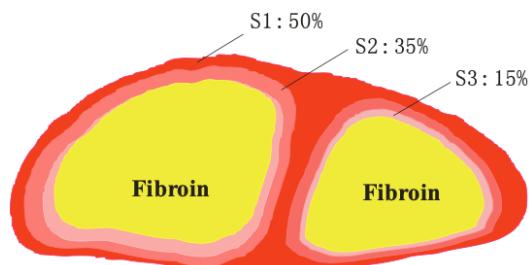


FIGURE 2.6. S1: outer sericin, S2: middle sericin, S3: inner sericin. Figure from Wang and Zhag [50].

The difference in solubility in water of the different layers of sericin is due to the significant difference in amino acid composition between the three layers as outlined in Table 2.1. From the outer layers to the inner, the percentage of non-polar (hydrophobic) amino acids increases from 27.90% to 46.02%. Conversely, the polar (hydrophilic) amino acid composition

TABLE 2.1. The amino acid composition (mol%) of silk fibroin (SF), whole sericin (Ser), outer sericin (S_o), middle sericin (S_m), and inner sericin (S_i) as described by Wang and Zhag [50]. Total polar amino acid composition below. * indicates a polar amino acid.

Amino acid	SF	Ser	S_o	S_m	S_i
Glycine	42.62	17.85	16.29	16.35	17.87
Alanine	33.38	6.70	5.20	6.13	11.58
Serine*	7.65	25.50	28.00	25.57	13.32
Tyrosine*	5.84	3.10	2.87	3.98	4.09
Valine	2.58	4.05	3.77	4.27	5.43
Aspartic acid*	1.79	18.38	17.97	17.08	15.83
Glutamic acid*	1.36	5.74	6.25	4.65	7.34
Threonine*	0.85	7.47	7.78	8.13	5.66
Phenylalanine	0.81	0.67	0.64	0.66	2.49
Isoleucine	0.72	1.02	0.79	1.17	3.76
Leucine	0.54	1.49	1.21	1.77	4.06
Proline	0.47	0.81	0.00	0.64	0.00
Arginine*	0.44	3.12	3.52	3.83	3.41
Lysine*	0.33	2.08	3.72	3.16	2.18
Cysteine*	0.26	0.38	0.69	0.95	0.75
Histidine*	0.21	1.32	1.32	1.69	1.38
Methionine	0.15	0.31	0.00	0.00	0.83
Total Polar:	7.65	24.94	28.00	25.57	13.32

decreases from 28.00% to 12.23% [50]. By comparison, the same paper determined the non-polar proportion of silk fibroin to be 80.75% and the polar to be 7.65%. The heterogeneous amino acid profile of sericin is at the core of the issues facing degumming processes. The water-solubility of the outer layers is highest, with decreasing solubility in the inner layers. The inner 15% of sericin is the most challenging to remove without causing damage to the silk fibroin fibers [51] due to its non-polar nature and similarity in amino acid profile to silk fibroin.

Wang and Zhag [50] also determined the molecular masses of the degummed sericin. The sericin obtained from the outer layer had the highest molecular mass, over 200kDa, as measured by SDS-PAGE (a process covered in section 2.3.1.1). Sericin of the middle layer a molecular mass of 20-100kDa, with that of the inner layer less than 20kDa. It's important to note here that this doesn't reflect the molecular weights of native sericin (within the silkworm or on untreated silk cocoons). It's rather a reflection of the cleaving that occurs during the

three stage degumming process that occurred during this analysis and indicates the molecular weight of the degummed layers of sericin.

2.3 Degumming

An ideal degumming agent will:

- (1) remove all sericin from the silk fibroin.
- (2) not degrade the silk fibroin.

The balance between these two competing priorities complicates degumming for biomedical applications [10], [36], [46]. Sericin removal is essential for its use in biomedical applications. Sericin-laden silk fibroin elicits an adaptive immune response from the body, whereas pure silk fibroin does not [11], [52], [53]. It's more resistant to solubilising into an aqueous solution and when solubilised, creates irregular hydrogels upon hydrogelated as in Figure 2.7 [46]. Degummed silk fibroin is more elastic, but has a lower tensile strength than the original bave [54], [55]

Conversely, overzealous degumming will result in damage of the SF which results in significant changes in its capacity for hydrogelation. As discussed in the previous section, the most difficult layer of sericin to remove is inner sericin due to it's chemical similarity to silk fibroin. Indeed, Ko, Yoon, Ki *et al.* [56] confirmed via SEM Imaging that some degumming methods can cause silk fibroin damage before complete degumming is achieved.

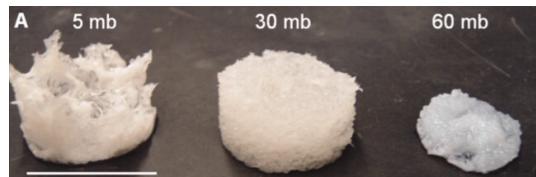


FIGURE 2.7. Hydrogel scaffolds made from SF degummed in 0.2% w/v Na_2CO_3 for 5, 30, and 60 mb (minutes boiled). 5 mb: incomplete degumming. 30 mb: complete degumming, some SF degradation. 60 mb: complete degumming, severe SF degradation. Figure from Wray, Hu, Gallego *et al.* [46].

Choosing a degumming method involves a careful balance between variables such as the agent, concentration, temperature, and duration. The specificity of degumming agents also plays a crucial role; non-specific agents like Na_2CO_3 are more likely to attack silk fibroin

leading to undesirable degradation, but specific agents that target certain amino acid bonds like proteases may not result in complete degumming, as we'll discuss further in Section 2.4.2.

Wray, Hu, Gallego *et al.* [46] demonstrated the effects of both under and over-degumming silk and proposed a model for SF degradation due to degumming. The researchers degummed cocoons in 0.2% w/v Na₂CO₃ for 5, 30, and 60 minutes. Figure 2.7 shows the effects of incomplete degumming at 5 mb (minutes boiled), complete degumming at 30 mb, and complete degumming with significant SF degradation at 60 mb. Section 2.4.1.2 will elaborate further on degumming with Na₂CO₃.

Excessive degradation also affects the chemical properties and suitability of silk fibroin for biomedical applications. Polymer materials with lower molecular weights, such as degraded SF, are more prone to enzymatic degradation and hydrolysis in the body. Wray, Hu, Gallego *et al.* [46] demonstrated that SF degradation can result in changes to the surface charges of silk films post-degumming, affecting how proteins in cell culture media adsorb to the silk surface.

Interestingly, while prolonged degumming leads to increased degradation of silk fibroin, there is no significant impact on the β -sheet content, indicating that these structurally critical

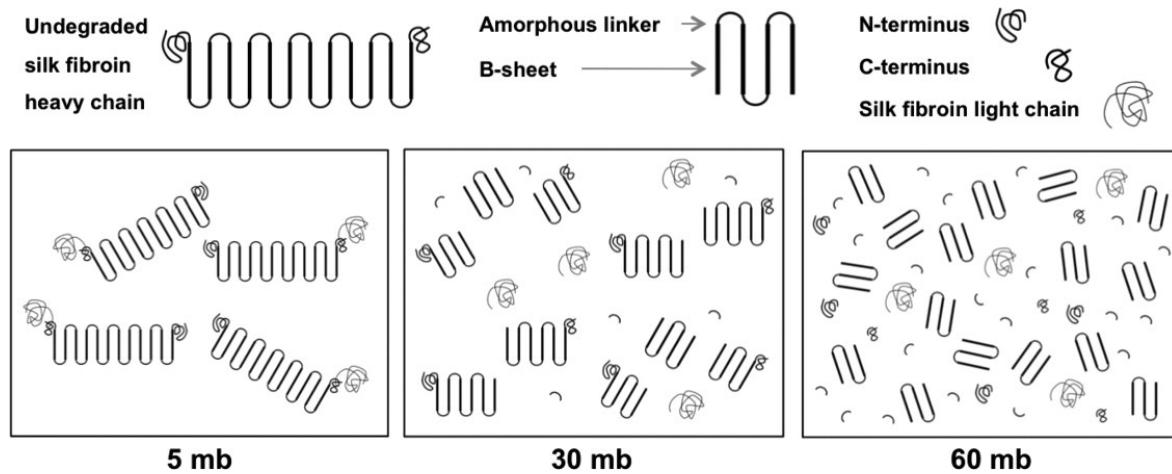


FIGURE 2.8. SF degradation model for degumming in 0.2% w/v Na₂CO₃ for 5, 30, and 60 minutes. Cleaving occurs in the hydrophilic amorphous regions and α helix turns while the hydrophobic β -sheet content remaining constant. Figure from Wray, Hu, Gallego *et al.* [46].

secondary structures remain intact during the degumming process. Figure 2.8 outlines the proposed mechanism of SF damage during degumming. The degradation occurs in the non-repetitive, amorphous regions, and the N- and C-terminal domains of the silk fibroin [46], [57].

2.3.1 Measuring Degumming

Researchers use several methods to determine the completeness of degumming and its effects on the remaining silk fibroin. Amino acid composition analysis and fluorescence spectroscopy are an accurate, though expensive, way to detect sericin residuals in silk solutions and to assess changes in silk fibroin [46]. A simple, yet effective method of measuring the amount of sericin removed from a sample is via the degumming ratio (DR), the mass loss percentage of the silk (m_0) after degumming (m_1) [48], [58].

$$DR(\%) = \frac{m_0 - m_1}{m_0} * 100 \quad (2.1)$$

Given the sericin layer comprises 25-30% of the mass of a cocoon shell, it's expected that effective degumming method will result in a degumming ratio in that range. While there will be some variation, it's expected that, a DR above 30% indicates degradation and mass loss of the silk fibroin and a DR below 25% conversely indicates incomplete sericin removal. While this ratio is a good starting point for determining the efficacy of the degumming method, it provides no indication of whether that method degrades the Silk Fibroin.

Degradation of silk fibroin is characterised by a decrease in its molecular weight as the protein chains are cleaved. When solubilised into an aqueous solution, the viscosity of the solution is often used as an indicator of SF damage [57]. Aqueous polymers with higher molecular weight produce more viscous solutions at the same concentration

Strain testing can provide information about the integrity of the protein chains in degummed silk. As mentioned, degummed silk is more elastic than the original bave, but degummed silk also has a lower ultimate fracture strain. Feng, Jiaming Lin, Lin *et al.* [39] found that raw

silk has a breaking strain of 25.2%, which decreased to 16.5% after degumming with 3 g/L papain and 8.7% when degummed with Na_2CO_3 . Table 2.2 allows further comparison.

2.3.1.1 SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate-poly(acrylamide) gel electrophoresis, is a technique used to determine the distribution of molecular weights within a protein sample [59], [60]. Researchers treat the protein sample with sodium dodecyl sulfate (SDS), a surfactant that denatures and coats proteins, imparting them with a negative charge that is proportional to their size.

This protein-surfactant mixture is then loaded into wells situated at the top of a polyacrylamide gel. Electrophoresis involves applying an electric field to the gel, causing the negatively-charged proteins in the wells to migrate towards the positive electrode at the opposite end of the gel. Proteins with smaller molecular weights will migrate more rapidly through the polyacrylamide matrix, thereby separating the proteins based on molecular weight during their passage through the gel. The proteins are then stained with a dye such as Coomassie Blue, yielding a series of distinct bands that correspond to different molecular weights as in figure 2.9. A marker lane containing proteins of known molecular weights undergoes SDS-PAGE alongside the samples.

As discussed in section 2.2.1, SF consists of 390kDa heavy protein chains, 26kDa light protein chains, and 30kDa glycoproteins found in a 1:1:6 ratio. An SDS-PAGE analysis of pure SF should then return clear bands around those regions, as shown in sample F in Figure 2.9. If an SDS-PAGE does not return those bands, or shows vibrant smears in other

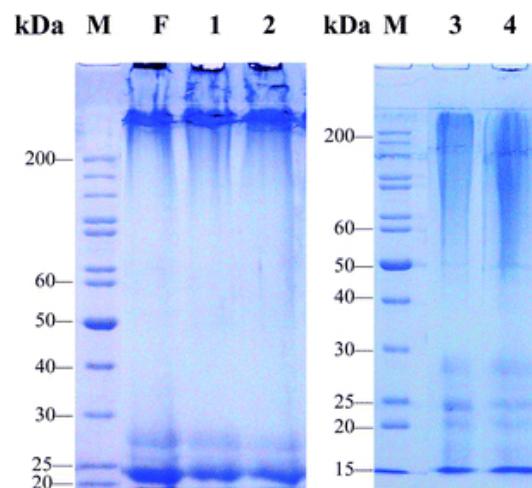


FIGURE 2.9. An example of SDS-PAGE analysis of SF after different degumming & solubilising methods. F: NSF, 1:Urea-LiBr, 2: Na_2CO_3 -LiBr, 3: Na_2CO_3 -LiBr, 4: Na_2CO_3 - CaCl_2 M:standard gradient marker. Figure from Wang and Zhang [61].

sections as in samples 3 and 4 of Figure 2.9, this shows either degradation to the SF chains, or residual sericin. Even if there is residual sericin, that will represent a small percentage of the degummed mass and will not change the bands. Of particular importance to degumming for hydrogelation is the smearing at the higher molecular weight region, as shown by samples 3 and 4 in Figure 2.9, indicates degradation of the SF heavy chains.

2.4 Degumming Methods

In this section, we will delve into a comprehensive exploration of the latest methods and agents used in degumming. Broadly speaking, these methods involve chemical, biological, and physical agents, each offering unique advantages and challenges as summarised in Table 2.2. By understanding the principles and outcomes of each degumming method, we aim to identify promising candidates that can potentially provide a low-cost, non-toxic, and efficacious method of degumming silk fibroin in small batches.

Chemical methods often involve such as soaps, alkaline solutions, or acids to dissolve sericin, while biological methods typically employ enzymes. Physical treatments encompass techniques such as steam, high-temperature and high-pressure, and ultrasonication, which apply mechanical and thermal energy to the system to enhance water hydrolysis of sericin or enhance other methods.

Each method carries its own advantages and disadvantages. Chemical methods offer a well-established, simple, and often cheap approach, but they may introduce undesirable chemical residues or result in degradation of the silk fibroin structure due to their non-specific mechanisms [55], [62], [63]. Biological methods are often less toxic and more gentle on the fibroin, but face challenges with enzyme stability, specificity, and cost [39], [64], [65]. Physical treatments rely on mechanical disruption, but their effectiveness and impact on the fibroin structure are often lacking [66]–[68]. Researchers are increasingly combining different degumming agents to create methods with a high degumming efficacy and minimal Silk Fibroin degradation.

The following table provides a detailed summary of various degumming methods applied to silk, after which we will dive into a discussion about the different agents and methods. The table makes distinction between the form of silk used: cocoons (all are *Bombyx mori*) or raw silk. In the textile industry, silk is often degummed post-weaving to maintain the integrity and uniformity of the fabric, ensuring smoother handling and a more appealing lustre in the final product [54], [69]. Raw silk is the name given to this not-degummed, woven silk. Ostensibly, raw silk should have a similar sericin content as the cocoon, but to date no studies have been performed to show this. Further categories have been explained in the glossary. To expand the available literature, degumming techniques used on raw silk have been included, though an important note of caution needs to be maintained when attempting to make direct comparisons across different studies and techniques. This thesis will later attempt to address these problems to allow better analysis of the literature in this field.

This table is best used as a reference guide for the coming sections and chapters. Though expansive, it does not represent the complete works of degumming science. I have included methods and studies that will be relevant for the coming discussions as well as important illustrative examples.

TABLE 2.2. An overview of different degumming methods, grouped by the primary type of agent, with notes of relevant information about the effect of these methods on silk fibroin or other relevant information. Further categories of Raw silk are expanded upon in the glossary.

Agent	Degumming Method	DR (%)	Type of Silk	Notes	Ref
Chemical					
CaOH	0.025% w/v; 100°C; 20 min; 2 cycles	26.78	Cocoons	No investigation of the SF	[70]
Citric acid	2% w/v; 100°C; 30 min	26.70	Cocoons	Higher MW than Na ₂ CO ₃ . Lower than HTHP.	[71]
Citric acid	2% w/v; 100°C; 30 min	27.00	Cocoons	Better MW and mechanical properties than Marseille Soap + Na ₂ CO ₃ . Similar to HTHP. Lower than urea.	[57]
Marseille soap	0.2% w/v; 100°C; 30 min; 5 cycles	25.00	Cocoons	Better mechanical properties than Na ₂ CO ₃ ; Similar to SAEW. Fracture strain 45.11%.	[68]
Marseille soap	0.2% w/v; 100°C; 30 min; 4 cycles	23.50	Raw silk	SDS-PAGE: Severe SF damage	[67]
Na ₂ CO ₃	0.005% w/v; 100°C; 30 min	27.92	Raw silk - 6A	SDS-PAGE + SEM: Slight SF damage	[62]
Na ₂ CO ₃	0.02% w/v; 100°C; 60 min	26.00	Cocoons		[72]
Na ₂ CO ₃	0.05% w/v; 100°C; 30 min; 3 cycles	23.43	Raw silk - reel		[39]
Na ₂ CO ₃	0.05% w/v; 100°C; 30 min; 3 cycles	22.93	Raw silk		[73]

TABLE 2.2. An overview of different degumming methods, grouped by the primary type of agent, with notes of relevant information about the effect of these methods on silk fibroin or other relevant information. Further categories of Raw silk are expanded upon in the glossary.

Agent	Degumming Method	DR (%)	Type of Silk	Notes	Ref
Na ₂ CO ₃	0.05% w/v; 100°C; 30 min; 3 cycles	23.52	Raw silk		[74]
Na ₂ CO ₃	0.05% w/v; 100°C; 30 min; 4 cycles	24.00	Raw silk	SDS-PAGE: severe SF damage	[67]
Na ₂ CO ₃	0.212 % w/v; 30 min	32.40	Cocoons	SDS-PAGE + SEM: Complete degumming, minimal SF damage	[43]
Na ₂ CO ₃	0.212 % w/v; 120 min	44.40	Cocoons	SDS-PAGE + SEM: Severe SF damage	[43]
Na ₂ CO ₃	0.5% w/v; 100°C; 30 min	32.64	Raw silk - 6A	SDS-PAGE + SEM: Moderate SF damage	[62]
Na ₂ CO ₃	0.5% w/v; 100°C; 30 min; 2 cycles	34.00	Cocoons		[50]
Na ₂ CO ₃	0.5% w/v; 100°C; 30 min; 2 cycles	33.00	Cocoons		[68]
Na ₂ CO ₃	0.5% w/v; 100°C; 30 min; 2 cycles	25.22	Cocoons	SDS-PAGE + SEM: severe SF damage	[75]
Na ₂ CO ₃	5% w/v; 100°C; 30 min	68.71	Raw silk - 6A	SDS-PAGE + SEM: severe SF damage	[62]
Na ₂ CO ₃ + Marseille soap	0.1% w/v; 1 % w/v; 98°C; 60 min	27.00	Raw silk - crêpe		[65]

TABLE 2.2. An overview of different degumming methods, grouped by the primary type of agent, with notes of relevant information about the effect of these methods on silk fibroin or other relevant information. Further categories of Raw silk are expanded upon in the glossary.

Agent	Degumming Method	DR (%)	Type of Silk	Notes	Ref
Na ₂ CO ₃ + sodium oleate	0.067% w/v; 0.1 % w/v; 100°C; 120 min	25.60	Cocoons	SEM: Slight SF damage	[56]
Na ₂ CO ₃ + sodium oleate	0.2% w/v; 0.3 % w/v; 100°C; 60 min	26.8	Cocoons		[71]
Na ₂ CO ₃ + sodium oleate	0.4% w/v; 0.6 % w/v; 100°C; 120 min	28.50	Cocoons	SEM: Moderate SF damage	[56]
NaHCO ₃ + US + Irgasol NA, then Marseille soap	0.5% w/v; 70W; 0.5% w/v; 60°C; 90 min, then 0.5% w/v; 100°C; 15 min	21.32	Raw silk - yarn	Fracture strain 15.01%	[54]
Urea	8.0 M; 90°C; 180 min	23.06	Raw silk	SF MW 126.4 kDa	[74]
Urea + mercaptoethanol	8.0 M; 5% (v/v); 80°C; 2 h	27.20	Cocoons	Better MW and mechanical properties than citric acid, HTHP, and Marseille soap + Na ₂ CO ₃	[57]
Urea + mercaptoethanol + Tris-SO4	8.0 M; 5% (v/v); 0.04 M; 80°C; 120 min	-	Cocoons	Better MW and mechanical properties than SAEW and Na ₂ CO ₃ . No DR. Fracture strain 45.11%.	[61]

TABLE 2.2. An overview of different degumming methods, grouped by the primary type of agent, with notes of relevant information about the effect of these methods on silk fibroin or other relevant information. Further categories of Raw silk are expanded upon in the glossary.

Agent	Degumming Method	DR (%)	Type of Silk	Notes	Ref
Biological					
Acidic protease	60 U/g of silk; pH 3; 50°C; 180 min	7.60	Raw silk - crêpe		[65]
Alkaline protease	1 U/g of silk; pH 10; 60°C; 240 min	17.60	Raw silk - crêpe		[65]
Alkaline protease	2 U/g of silk; pH 10; 65°C; 240 min	24.00	Raw silk - crêpe		[65]
Bromelain	4 g/L; pH 6.4; 40°C; 60 min; 2 cycles	21.76	Raw silk	SEM: Incomplete degumming	[73]
Bromelain	4 g/L; pH 6.4; 40°C; 60 min; 2 cycles	23.14	Raw silk	SF MW 142.2 kDa. Produced hydrogels with good biocompatibility and compressive strength.	[74]
Cocoonase	120 µg/mL; pH 8; 40°C; 60 min; 39.00 cycles	39.00	Cocoons	SEM: 98% sericin removed, no SF damage. No data provided.	[76]
Metalloprotease	490 U/mL; 15°C; 12 h	23.47	Raw silk	Fracture strain 21.78%	[75]
Metalloprotease	490 U/mL; 25°C; 12 h	24.03	Raw silk	Fracture strain 21.83%	[75]

TABLE 2.2. An overview of different degumming methods, grouped by the primary type of agent, with notes of relevant information about the effect of these methods on silk fibroin or other relevant information. Further categories of Raw silk are expanded upon in the glossary.

Agent	Degumming Method	DR (%)	Type of Silk	Notes	Ref
Metalloprotease	490 U/mL; 35°C; 12 h	24.90	Raw silk	Fracture strain 20.89%	[75]
Metalloprotease	490 U/mL; 45°C; 12 h	22.90	Raw silk	Fracture strain 20.78%	[75]
Papain	0.1 U/g silk; pH 6; 65°C; 240 min	19.00	Raw silk - crêpe		[65]
Papain	3 g/L; 85°C; 60 min	22.73	Raw silk - 6A	Fracture strain 16.5%	[39]
Papain	4 g/L; pH 6.6; 5 °C; 60 min; 2 cycles	23.85	Raw silk	SEM: Incomplete degumming. SF MW 162.5 kDa.	[73]
Subtilisin	4 g/L; pH 7; 50°C; 60 min; 2 cycles	22.30	Raw silk	SEM: Incomplete degumming. SF MW 166.5 kDa.	[73]
Trypsin	4 g/L; pH 6.8; 38°C; 60 min; 2 cycles	24.53	Raw silk	SEM: Incomplete degumming. Severe SF damage. SF MW 168 kDa.	[73]
Physical					
H ₂ O	100°C; 60 min	13.10	Cocoons	No SF analysis	[50]
H ₂ O	100°C; 60 min	11.40	Cocoons	No SF analysis	[70]
HTHP H ₂ O	115°C; 60 min	27.00	Cocoons	No SF analysis	[50]

TABLE 2.2. An overview of different degumming methods, grouped by the primary type of agent, with notes of relevant information about the effect of these methods on silk fibroin or other relevant information. Further categories of Raw silk are expanded upon in the glossary.

Agent	Degumming Method	DR (%)	Type of Silk	Notes	Ref
HTHP H ₂ O	120°C; 30 min	27.00	Cocoons	No SF analysis	[50]
HTHP H ₂ O	125°C; 10 min	27.00	Cocoons	No SF analysis	[50]
HTHP H ₂ O	120°C; 30 min	26.50	Cocoons	Higher MW than citric acid and Na ₂ CO ₃	[71]
HTHP H ₂ O	120°C; 120 min	28.05	Cocoons	No SF analysis	[70]
HTHP H ₂ O	130°C; 60 min	22.60	Cocoons	SDS-PAGE + SEM: Removed 80% of sericin, no SF damage	[77]
Steam	140 kPa; 125°C; 120 min	24.00	Raw silk	SDS-PAGE: Severe SF damage	[67]
Steam	106 kPa; 120°C; 30 min	26.20	Cocoons	Autoclave. Better MW and mechanical properties than Marseille soap + Na ₂ CO ₃ . Similar to citric acid. Lower than urea.	[57]
Steam	106 kPa; 120°C; 30 min	31.00	Cocoons	Autoclave. SDS-PAGE + SEM: Complete degumming, slight SF damage	[43]
Ultrasonication (US)	60°C; 60 min	25.90	Cocoons	SDS-PAGE + SEM: incomplete degumming, slight SF damage	[43]
Mixed					
NaHCO ₃ +	0.5% w/v;	15.80	Raw silk - yarn	Fracture strain 17.97%	[54]

TABLE 2.2. An overview of different degumming methods, grouped by the primary type of agent, with notes of relevant information about the effect of these methods on silk fibroin or other relevant information. Further categories of Raw silk are expanded upon in the glossary.

Agent	Degumming Method	DR (%)	Type of Silk	Notes	Ref
US +	70W;				
Irgasol NA +	0.5% w/v;				
alcalase	0.1% w/v; 55°C; 30 min				
NaHCO ₃	0.5% w/v;	17.21	Raw silk - yarn	Fracture strain 13.68%	[54]
US +	70W;				
Irgasol NA +	0.5% w/v;				
savinase	0.1% w/v; 55°C; 40 min				
NaHCO ₃	0.5% w/v;	22.28	Raw silk - yarn	Fracture strain 14.12%	[54]
US +	70W;				
Irgasol NA,	0.5% w/v; 60°C; 15 min,				
then alcalase	then 0.1% w/v; 55°C; 30 min				
NaHCO ₃	0.5% w/v;	20.89	Raw silk - yarn	Fracture strain 11.94%	[54]
US +	70W;				
Irgasol NA,	0.5% w/v; 60°C; 15 min,				
then savinase	then 0.1% w/v; 55°C; 30 min				
NaHCO ₃ +	0.5% w/v;	22.29	Raw silk - yarn	Fracture strain 10.38%	[54]
US +	70W;				

TABLE 2.2. An overview of different degumming methods, grouped by the primary type of agent, with notes of relevant information about the effect of these methods on silk fibroin or other relevant information. Further categories of Raw silk are expanded upon in the glossary.

Agent	Degumming Method	DR (%)	Type of Silk	Notes	Ref
Irgasol NA, then alcalase +	0.5% w/v; 60°C; 15 min, then 0.1% w/v;				
savinase	0.1% w/v; 55°C; 30 min				

2.4.1 Chemical

Chemical degumming of silk, utilizing agents like sodium carbonate, citric acid, and urea, operates through distinct mechanisms to effectively remove sericin while preserving the integrity of silk fibroin. Surfactants like soaps improve the wettability of silk, allowing water penetration into the more hydrophilic inner sericin layers. Alkali and acids facilitate the breakdown of sericin through hydrolysis of peptide bonds. Urea, a chaotropic agent, disrupts the hydrogen bonds in sericin, enhancing its solubility in water. In general, chemical agents are cheap and highly customisable in concentration, making them attractive candidates for a lab-based method.

2.4.1.1 Soap

The textile industry has employed soap for centuries to perform degumming. Soap molecules are surfactants. They have a hydrophilic head and a hydrophilic tail, allowing them to interact well with both polar and non-polar substances. When mixed in water, they improve the wettability of silk, facilitating water penetration into the less polar inner sericin layer, thereby improving degumming results [48], [55].

Yuksek, Kocak, Beyit *et al.* [55] compared the efficacy of three different soaps - turpentine, daphne, and Marseille, on raw silk fabric degumming. They treated the fabric for 60 minutes at 95°C using a 0.7 % w/w soap solution in a 1:30 mass ratio silk to solution. Turpentine soap yielded the highest DR of 25%, indicating incomplete degumming.

They then repeated the experiment with the same soap solutions in an ultrasonic bath at 20 kHz for 20 minutes at 60°C. Despite the shorter time and lower temperature, turpentine soap under ultrasonic agitation yielded a DR of 28.9 %, again the highest of the soaps. Ultrasonication did also increase the DR using daphne and Marseille soaps by 1-2%, though the use of neither resulted in a DR above 25%. This study's findings does hold significance as they demonstrate that the combination of a surfactant, such as turpentine soap, with ultrasonic agitation can enhance degumming results without causing detectable damage to the silk fibroin.

The use of soap-based degumming in silk processing, especially for biomedical applications, is significantly hindered by inconsistencies in soap formulations. Variations in ingredients and their sources, such as the volatile turpentine in turpentine soap or the different oils and regional formulations for Marseille soap, directly impact the degumming process. Additionally, small variations in batch manufacturing can cause sizeable changes in the pH level of the soap solution, which can significantly change the degumming mechanism, from a surfactant/wetting approach to alkaline hydrolysis, detailed further in Section 2.4.1.2. Degumming with soap does not have the consistency required for a scientific method.

2.4.1.2 Sodium Carbonate

Sodium carbonate (Na_2CO_3) has long been the standard chemical agent for degumming silk in the textile industry. Typically, the process involves boiling the silk in a 0.05 - 0.5 % w/v solution for 30 minutes, sometimes several times, a method known for its efficiency and cost-effectiveness. However, despite its widespread use in the textile industry, sodium carbonate degumming has notable drawbacks, particularly when silk fibroin's integrity is a priority, as in biomedical applications.

The mechanism of sodium carbonate in degumming involves alkaline hydrolysis, where it hydrolyses the amide bonds of sericin [37]. While effective in sericin removal, this non-specific action often leads to inadvertent damage to the silk fibroin itself. Many studies have shown that degumming with sodium carbonate can result in reduced mechanical properties of silk fibroin, such as lower tensile strength, elasticity, and fracture strain [39], [43], [46], [61], [62], [67], [75].

Many attempts have been made to modify the duration of degumming and concentration of the solution to limit SF damage. Shorter degumming times remove less sericin but limit fibroin damage, whereas extended periods increase sericin removal but also lead to considerable fibroin damage [43]. Similarly, lower concentrations limit sericin removal and fibroin damage [62]. Attempts have been made to improve the degumming ratio of solutions of lower concentrations of sodium carbonate, such as through the addition of Marseille soap [65] or

even using sodium bicarbonate, a less basic substance, alongside non-ionic surfactants and ultrasonication [54], though with little success.

At this point it may be tempting to compare the Degumming Ratios of the many different sodium carbonate methods in Table 2.2, but the slight differences between cocoon and raw silk sources prevents that. Thankfully, Allardyce, Rajkhowa, Dilley *et al.* [48] tested 18 different combinations, not listed in Table 2.2 for brevity sake, of temperature, concentration, and duration, concluding that using a 0.2% w/v solution at 98 °C for 30 minutes was the best method for degumming silk for biomedical applications. However, they also note that this method does too lead to SF degradation.

Despite the evident drawbacks of sodium carbonate, it remains a common control in many studies due to its established methodology and low cost. However, the lack of standardisation in testing methods and the use of different silk forms (cocoons vs. raw silk) in studies pose challenges in directly comparing results across different research works. This thesis will help standardise the literature for sodium carbonate, allowing more direct comparison without undertaking large replication study like Allardyce, Rajkhowa, Dilley *et al.* [48].

2.4.1.3 Urea

Urea, or carbamide ($\text{CH}_4\text{N}_2\text{O}$), functions as a degumming agent through a unique chemical action. As a by-product of protein metabolism and a weak organic base with a pH of 7.05 in a 1.0 M solution, urea acts primarily as a chaotropic agent. This means it disrupts the hydrogen bonds and hydrophobic interactions that stabilise proteins like sericin thereby enhancing its solubility in water [37], [58].

Urea degumming of silk, using an 8.0 M solution at 80°C to 90°C, effectively removes sericin while preserving silk fibroin. Wang, Wang, Pan *et al.* [74] reported successful sericin removal with minimal fibroin damage, achieving a degumming ratio of 23.06 %. Wang, Yang, Li *et al.* [44] found that urea-treated silk retained higher molecular weight than sodium carbonate-treated silk, indicating less structural damage. Kim, Hyun Ju Kim, Hyun-Ju Kim *et*

al. [57] added 2-mercaptoethanol, a reducing agent that targets disulfide bonds, to improve urea's performance, increasing the DR to 27.2 %.

As a non-toxic substance, further investigation of urea as a degumming agent for biomedical applications is warranted.

2.4.2 Biological

The textile industry began investigating the use of biological agents such as enzymes in the late 1990s to deal with the environmental impact of the large amounts of alkaline waste water discarded after industrial degumming [78]–[80]. Proteases are, in simple terms, enzymes that primarily act on the peptide bonds found in proteins, peptides, and other related molecules. Unlike non-specific chemical agents like Na_2CO_3 , some proteases can target certain peptide bonds, such as trypsin that selectively targets the bond between lysine and arginine [72]. The selectivity of proteases may lead to enhanced degumming methods that specifically target sericin while preserving silk fibroin.

2.4.2.1 Cocoonase

Cocoonase is a protease produced by the *Bombyx mori* silkworm to soften the cocoon for escape after metamorphosis. Unajak, Aroonluke and Promboon [76] extracted this enzyme and expressed it in yeast to produce recombinant cocoonase, which they used to degum 'silk sheets'. The researchers placed *Bombyx mori* larvae on waxed paper and allowed them to spray out silk, forming a sheet. Their method yielded a DR of 39%, significantly above the expected ratio of sericin to silk fibroin in freshly spun silk. Analysis of the degummed SF by SEM and OPA assay indicated "cocoonase had no effect on fibroin fiber."

Strangely, similar analysis on the control degummed with Na_2CO_3 also "showed no sign of destruction or damage to the surface of the fibroin fiber." This conflicts strongly with the rest of the literature. A big limitation of this paper is that it didn't include key results to contextualise their findings, such as the degumming ratio of the control. A possible reason for such a large degumming ratio with no fibroin damage may have to do with the way

the silkworms sprayed the thread. A plausible explanation is that the silkworms excreted non-cocoon silk, which contains 40% sericin. It's plausible that on wax paper, the lack of adhesion meant the silkworm never tried spinning a cocoon, and was instead stuck in a cycle of attempting adhesion. Further research using whole cocoons or raw silk is needed to compare the use of cocoonase to other degumming agents. The difficulty of producing this enzyme is prohibitive investigation in this thesis.

2.4.2.2 Trypsin

Trypsin is a common protease with evolutionary similarity to cocoonase [81]. It's a digestive enzyme that acts directly on lysine and arginine, both found in higher concentration in sericin than silk fibroin, except when either is adjacent to proline. Given the negligible amount of proline in both SF and sericin, this is unlikely to have statistical significance. Nultsch, Bast, Näf *et al.* [72] degummed silk cocoons in 1% w/v trypsin at pH 8 for 37°C for two hours, yielding a low DR of 20%. The trypsin treatment also degraded the silk fibroin as observed by mechanical testing and via SEM. Liu, Huang, Pan *et al.* [73] also retested trypsin with different conditions. Though the DR improved, significant damage to SF was again observed. SEM showed the degummed SF surface as rough and grooved with fibrils peeled from the surface. Trypsin appears to be too aggressive for use as a degumming agent.

2.4.2.3 Alkali Proteases

Alkali proteases function optimally in an alkaline environment. They are common in modern laundry detergents to aid in removing tough protein stains. The added mechanism of alkali hydrolysis may help remove the hydrophobic inner sericin that proteases struggle with.

Freddi, Mossotti and Innocenti [65] degummed raw silk using two of these common industrial alkali proteases (3374-L and GC 897-H), a neutral protease (papain) and an acidic protease (EC 3.4 23.18(D)). The acidic protease yielded a low DR of 7.6% after 180 min. Conversely, the alkali proteases yielded DRs of 17.6% and 24% respectively, against 27% for the Na₂CO₃-Marseille soap control DR. The concentration of the second protease was double that of the first which may explain the better yield.

Mahmoodi, Arami, Mazaheri *et al.* [54] incorporated alkali proteases into a novel multi-step degumming method. They first treated raw silk yarn in a solution of 5 g/L NaHC₀₃ and 5 g/L Irgasol NA at 60C for 15 min in a 70W ultrasonic bath. The DR after 15 minutes was 12%. Following this, they treated the samples with 1g/L of alcalase, savinase, or an equal mixture of both. After a further 30 minutes, the three samples yielded similar DRs of 22.28%, 20.89%, and 22.29% respectively. SEM images showed “perfect degumming and no sign of destruction and damage to the surface of the yarn.” The use of the much milder NaHC₀₃ over the common Na₂C₀₃ likely contributed to preserving the SF. Unfortunately, the alkali-soap control also used NaHC₀₃, yielding a DR of 13.75, significantly below the methods tested. This makes it difficult to compare these results to other degumming methods without repeating with a more standard control.

2.4.2.4 Neutral Proteases

Neutral proteases have an advantage of more mild degumming conditions, helping prevent any non-specific alkali hydrolysis [39]. Zhang, Li, Liu *et al.* [75] investigated the use of a crude sample of a halotolerant metalloprotease produced by *Vibrio* sp. LA-05 for degumming. These salt-tolerant (halotolerant) proteases use a metal ion in cleaving peptide bonds. A crude enzyme sample refers to one that still contains other components from the growth medium, such as proteins, cellular debris, and salts.

To explore the impact of temperature and duration on metalloprotease degumming, Zhang, Li, Liu *et al.* [75] measured the DR of raw silk samples treated at temperatures of 15°C, 25°C, 35°C, and 45°C after 1, 3, 6, 9, and 12 hours. All four temperature settings ultimately yielded a DR above 23% after 12 hours, with the higher-temperatures accelerating the protease activity [82]. Degumming at 35°C for 12 hours yielded a DR $24.90 \pm 0.20\%$ which equals that of the aggressive Na₂CO₃ control yield of 25.22%.

Mechanical testing demonstrated superior results from the metalloprotease. The control experienced failure at 0.76N with an elongation of 17.89%. All the protease-degummed fibers exhibited better results, with the 35°sample breaking at 0.79N and with 20.89% elongation. Though an good degumming agent, the difficulty of in-house production and storage of such

an enzyme renders it inappropriate for this thesis. Instead let us turn our attention to more common proteases.

2.4.2.5 Papain

Feng, Jiaming Lin, Lin *et al.* [39] explored the potential of papain, a protease found naturally in papaya fruit. Papain exhibits a preference for targeting peptide bonds involving arginine and lysine, though can also break of carboxyl groups of histidine, glutamine, leucine, tyrosine, glycine, and glutamic acid [73]. Papain is a promising degumming agent as sericin contains higher levels of arginine and lysine than silk fibroin, meaning papain should selectively target sericin. The high molecular weight of papain (23kDa) also makes it difficult to penetrate into the fibroins, helping prevent SF damage.

The study identifies optimal degumming conditions at a concentration of 3.0 g/L papain, with treatment conducted at 85°C for 60 minutes. Under these conditions, papain achieves a DR of 23%, comparable to the results of conventional degumming of the raw silk fabric. SDS-PAGE analysis showed no major degradation of silk fibroin. The choice of temperature seems high for papain, potentially reducing its efficacy. Liu, Huang, Pan *et al.* [73] yielded a better result using the same process with a concentration of 4g/L at 55°C, but noted that SEM imaging showed incomplete degumming and some SF damage. The specificity of papain may not be appropriate for the complete removal of inner sericin, whose arginine and lysine levels approach that of fibroin.

2.4.2.6 Bromelain

Pineapples, like papayas, produce a protease called bromelain shows a preference for targeting lysine, arginine, and glutamic acid [74]. Glutamic acid is another amino acid found in higher concentration in all three layers of sericin, in an even higher ratio than lysine or arginine. A protocol of degumming raw silk twice using 4g/L at pH 6.4 and 40°C for 60 minutes shows promising preliminary results.

Wang, Wang, Pan *et al.* [74] produced hydrogel scaffolds from bromelain-degummed silk fibroin. Compared to scaffolds produced from urea and Na_2CO_3 methods, the bromelain-degummed scaffolds showed slower enzyme degradation in vitro and “significantly higher” proliferation of human umbilical vein vascular endothelial (HUVEC) when seeded. SDS-PAGE showed a average molecular weight of 142.2 kDa, indicating minimal heavy chain cleaving. Correspondingly, the compression strength and compression modulus of bromelain-SF scaffold were significantly higher than those of urea- and Na_2CO_3 -SF scaffolds.

In a complementary study, Liu, Huang, Pan *et al.* [73] compared bromelain to trypsin and papain. Among all tested methods, bromelain degumming retained the highest proportion of high molecular-weight segments with an average molecular weight of 170 kDa, indicating minimal fibroin damage. Amino acid analysis revealed that bromelain achieved degumming ratios comparable to Na_2CO_3 , though amino acid analysis showed that the degummed SF still contained 0.76% sericin, slightly higher than the 0.33% in the control. Bromelain shows promise as a effective,

2.4.3 Physical

The structure of sericin, unlike silk fibroin, does not form the tightly packed crystalline β -sheets that contribute to SF’s strength. Sericin’s less ordered network of intermolecular interactions, including hydrogen bonds, hydrophobic interactions, and Van der Waals interactions, is still robust enough to for the middle and inner layers to remain intact in 100 °C water. As we have discussed, this resistance to water solvation can be overcome with the use of alkali or enzymes that break peptide bonds and reducing molecular chain length to enhance solubility.

Physical treatments rely on mechanical disruption of these bonds and improving the penetration of water into the more hydrophobic layers. The addition of energy in the form of temperature and pressure is used.

2.4.3.1 High Temperature, High Pressure (HTHP) Water

As discussed in Section 2.2.2, Wang and Zhag [50] found 50% of sericin can be removed by boiling in water at 100°C. By increasing the pressure of the system to 2 atm, they were able keep water in it's liquid phase past boiling, removing a further 35% of the sericin 120 °C water.

Wang and Zhag [50] also determined a relationship between temperatures beyond 100°C and duration. With an array of temperatures from 100 °C to 125 °C and durations from 5 to 120 minutes, they determined that the maximum degumming ratio for water was around 27%. The boiling point sample never reached that, though as the temperature increased, that maximum occurs faster and faster. There was no difference in the degumming ratio for the 125 °C sample at 10 minutes and 120 minutes. This suggests that water degumming along will not degrade, or at least dissolve away any silk fibroin, aligning with other evidence of it's thermal stability [48]. These results have since been confirmed by further testing at 130 °C for 120 minutes [70].

Kalita, Allardyce, Sankaranarayanan *et al.* [77] performed similar testing at 130 °C for 60 minutes, with SDS-PAGE and SEM imaging confirming an estimated 80% of sericin was removed with a DR of 22.60%. Given the similar percentage (80 vs 85% for Wang and Zhag [50]), this is likely due to a lower sericin content for the cocoons used in this experiment. This example serves as an important reminder that direct comparison of degumming ratios between papers is difficult due to the natural variations in sericin level.

Though HTHP water degumming alone cannot remove all the sericin, it may be a useful tool to remove most of it. This may potentially reduce the amount of silk fibroin damage that occurs with a more aggressive method by reducing the time required for complete degumming.

2.4.3.2 Steam

The evidence for the use of steam, rather than HTHP water is mixed. An autoclave is often used as a convenient vessel for steaming. Though the degumming ratios for steam are similar to that of HTHP water, SDS-PAGE and SEM imaging show higher levels of SF damage [43].

In particular, severe damage was detected by SDS-PAGE when the pressure was increased to 140kPa [67]. Steaming in an autoclave may be a more convenient, though less effective alternative to degumming with HTHP water.

2.4.3.3 Ultrasonication

Ultrasonication (US) is best used to enhance results in a liquid medium through the phenomenon of cavitation [43], [54]. This process occurs when ultrasound waves are applied to a liquid, leading to the formation, rapid oscillation, and violent collapse of micro-bubbles under high acoustic pressure. These collapses near solid surfaces generate micro-jets and shock waves. These collapses near solid materials, such as silk fibers, create micro-jets and shock waves. This action significantly enhances micro-mixing in the liquid, which improves the distribution and penetration of degumming agents into the silk.

2.5 Fiber dissolution

Unlike with the degumming process, the literature is clear on the best current method for dissolving silk fibroin, without causing SF damage.

Traditionally, an 8.0 M LiBr solution has been the go-to solvent for silk dissolution Cristian Lujerdean, Baci, Cucu *et al.* [17] and Feng, Jiaming Lin, Lin *et al.* [39]. However, this method comes with its challenges. Lithium bromide's SDS alerts that it is acutely toxic, corrosive, irritating, and sensitising to skin, and can cause serious eye damage if exposed.

More recently, CaCl₂ has emerged as an alternative solvent. Research by Cheng, Wang, Wu *et al.* [83] confirms that regenerated silk fibroin (RSF) prepared using a ternary reagent of CaCl₂, ethanol, and water retains the same morphology and amino acid composition as that prepared using LiBr. However, a slight caveat is the small amount of calcium residue remaining in the RSF even after extensive dialysis. Despite this, the residual calcium does not significantly affect the structure and biological performance of RSF, including its cytotoxicity, blood

compatibility, and antibacterial properties, making ternary reagents a promising alternative for silk dissolution.

Looking to the future, ionic liquids show potential as an innovative dissolution system for silk. Ionic liquids typically consist of large organic cations and organic or inorganic anions. The unique properties of ionic liquids could offer new avenues for effective and efficient silk fiber dissolution, expanding the possibilities for silk processing and application Hang Heng, Qianqian Deng, Yipeng Yang *et al.* [84].

2.6 Limitations of the Literature

In concluding this literature review, I've identified a significant gap in the current research on silk degumming, which I aim to address in this thesis. A key challenge in this field is the difficulty in directly comparing degumming ratios between studies, largely due to the natural variation in sericin levels in silk. This problem is exacerbated by the use of various types of raw silk and cocoons in different experiments, all which seem to have markedly different sericin levels.

To tackle this issue, I propose an innovative approach. Given the impracticality of repeating every experiment in the field, I've chosen to focus on a select few experiments commonly used as 'controls' in multiple studies.

Firstly, I plan to assess how different degumming methods performed relative to their respective controls. For example, if a study's control method of 0.5% Na_2CO_3 results in a DR of 25% and within the same paper, a urea based method yields a DR of 27.5%, then I can say that the urea method removes 2.5% more mass than it's control.

If I then repeat that control experiment using my cocoons, which will have a unique sericin level, and find it yields a DR of 30%, that means the urea based method will likely yield a DR of 32.5%.

Further, if I perform the same control experiment with raw silk and yield a DR of 20%, that provides evidence that there's likely a significant difference in the level of sericin in my cocoons and raw silk.

This comparative analysis is crucial as it allows me to bridge the gap between different silk types and degumming methods. Ultimately, my aim is to enable a more coherent and comprehensive understanding of the current literature, facilitating meaningful conversions between studies and silk types. By doing so, I hope to provide a more robust and sound analysis of silk degumming methods, offering valuable contributions to the broader field of silk research.

The controls chosen for investigation are:

TABLE 2.3. The controls chosen for replication and the number of methods they're linked to.

Agent	Degumming Method	# of Linked Methods	Ref
Na ₂ CO ₃	0.05% w/v; 100°C; 30 min; 3 cycles	7	[39], [73], [74], [76]
Na ₂ CO ₃ + sodium oleate	0.2% w/v; 0.6 % w/v; 100°C; 120 min	2	[71]
Na ₂ CO ₃	0.5% w/v; 100°C; 30 min; 2 cycles	9	[50], [68], [75]
CaOH	0.025% w/v; 100°C; 20 min; 2 cycles	2	[70]

We will begin with preliminary tests to determine an adequate bath ratio. From there, we will perform other experiments to help us understand the relationship between sericin solubility and the variables of temperature, time, and surfactant use.

CHAPTER 3

Methods

The methods for preparing silk, degumming testing, and mathematical analysis are detailed in this section. All chemicals used were sourced from Merck. All water used was drawn from a Milli-Q water system inside the lab.

A makeshift drying oven was created with a Homemaker 35 Litre Large Oven, within which a tray of silica desiccant gel beads was placed. The oven operated at 100 °C with a 60 minute timer. The oven was turned on for 3 consecutive hours per day. For the remainder of the time, the drying silk remained in the oven with the silica desiccant gel beads. Preliminary testing showed that with this protocol, the mass of boiled cocoons stabilised after 48 hours, suggesting complete drying.

3.1 Preparing the Silk

3.1.1 Preparing the Cocoons

Bombyx mori silkworm waste cocoons were purchased from Everything Silkworms, a Melbourne, Australia based silkworm supplier. To prepare the cocoons, the outsides were lightly brushed with a paper towel to remove excess floss. The holed end was cleaved and the rest of the cocoon was halved with scissors, discarding the pupa shell remaining within. The inner floss lining, shown in Figure 3.1b was removed by rubbing vigorously with a paper towel. Excessively stained or soiled sections, as in Figure 3.1c of the cocoon were excised. The cocoons halves were cut in half again, resulting in cocoon quarters.

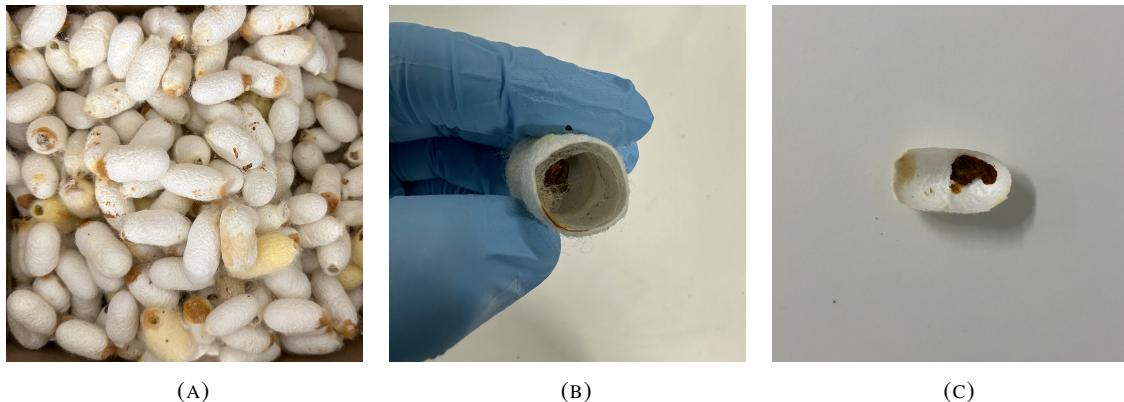


FIGURE 3.1. (A) *Bombyx mori* cocoons with visible holes caused by emerging moths; (B) a cleaved cocoon, showing the inner floss layer; and (C) a halved cocoon, showing excess staining and soiling to be excised before degumming.

3.1.2 Preparing the Raw Silk Yarn

Raw *Bombyx mori* silk yarn was purchased from Nundle Woolen Mill. The yarn was cut into 1 cm segments so the length of the raw silk fibers roughly matched the longest length of the cocoon quarters.

3.2 Preliminary Testing

3.2.1 Determining the Bath Ratio

The aim was to determine the Bath Ratio (the ratio of silk mass to degumming solution volume) for the experiments. The key variable was the evaporation rate of the degumming solution, ensuring that the cocoons are still fully submerged at the end of the degumming time. In short, approximately 0.5 g of cocoons were prepared described in section 3.1. The required amount of Milli-Q water, as per Table 3.1, was heated in a 100mL beaker on a hotplate until the required temperature was reached.

The samples were then added to the beakers and lightly stirred with a stirring rod to ensure complete wetting. A magnetic stirrer was used at 200 rpm to mix the samples throughout the process. Aluminium foil was lightly wrapped around the top of the beaker to prevent excessive evaporation. The samples were visually observed to ensure that no beakers fully

evaporated. If the volume became dangerously low, the beaker was removed from the hotplate. After the required time, the beakers were visually observed to determine if the remaining liquid volume was sufficient to keep the cocoons fully submerged.

TABLE 3.1. Preliminary testing for determining the Bath Ratio for future experiments.

Bath Ratio	Cocoon mass (g)	Volume (mL)	Time (s)
1:25	0.5	12.5	30
1:50	0.5	25	30
1:100	0.5	50	30
1:150	0.5	75	30

3.2.2 Variable Testing

Initial testing was performed to better understand the relationship between sericin solubility and the variables of temperature, time, and surfactant use.

Test IDs were developed for easy reference to different tests. The goal with the Test IDs was to create a short name that encapsulates the important variables involved in the test, allowing the reader to understand the results and discussion without needing to constantly referencing the methods section. They are formatted as follows:

- If the test uses pure water, as in Sections 3.2.2.1 and 3.2.2.2, the format is variable-temperature-repeats. For example, for the temperature variable testing, a test at 100 °C with two degumming cycles will be temp-100-2.
- If the test involves a solution, as in Sections 3.2.2.3 and 3.3, the format is concentration-agent. For example, for 0.05% w/v Sodium Carbonate the Test ID will be 0.05-Na₂CO₃ and for 0.025% w/v Calcium Hydroxide, the Test ID will be 0.025-CaOH.

Notes:

- Cycles are inclusive of the first cycle. For example, the process for a test with one cycle is performed once. The process for a test with two cycles is performed twice.

- To maintain consistency with the test protocols in section 3.3, two cycle of 30 minutes, instead of one cycle of 60 minutes, were performed during the water-only variable testing.

3.2.2.1 Temperature

Cocoon and raw silk samples were tested under the conditions listed in Table 3.2. In short, approximately 0.5 g of cocoon and raw silk samples were prepared as described in section 3.1, then weighed with the precise mass recorded. 75mL Milli-Q water was heated in a 100mL beaker on a hotplate until the required temperature was reached, except in the case of 23 °C, the room temperature of the lab, where the beaker was not heated on the hotplate.

TABLE 3.2. Conditions used for testing the effect of temperature on the solubility of sericin.

Test ID	Agent	Temp (°C)	Time (min)	Cycles
temp-23-2	H ₂ O	23	30	2
temp-50-2	H ₂ O	50	30	2
temp-100-2	H ₂ O	100	30	2

The samples were then added to the beakers and lightly stirred with a stirring rod to ensure complete wetting. A magnetic stirrer was used at 200 rpm to mix the samples throughout the process. Aluminium foil was lightly wrapped around the top of the beaker to prevent excessive evaporation. After the first cycle, the samples were removed from the water with tongs and the liquid strained to prevent mass loss. The samples were rinsed with copious amounts of room temperature Milli-Q water. The above process was repeated as required. After the final cycle, the samples were squeezed with gloves hands of excess water, spread onto aluminium foil sheets and dried in the drying oven for 48 hours. The dried sample was weighed to determine the mass loss and the degumming ratio was calculated as per equation 2.1.

3.2.2.2 Time

Cocoon and raw silk samples were tested under the conditions listed in Table 3.3 using the method described in section 3.2.2.1.

TABLE 3.3. Conditions used for testing the effect of degumming time on the solubility of sericin

Test ID	Agent	Temp (°C)	Time (min)	Cycles
time-23-1	H ₂ O	23	30	1
time-23-2	H ₂ O	23	30	2
time-100-1	H ₂ O	100	30	1
time-100-2	H ₂ O	100	30	2

3.2.2.3 Surfactants

One of the tests performed in Section 3.3 calls for the use of a surfactant, sodium oleate, to improve the wettability of silk. To determine the effect of this surfactant, that test was repeated without the surfactant. Cocoon and raw silk samples were tested under the conditions listed in Table 3.3 using the method described in section 3.3.

TABLE 3.4. Conditions used for testing the effect of a surfactant on the solubility of sericin

Test ID	Agent	Temp (°C)	Time (min)	Cycles
0.2-Na ₂ CO ₃	0.20% Na ₂ CO ₃	100	30	2
0.2-Na ₂ CO ₃ -SO	0.20% Na ₂ CO ₃ + 0.30% sodium oleate	100	30	2

3.3 Replicating the Controls

Cocoon and raw silk samples were tested under the conditions listed in Table 3.5. In short, approximately 0.5 g of cocoon and raw silk samples were prepared as described in section 3.1, then weighed with the precise mass recorded.

To create the solutions, 75mL of Milli-Q water was added to a 100mL beaker. The corresponding mass of dry solute was calculated using Table 3.5, weighed, then added to the beaker and stirred gently to dissolve. The beaker containing the solution was heated on a hotplate until the required temperature was reached.

TABLE 3.5. Conditions as per Table 2.2 used for replicating the control experiments with both cocoons and raw silk

Test ID	Agent (w/v)	Temp (°C)	Time (min)	Cycles	Ref
0.05-Na ₂ CO ₃	0.05% Na ₂ CO ₃	100	30	3	[39], [73], [74], [76]
0.2-Na ₂ CO ₃ -SO	0.20% Na ₂ CO ₃ + 0.30% sodium oleate	100	30	2	[71]
0.5-Na ₂ CO ₃	0.50% Na ₂ CO ₃	100	30	2	[50], [68], [75]
0.025-CaOH	0.025% CaOH	100	30	2	[70]

The samples were then added to the beakers and lightly stirred with a stirring rod to ensure complete wetting. A magnetic stirrer was used at 200 rpm to mix the samples throughout the process. Aluminium foil was lightly wrapped around the top of the beaker to prevent excessive evaporation. After the first cycle, the samples were removed from the water with tongs and the liquid strained to prevent mass loss. The samples were rinsed with copious amounts of room temperature Milli-Q water. The above process was repeated, using the same volume and concentration of solutions, as required. After the final cycle, the samples were squeezed with gloves hands of excess water, spread onto aluminium foil sheets and dried in the drying oven for 48 hours. The dried samples were weighed to determine the mass loss and the Replicated Degumming Ratios (RDR)s for both cocoons and raw silk were calculated as per equation 2.1.

3.4 Standardising the Degumming Ratios

Firstly, the original degumming ratios for both the control methods and the tested methods (referred to as 'linked methods') were gathered from the papers referenced in Table 3.5. For example, returning to Table 2.3, there are two linked methods for the 0.025-CaOH Original Control. From the original paper by Zhao and Zhang [70], the degumming ratios for each of those three methods (one control, two linked) were as follows:

TABLE 3.6. Degumming ratios for the three methods (one control, two linked) tested in one paper [70].

Agent	Degumming Method	DR (%)
CaOH	0.025% w/v; 100°C; 20 min; 2 cycles	26.78
H ₂ O	100°C; 60 min	11.40
HTHP H ₂ O	120°C; 120 min	28.05

Next, the Relative Degumming Efficacy (RDE), the degumming ratio of each linked method to the degumming ratio of its Original Control, is calculated as follows:

$$\text{RDE} = \frac{\text{Linked Method DR}}{\text{Original Control DR}} \quad (3.1)$$

Given that CaOH was used as the control for this study, the RDE of H₂O and HTHP H₂O is:

TABLE 3.7. Relative degumming efficiency (RDE) calculated for both the methods linked to the 0.025-CaOH control.

Agent	Degumming Method	DR (%)	CDR (%)	RDE
0.025-CaOH				
H ₂ O	100°C; 60 min	11.40	26.78	0.4257
HTHP H ₂ O	120°C; 120 min	28.05	26.78	1.047

From there, the key assumption is that the RDE will remain the same no matter the sericin content of the cocoon and no matter the type of raw silk. Given the Replicated Degumming

Ratios (RDR)s measured in Section 3.3 for each of the controls with both the cocoons and raw silk, the Standardised Degumming Ratio (SDR) for each linked method is calculated as follows:

$$\text{SDR} = \text{RDR} * \text{RDE} \quad (3.2)$$

Thus for the CaOH linked methods, the Standardise Degumming Ratios for their expected performance on the batch of cocoons and silk in this paper are calculated:

TABLE 3.8. An example of the Standardised Degumming Ratios (SDR)s calculated for both the methods linked to the 0.025-CaOH control.

Agent	Degumming Method	RDR (%)		SDR (%)	
		RDE	Cocoons	Raw Silk	Cocoons
0.025-CaOH					
H ₂ O	100°C; 60 min	0.4257	36.93	16.27	15.72 6.93
HTHP H ₂ O	120°C; 120 min	1.047	36.93	16.27	38.68 17.04

This process was repeated for each linked method for each control referenced in Table 3.5.

CHAPTER 4

Results

4.1 Preparing the Silk

4.1.1 Preparing the Cocoons

Generally, the preparation method resulted in clean, consistent cocoon shell pieces. The cocoons were reasonable consistent in size, facilitating a consistent quartering of the cocoons. Some shells were significantly more soiled than others, resulting in some small pieces due to large excised portions. Given the concave surface of the pieces, some had a habit of sticking together like two bowls. For some shells, the floss was particularly difficult with the paper towel.

4.1.2 Preparing the Raw Silk Yarn

The raw silk yarn preparation resulted in consistent pieces that degummed well. The raw silk had a slight lustre and was smooth to the touch. Visual inspection indicated that it was more shiny than expected for raw silk. The pieces were short enough that they could ‘unravel’, preventing excessive knotting or bunching.

4.2 Preliminary Testing

4.2.1 Determining the Bath ratio

The 1:25 and 1:50 bath ratio tests were ended early to prevent the beaker from breaking as the liquid level dropped dangerously low. The 1:100 test did have remaining liquid at the end of the test, but the level was low enough that some of the cocoon was above the water line, thus it was deemed inappropriate. The 1:150 bath ratio had enough liquid volume to keep the cocoons submerged for the entire duration and was adopted as the bath ratio for all future tests.

4.2.2 Variable Testing

4.2.2.1 Temperature

An increase in temperature corresponded to an increase in the Degumming Ratio for both cocoons and raw silk in Milli-Q water. For both silk forms, there was a significant increase in DR when the temperature was increased from 50 °C to 100 °C. The DR for cocoons for all temperatures was significantly higher than for raw silk. Unexpectedly, the DR for raw silk was slight lower at 50 °C than at 23 °C.

TABLE 4.1. The mass of raw silk and cocoons before and after degumming and the corresponding degumming ratio. The degumming ratio increased with increasing temperature. The degumming ratio of cocoons was higher than that of raw silk.

Test ID	Initial Mass (g)		Final Mass (g)		Degumming Ratio	
	Cocoon	Raw Silk	Cocoon	Raw Silk	Cocoon	Raw Silk
temp-23-2	0.5078	0.5018	0.4754	0.4824	6.38%	3.86%
temp-50-2	0.5057	0.4946	0.4689	0.4760	7.28%	3.75%
temp-100-2	0.4938	0.5049	0.4139	0.4568	16.19%	9.53%

4.2.2.2 Time

An increase in degumming time corresponded to an increase in the Degumming Ratio for both cocoons and raw silk in Milli-Q water at both 23 °C and 100 °C. The increase was more noticeable at a higher temperature, which aligns with the results from previous testing in table 4.1. The DR for cocoons for all times was significantly higher than for raw silk.

TABLE 4.2. The mass of raw silk and cocoons before and after degumming and the corresponding degumming ratio. The degumming ratio increased with increasing degumming time. The degumming ratio of cocoons was higher than that of raw silk.

Test ID	Initial Mass (g)		Final Mass (g)		Degumming Ratio	
	Cocoon	Raw Silk	Cocoon	Raw Silk	Cocoon	Raw Silk
time-23-1	0.4911	0.4929	0.4614	0.4790	6.05%	2.83%
time-23-2	0.5078	0.5018	0.4754	0.4824	6.38%	3.86%
temp-100-1	0.4973	0.5009	0.4396	0.4650	11.60%	7.16%
temp-100-2	0.4938	0.5049	0.4139	0.4568	16.19%	9.53%

4.2.2.3 Surfactants

The use of sodium oleate as a surfactant very slightly increased the the degumming ratio of the Na₂CO₃ solution.

TABLE 4.3. The mass of raw silk and cocoons before and after degumming and the corresponding degumming ratio. The degumming ratio for the solution increased slightly in the presence of a surfactant to aid in wetting the silk.

Test ID	Initial Mass (g)		Final Mass (g)		Degumming Ratio	
	Cocoon	Raw Silk	Cocoon	Raw Silk	Cocoon	Raw Silk
0.2-Na ₂ CO ₃	0.5274	0.5307	0.3272	0.4645	37.96%	12.47%
0.2-Na ₂ CO ₃ -SO	0.5183	0.5116	0.3212	0.4460	38.03%	12.82%

4.3 Replicating the Controls

For both cocoons and raw silk, the degumming ratios increased with increasing Na_2CO_3 concentration. There was a significant difference between the Standardised Degumming Ratios (SDR)s of the cocoons and raw silk. This difference was remarkably constant at 25% for the Na_2CO_3 solutions, with the difference still a large 20% for the CaOH solution.

There were noticeable morphological changes to the cocoons in these solutions, with the cocoon structure breaking down fully into a cotton ball consistency. The raw silk underwent a similar change, though the fibrous structure was still visible.

TABLE 4.4. The mass of raw silk and cocoons before and after degumming with several key methods from the literature and the corresponding Replicated Degumming Ratio (RDR). An increasing concentration of Na_2CO_3 corresponded to an increased degumming ratio.

Test ID	Initial Mass (g)		Final Mass (g)		RDR (%)	
	Cocoon	Raw Silk	Cocoon	Raw Silk	Cocoon	Raw Silk
0.05- Na_2CO_3	0.5044	0.4984	0.3300	0.4516	34.58	9.40
0.2- Na_2CO_3 -SO	0.5183	0.5116	0.3212	0.4460	38.03	12.82
0.5- Na_2CO_3	0.5091	0.5035	0.3043	0.4271	40.23	15.18
0.025- CaOH	0.5126	0.5041	0.3233	0.4221	36.93	16.27

4.4 Standardising the Degumming Ratios

This table groups the methods based on the control method used in that particular paper. The same control across several different papers yields several different degumming ratios. The relative degumming efficacy (RDE) for all but three methods were below 1, indicating that the controls used in the literature often have a higher degumming ratio than the methods being tested.

There is a sizeable difference between the method's original literature-based DR and the calculated SDR for cocoons due to the high Replicated DR for Cocoons. Similarly, the raw silk SDR is low due to the low Replicated DR.

TABLE 4.5. Standardised Degumming Ratios (SDRs) to determine the expected efficacy of each method on the batch of cocoons and raw silk tested in this thesis. R/C reminds the reader whether the original method was tested on Raw silk or Cocoons. The Control Degumming Ratio (CDR) refers to the degumming ratio of the control method according to the literature. The Relative Degumming Efficacy (RDE) is calculated using Equation 3.1. The Replicated Degumming Ratios (RDR) are drawn from Table 4.4.

Ref	Agent	Degumming Method	DR (%)			RDE	RDR (%)			SDR (%)
			C	R/C	CDR (%)		Cocoon	R Silk	R Silk	
0.05-Na₂CO₃										
[73]	Bromelain	4 g/L; pH 6.4; 40°C; 60 min; 2 cycles	21.76	R	22.93	0.9490	34.58	9.40	32.82	8.920
[74]	Bromelain	4 g/L; pH 6.4; 40°C; 60 min; 2 cycles	23.14	R	23.52	0.9838	34.58	9.40	34.02	9.248
[39]	Papain	3 g/L; 85°C; 60 min	22.73	R	23.43	0.9701	34.58	9.40	33.55	9.119
[73]	Papain	4 g/L; pH 6.6; 5 °C; 60 min; 2 cycles	23.85	R	22.93	1.040	34.58	9.40	35.97	9.777
[73]	Subtilisin	4 g/L; pH 7; 50°C; 60 min; 2 cycles	22.30	R	22.93	0.9725	34.58	9.40	33.63	9.142
[73]	Trypsin	4 g/L; pH 6.8; 38°C; 60 min; 2 cycles	24.53	R	22.93	1.070	34.58	9.40	36.99	10.056
[74]	Urea	8.0 M; 90°C; 180 min	23.06	R	23.52	0.9804	34.58	9.40	33.90	9.216

TABLE 4.5. Standardised Degumming Ratios (SDRs) to determine the expected efficacy of each method on the batch of cocoons and raw silk tested in this thesis. R/C reminds the reader whether the original method was tested on Raw silk or Cocoons. The Control Degumming Ratio (CDR) refers to the degumming ratio of the control method according to the literature. The Relative Degumming Efficacy (RDE) is calculated using Equation 3.1. The Replicated Degumming Ratios (RDR) are drawn from Table 4.4.

Ref	Agent	Degumming Method	DR (%)			CDR (%)	RDE	RDR (%)			SDR (%)
			C	R/C	Cocoon			Cocoon	R Silk	Cocoon	
0.2-Na₂CO₃-SO											
[71]	Citric acid	2% w/v; 100°C; 30 min	26.70	C	26.8	0.9963	38.03	12.82	37.89	12.77	
[71]	HTHP	120°C; 30 min	26.50	C	26.8	0.9888	38.03	12.82	37.60	12.68	
0.5-Na₂CO₃											
[50]	H ₂ O	100°C; 60 min	13.10	C	34.00	0.3853	40.23	15.18	15.50	5.85	
[50]	H ₂ O	115°C; 60 min	27.00	C	34.00	0.7941	40.23	15.18	31.95	12.05	
[50]	H ₂ O	120°C; 30 min	27.00	C	34.00	0.7941	40.23	15.18	31.95	12.05	
[50]	H ₂ O	125°C; 10 min	27.00	C	34.00	0.7941	40.23	15.18	31.95	12.05	
[68]	Marseille soap	0.2% w/v; 100°C; 30 min; 5 cycles	25.00	C	33.00	0.7576	40.23	15.18	30.48	11.50	
[75]	Metalloprotease	490 U/mL; 15°C; 12 h	23.47	R	25.22	0.9306	40.23	15.18	37.44	14.13	
[75]	Metalloprotease	490 U/mL; 25°C; 12 h	24.03	R	25.22	0.9528	40.23	15.18	38.33	14.46	

TABLE 4.5. Standardised Degumming Ratios (SDRs) to determine the expected efficacy of each method on the batch of cocoons and raw silk tested in this thesis. R/C reminds the reader whether the original method was tested on Raw silk or Cocoons. The Control Degumming Ratio (CDR) refers to the degumming ratio of the control method according to the literature. The Relative Degumming Efficacy (RDE) is calculated using Equation 3.1. The Replicated Degumming Ratios (RDR) are drawn from Table 4.4.

Ref	Agent	Degumming Method	DR (%)			RDE	RDR (%)			SDR (%)
			R/C	CDR (%)	Cocoon		R Silk	Cocoon	R Silk	
[75]	Metalloprotease	490 U/mL; 35°C; 12 h	24.90	R	25.22	0.9873	40.23	15.18	39.72	14.99
[75]	Metalloprotease	490 U/mL; 45°C; 12 h	22.90	R	25.22	0.9080	40.23	15.18	36.53	13.78
0.025-CaOH										
[70]	H ₂ O	100°C; 60 min	11.40	C	26.78	0.4257	36.93	16.27	15.72	6.93
[70]	HTHP	120°C; 120 min	28.05	C	26.78	1.047	36.93	16.27	38.68	17.04

CHAPTER 5

Discussion

5.1 The Ethics of Silk as a Biomaterial

In the field of biomedical ethics, a notable disparity exists between the ethical considerations given to the use of animals in clinical trials and the use of animal products as biomaterials. The ethical focus on animal testing in clinical trials is driven by concerns about animal welfare and the moral obligations to minimise harm. In contrast, the use of animal-derived materials, such as silk fibroin, has not received the same level of ethical scrutiny [85], [86]. This could be due to a perception that once the material is extracted, it does not involve ongoing animal suffering or direct harm. Additionally, the processes involved in obtaining materials like silk are often less visible to researchers and end-users, leading to less ethical consideration.

Traditional sericulture involves the boiling of silk moths inside their cocoons to avoid the moths damaging the silk as they emerge [87]. The alternative is the use of waste cocoons, cocoons from which the moth has been allowed to emerge. The use of waste cocoons in Regenerated Silk Fibroin (RSF) production aligns with the principles of ethical and sustainable biomaterials research. These cocoons, byproducts of the silkworm moth's lifecycle, offer a resource that does not necessitate the intentional harming of silkworms.

However, a broader issue on the use of silk remains. *Bombyx mori* has been so thoroughly cultivated that it now exists in a state of complete dependency on human care. The moth has become blind, is incapable of flight, and lives only a few days, laying about 500 eggs before its life cycle concludes [88]. This level of domestication poses ethical questions different from those associated with less domesticated or wild species. It's not just about the harm or

suffering caused during silk harvesting, but also about the ethical implications of maintaining a species in a state of such dependency for silk production.

This nuanced situation where the ethical concerns extend beyond the immediate process of silk harvesting to the entire lifecycle of the organism, parallels the moral considerations in clinical animal trials. In both contexts, the key ethical imperative is to minimise harm and ensure responsible stewardship. However, while the focus in animal trials is often on avoiding pain and distress during experimentation, the concern with silk production revolves around the broader implications of breeding and sustaining a species solely for human use.

This scenario demands a holistic ethical approach in biomaterials, akin to the rigour in animal trials, where the welfare of *Bombyx mori* and other animals used for biomaterials is considered throughout their lifecycle. Such an approach ensures that the use of animal-derived products like silk fibroin aligns with ethical principles, balancing scientific and economic benefits with humane and sustainable practices.

5.1.1 The Suitability of Waste Cocoons

The difference is that the moth has been allowed to emerge. As discussed in Section 2.4.2.1, this involves the use of cocoonase to soften the shell. This cocoonase is responsible for the yellowing of the cocoon, visible in Figure 3.1a. Those sections were excised during preparation of the shell to prevent the presence of excess cocoonase that might have affects the results. Still, it's possible that waste cocoons may not be suitable for biomedical research, where consistency is key.

There is limited research on how waste cocoons might affect the properties of Regenerated Silk Fibroin (RSF). There has been some research on the use of waste cocoons in the textile industry to produce ‘peace silk’, with findings demonstrating that the differences aren’t as significant as expected [87]. Once the cocoonase has degumming the local region of the cocoon, the moth pushes aside the silk fibers rather than biting through them. Given that the limited testing of cocoonase as a degumming agent in its own right has shown no effect on fibroin [76], it seems unlikely that the silk fibroin of waste cocoons is likely to be degraded.

Waste cocoons do result in greater excising of the shell to remove the cocoonase-affect regions. The pupal shell is also left within the cocoon upon the moth's emergence. It was very brittle and crumbled very easily, spreading a small amount of visible dark brown dust throughout the inside of the cocoon. This was largely trapped in the inner floss and was easily removed with it. Occasionally, part of the pupal shell was stuck to the inside of the shell, as in Figure 3.1c, that was essential to cut out.

Further testing comparing waste cocoons with conventional cocoons is warranted to determine their suitability.

5.2 Preparing the Silk

There were several challenges involved in preparing the silk. The removal of the floss, the cotton-candy like inner and outer layers of the cocoon within which dirt and debris is easily trapped, was tedious and difficult. It pulled away clearly in some instances, but for others, was stubborn stuck to the cocoon. This often resulted in cocoons or pieces of cocoons being thrown away for scientific consistency. The paper towel, while providing a slight rough surface on which to 'catch' the floss, wasn't suitable. A brush, like a toothbrush, is recommended for future attempts.

Another issue is the shape of the cocoon and the consistency of the pieces once they were quartered and had the soiled sections excised. Though the quartered pieces were roughly similar 1 cm x 1 cm concave squares as often used in the literature [48], [50], [77], there was some noticeable difference in the size once excised. A better protocol to help ensure more consistent pieces is recommended. Cutting the cocoons into smaller pieces of a deliberate geometry (i.e. 0.5 cm x 1 cm) may be a better option.

Smaller pieces will also help with the tendency for the shells to stick together during the initial minutes of degumming. The concave shape of the shells meant that the pieces easily stick together like stacked bowls. This limited the penetration of the degumming liquid which

may have affected the efficacy of a particular method, even with the mixing provided by the magnetic stirring rod.

The preparation of the raw silk yarn, by comparison, was easy. I think there's a case to be made for the use of raw silk instead of cocoons as a source of silk fibroin in the lab. The pieces are more consistent, with the fibers easily untangled to maximise surface area. There's also less sericin content raw silk, as we will discuss in later sections, reducing the amount of degumming necessary.

5.3 Preliminary Testing

The preliminary bath ratio testing revealed some notable deviations from the ratios referenced in the literature. Despite being larger than commonly used ratios in previous studies [48], [63], [67], [68], these experiments indicated that smaller liquid volumes were not sufficient to maintain enough liquid through the boiling phase. This discrepancy could be attributed to the smaller mass of silk mass used per test in this test, necessitated by a limited supply of cocoons.

Interestingly, a method by Yuksek, Kocak, Beyit *et al.* [55] recommend 2L of solution for 5 grams of silk, a much higher ratio of 1:400. Such high bath ratios are advantageous in maintaining constant temperature upon adding the silk, providing ample space for mixing, stabilising the pH, and ensuring sufficient reactant availability without using higher concentrations. 2L of openly boiling water is however, a sizeable safety concern, both during the process, but also for extracting the silk after degumming.

Wang, Cao and Zhang [89] looked at the effect of the bath ratio on the degumming ratios. It was observed little difference in the effect of the bath ratio above the threshold of 1:80 for their experiment, though they did not state the mass of silk or volume of liquid being used. Together with the results from this test, it seems better to err on the side of a higher bath ratio, at minimum 1:150 as used in this paper. Such a level provides many of the benefits of a larger volume of liquid, balanced against the dangers of large volumes of boiling solutions.

5.3.1 Temperature, Time, and Sericin Solubility

Preliminary variable testing for both temperature and time showed that higher temperatures result in more degumming both cocoons and raw silk in water. The same observation holds for increasing degumming durations at constant temperatures too.

This study highlights a notable distinction in the composition of cocoons and raw silk. A key observation is the consistently lower degumming ratio in raw silk across all the experiments, suggesting different sericin contents for cocoons and raw silk. To produce raw silk, the cocoons need to be softened, often in warm water, slightly before the silk threads can be pulled [54], [69]. Though raw silk has not been formally degummed, it's clear that a significant portion of the most soluble outer sericin has been lost. Given that makes up 12.5-15% of the mass of the cocoon, a figure below that is expected value for the total difference in sericin content between cocoons and raw silk. The difference between the raw silk and cocoons at the maximum time and temperature was 6.5%, however, as we will discuss later in Section 5.4, it doesn't seem that this represents the full difference in sericin content between the two.

Looking at the cocoons, after 60 total minutes of degumming in boiling water, the mass loss was 16.19%. That's already above the high end of the expected outer sericin content, and above the level Wang and Zhag [50] found at 60 minutes in their seminal work. Interestingly, they found that further degumming to 120 minutes increased the degumming ratio to 14.9%. It seems highly likely that further degumming to 120 minutes would produce similar results. This suggests these cocoons have either a higher outer sericin content, or a very high total sericin content.

As previously discussed, by increasing the temperature to 125 °C, Wang and Zhag [50] determined a hard cap of 27% on the amount of sericin able to be removed from their cocoons by water degumming alone. Replicating this experiment with raw silk and cocoons may have provided a clearer answer on the difference in water-soluble sericin, but the means to do this safety was beyond the lab's capabilities.

In hindsight, the decision to limit boiling times in these experiments may have restricted our understanding of the full extent of sericin content variability between these cocoons and raw silk. The choice was made to try and maintain as much consistency with the following replication studies so that more insightful conclusions could be drawn about the effects of the degumming agents with respect to the times and temperatures. Moving forward, a concurrent experiment that boils both cocoons and raw silk for substantially longer times may help determine the sericin difference between the silks. Repeating the experiment for up to 240 minutes with the protocol by Wang and Zhab [50], with measurements recorded at 30 minute intervals will provide a clearly picture without the need for higher than boiling point temperatures.

5.3.2 Surfactants and Sericin Solubility

One of the replicated controls tested included sodium oleate, a surfactant, as part of it's method [71]. To understand how the inclusion of a surfactant could modify the degumming process, the experiment was repeated without the sodium oleate. The experiment's results revealed a slight increase in the degumming ratio with the addition of sodium oleate to the Na_2CO_3 solution. This increment, although minimal, suggests that the surfactant had some impact on the degumming efficiency. The presence of the surfactant likely enhanced the wettability of the sericin, particularly the more hydrophobic inner sericin, allowing for more effective penetration of the degumming solution.

The relatively small effect of sodium oleate on the degumming ratio can be attributed to the already high degumming efficiency of the Na_2CO_3 solution. This observation suggests that the utility of a surfactant like sodium oleate may be more pronounced in less effective degumming agents or in solutions with lower inherent degumming ratios.

Reflecting on the outcomes, it would have been valuable to conduct a parallel experiment to examine the impact of the surfactant with a less effective degumming agent, such as pure water. However, limitations in resources, particularly the cost of sodium oleate and its limited supply the lab, restricted this aspect of the study. Such an experiment could have provided a

clearer understanding of the surfactant's role in isolation and its comparative effectiveness against a baseline.

5.4 Replicating the Controls

5.4.1 The Sericin Content of our Cocoons

It is evident that degumming agents, and especially Na_2CO_3 , have a large effect on the degumming process. This is highlighted by the high degumming ratios observed in cocoons, with the most concentrated Na_2CO_3 solution yielding a degumming ratio of over 40%. This result is significantly above the degumming ratios observed in boiling water and indeed significantly higher than their literature-drawn Control Degumming Ratios (CDR). For example, looking at Table 5.1, the 0.5- Na_2CO_3 protocol on cocoons yielded a degumming ratio of 33.00% and 34.00% in it's original papers.

There were a few minor differences between this experiment for 0.5- Na_2CO_3 and the protocols in the literature. Wang and Zhag [50] used a 1:50 bath ratio, which we earlier deemed inappropriate for this experiment. They may used a higher mass of cocoons, resulting in a higher volume of water that did not boil entirely throughout testing or had had a better way to prevent evaporation than our makeshift aluminium foil lids. It seems unlikely that methodological issues are at fault here given the remarkable consistency of the remarkably high degumming ratio for cocoons across all the concentrations.

Far more likely is that the sericin content of the batch of cocoons used in this experiment was higher than the normal range, likely around 35%. The first piece of evidence for this claim is how the rest of the Replicated Degumming Ratios (RDR) compare to their CDRs. All the RDRs that had a cocoon CDR reference are 6 - 11% higher than their CDR, showing consistency in the higher degumming ratios for cocoons across the tests. Further, as previously discussed, the water degumming results from Table 4.1 show a much higher degumming ratio than the expected 12.5-15%. If we consider the degumming ratio of 16.19% in test temp-100-2 is around 50% of the total sericin mass, then a total sericin content for these

TABLE 5.1. A comparison of the Control Degumming Ratios (CDR) on either cocoons or raw silk as specified in each paper and the experimentally measured Replicated Degumming Ratios (RDR).

Test ID	Reference	CDR (%)		RDR (%)	
		Cocoon	R Silk	Cocoon	R Silk
0.05-Na ₂ CO ₃	Feng, Jiaming Lin, Lin <i>et al.</i> [39]		22.73	34.58	9.40
	Liu, Huang, Pan <i>et al.</i> [73]		22.93	34.58	9.40
	Wang, Wang, Pan <i>et al.</i> [74]		23.52	34.58	9.40
0.2-Na ₂ CO ₃ -SO	Kim and Um [71]	26.80		38.03	12.82
0.5-Na ₂ CO ₃	Wang and Zhag [50]	34.00		40.23	15.18
	Cao, Wang and Zhang [68]	33.00		40.23	15.18
	Zhang, Li, Liu <i>et al.</i> [75]		25.22	40.23	15.18
0.025-CaOH	Zhao and Zhang [70]	26.78		36.93	16.27

cocoons of around 36% is likely. This is high, though a sericin content of up to 35% is reported in the literature [49], so not outside the realm of possibility. It's well established that diet and environmental factors can have a large impact on the quality of the cocoon, including it's sericin content [90], [91].

Unfortunately, details concerning the specific conditions of the silkworms' lifecycle are seldom reported in research papers, and were not available from our supplier, leading to challenges in reproducibility and standardisation. This points to a broader issue in the use of animal products as biomaterials: the inherent variability that comes with biological systems. Unlike synthetic materials, where consistency can be tightly controlled, the biological nature of silk means that each batch may respond differently to the same degumming protocols due to natural variations. This variability underscores the necessity for comprehensive documentation of all factors involved in biomaterial production, including the silkworms' diet and rearing conditions.

5.4.2 The Sericin Content of Raw Silk

The decision to replicate these specific controls from the literature was intentional and strategic. These controls were commonly cited across multiple studies, allowing for a broad comparison of degumming methods when standardised. Moreover, by selecting controls with varying sodium carbonate concentrations, we could examine the impact of agent concentration on degumming. The inclusion of a surfactant in one control provided an important comparison of the different degumming strategies, although the limitations in materials and resources prevented extensive experimentation with this variable. The other key aim was to compare the degumming ratios of raw silk and cocoons, allowing further comparison with the studies that degummed with raw silk.

The results show a consistent 25% gap in the degumming ratios of the cocoons and raw silk for the Na_2CO_3 solutions, and a slightly reduced 20% difference for the CaOH solution. Such a stark difference in degumming ratios was not expected and is not in line with previous studies using raw silk. The RDR of 9.40% for the 00.5- Na_2CO_3 protocol was less than half that of all three corresponding CDRs from the literature [39], [73], [74], with similar results for the 0.5- Na_2CO_3 protocol. Interestingly, temp-100-2 yielded a slightly higher degumming ratio of 9.53% than that of the 00.5- Na_2CO_3 protocol. Though that final result is somewhat confusing, the rest of the results suggest that the raw silk used in these experiments has undergone processing that has significantly reduced its sericin content. The impact of such processing is often not adequately detailed in research papers, leading to challenges in replication and comparison across studies.

The discernible difference between the degumming ratios of cocoons and raw silk once again underscores a critical aspect of silk research — the variability of silk as a biomaterial due to its natural origin and processing history. Comprehensive documentation of the processing involved is essential. Such transparency is not just a matter of academic rigour; it has practical implications for the consistency and reliability of results in silk research. Unfortunately, this information isn't always easy to obtain, as we found out first-hand.

To address these challenges, future research in the field of silk degumming and silk-based biomaterials should prioritise the establishment of standardised protocols for reporting silk sources and processing methods. By documenting these factors, researchers can ensure that their findings are replicable and that comparisons across different studies are valid. This standardisation will benefit the development of more effective degumming protocols, tailored to the specific characteristics of the silk being used.

5.5 Standardising Degumming Research

The primary objective of this thesis is to develop an optimal degumming method that effectively removes sericin without compromising the integrity of silk fibroin. The establishment of Standardised Degumming Ratios (SDR) represents a pivotal step towards achieving this goal. By creating the concept of Relative Degumming Efficacy (RDE), we have devised a framework for standardising degumming analysis for cocoons of varied sericin contents and raw silk types. This standardisation is critical in addressing the fragmented literature in silk degumming and enabling meaningful comparisons between different methods.

Given the conclusion that our cocoons have an estimated sericin content of 35%, and using the SDRs from Table 4.5, we can begin to draw some conclusions regarding the suitability of the methods that are expected to achieve complete degumming. As we've discussed at length in the literature review, there is no silver bullet method and each involves a balance between the efficacy of sericin removal and silk fibroin preservation. At this point, an important question and distinction can be drawn. *At what point does silk fibroin damage become detrimental to hydrogel formation?*

Returning to Section 2.3, Figure 2.8 provides some guidance on the extreme end of silk fibroin damage. Boiling for 60 minutes in 0.2% Na₂CO₃ results in too much damage. Frustratingly, Wray, Hu, Gallego *et al.* [46] did not measure the mass loss in their experiments, so the respective degumming ratio for this is unknown. We did however, during the surfactant variable testing, perform a similar method of 0.2% Na₂CO₃, but for 30 minutes, repeated

twice. From Table 4.3, this yielded a DR of 37.96% which we can roughly assume to result in too much degumming.

This reduces the list of suitable degumming methods significantly. Only five methods in Table 4.5 meet the criteria of $35\% < \text{DR} < 37.96\%$.

Papain and Trypsin, both used at a concentration of 4 g/L, present as strong enzymatic options for silk degumming. These enzymes promise to offer a more specific approach, potentially advantageous for preserving the integrity of silk fibroin compared to chemical methods. Trypsin, in the current protocols tested across the literature, does not preserve silk fibroin [72], [73] well. Papain, with it's significantly higher molecular weight, but similar specificity to trypsin, tends to be more gentle and is worth further investigation.

The 2% w/v citric acid and 120°C for 30 minutes HTHP protocols are both drawn from the same paper by Kim and Um [71]. They noted that the HTHP method resulted in a higher molecular weight than citric acid, but provided no information about what temperature or pressure they used, not any other details about that method.

Lastly, we have the 15 °C metalloprotease protocol from Zhang, Li, Liu *et al.* [75]. The metalloprotease protocols resulted in degummed fibers with good mechanical properties, a strong indicator of silk fibroin integrity. Unfortunately, these particular metalloproteases were difficult to produce and limited in their storage capability. Though the evidence suggests that they are worth further investigation, the difficult of using them conflicts with the key aim of designing a method that can be done easily.

5.6 Recommendations for Future Testing

Further investigation will help provide some much needed answers to knowledge gaps. Time and resource limits prevented some simple questions from being answered, which limited the successful outcome of this thesis. Firstly, I would strongly recommend repeating the experiments in this paper to achieve better statistical rigour. Each individual experiment in this paper took *hours* to complete, performed twice for each type of silk, with two days of

drying in limited drying oven space. Due to time constants and being unable to multiplex my experiments, I had to choose between scope and statistical rigour, and chose scope for a more qualitative analysis.

It seems likely that acid and alkali hydrolysis on it's own is too non-specific to adequately avoid silk fibroin damage with complete degumming. However, replicating the experiments by Wray, Hu, Gallego *et al.* [46] with more granular time steps, measured degumming ratios, and the formation of hydrogels to demonstrate hydrogelation performance would provide a strong reference point for further experiments.

There appears to be a strong case for a multi-step multi-agent degumming process. At risk of citing the paper once too many times, [50] provided significant evidence that with the right temperature and time, a significant proportion of sericin can be removed with no damage to silk fibroin. If that is indeed true, then the problem of degumming across three different sericin proteins with three different solubilities is reduced to the problem of removing only inner sericin. Replicating these results is the first step in producing a multi-step protocol.

If indeed, it is possible to remove the outer and middle sericin without silk fibroin damage, then pH neutral surfactants may help improve the penetration of degumming agents into the hydrophobic inner sericin. This combined with an agent such as papain that is unable to effectively penetrate deep into the fibers due to it's size, may provide a gentle enough and specific enough approach to completely degum silk with minimal silk fibroin degradation.

CHAPTER 6

Conclusion

This thesis, while not achieving its primary goal of designing a low-cost, non-toxic method for aqueous silk fibroin solution production in the lab, has nonetheless made significant contributions to the field. The development of Standardised Degumming Ratios (SDR) and Relative Degumming Efficacy (RDE) represents a major advancement. These metrics provide a much-needed framework for standardising degumming analysis across the natural variability of different cocoon batches and between cocoons and raw silks, thereby addressing a critical gap in the literature. This approach paves the way for more systematic and comparable research in silk degumming, fostering a deeper understanding of the process and its outcomes.

The insights gained in this research highlight the delicate balance required between effective sericin removal and preservation of silk fibroin integrity. The identification of potential methods that may achieve complete degumming without significant fibroin damage opens new avenues for future research. However, the variability inherent in biological materials like silk, coupled with the diverse methodologies used in the literature, underscores the challenge of standardising silk degumming procedures. This research also brings to light the ethical considerations surrounding the use of animal products in biomaterials. The reliance on silk, a natural polymer with significant biological variability, highlights the potential limitations in its viability as a biomaterial. Future research should not only focus on the scientific aspects of silk processing but also consider the ethical implications of using animal-derived products, aiming for methods that are humane, sustainable, and mindful of the natural inconsistencies of such materials.

6.1 Future outlook

For future research, several key areas have been identified that can build upon the findings of this thesis:

Exploring silk fibroin properties on hydrogel formation: Future studies should investigate the specific properties of silk fibroin that are crucial for effective hydrogel formation. Understanding how different degumming and solubilising methods impact these properties will be instrumental in refining the process for hydrogel applications.

Using sericin waste as a biomaterial: Sericin is a potential biomaterial in its own right [10], [92]. Extracting and using the sericin byproducts from degumming will result in a greater value add to any lab's research and improve the environmental footprint of degumming.

Extended boiling point degumming: To replicate and extend the findings of Wray, Hu, Gallego *et al.* [46], conducting degumming at the boiling point for prolonged periods could provide valuable data on the upper limit of boiling water's ability to degum silk. Finding a way to remove sericin to the same effect as HTHP methods without needing higher temperatures than boiling point will make it easier to perform in a simple lab environment.

Multi-step multi-agent degumming processes: The prospect of a multi-step, multi-agent degumming process deserves exploration. This approach could address the challenge of removing different sericin types with varying solubilities, potentially leading to a more effective and fibroin-preserving method. Investigating the use of pH-neutral surfactants to enhance the penetration of degumming agents into the hydrophobic inner sericin, combined with specific enzymatic agents like papain, could offer a more gentle yet effective degumming approach.

Standardisation and documentation: Emphasis on standardising methodologies and documenting all variables, including silk sources, environmental conditions, and processing methods, will be vital. This will aid in replicability and comparability of future research, ensuring that findings are reliable and applicable across different studies.

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