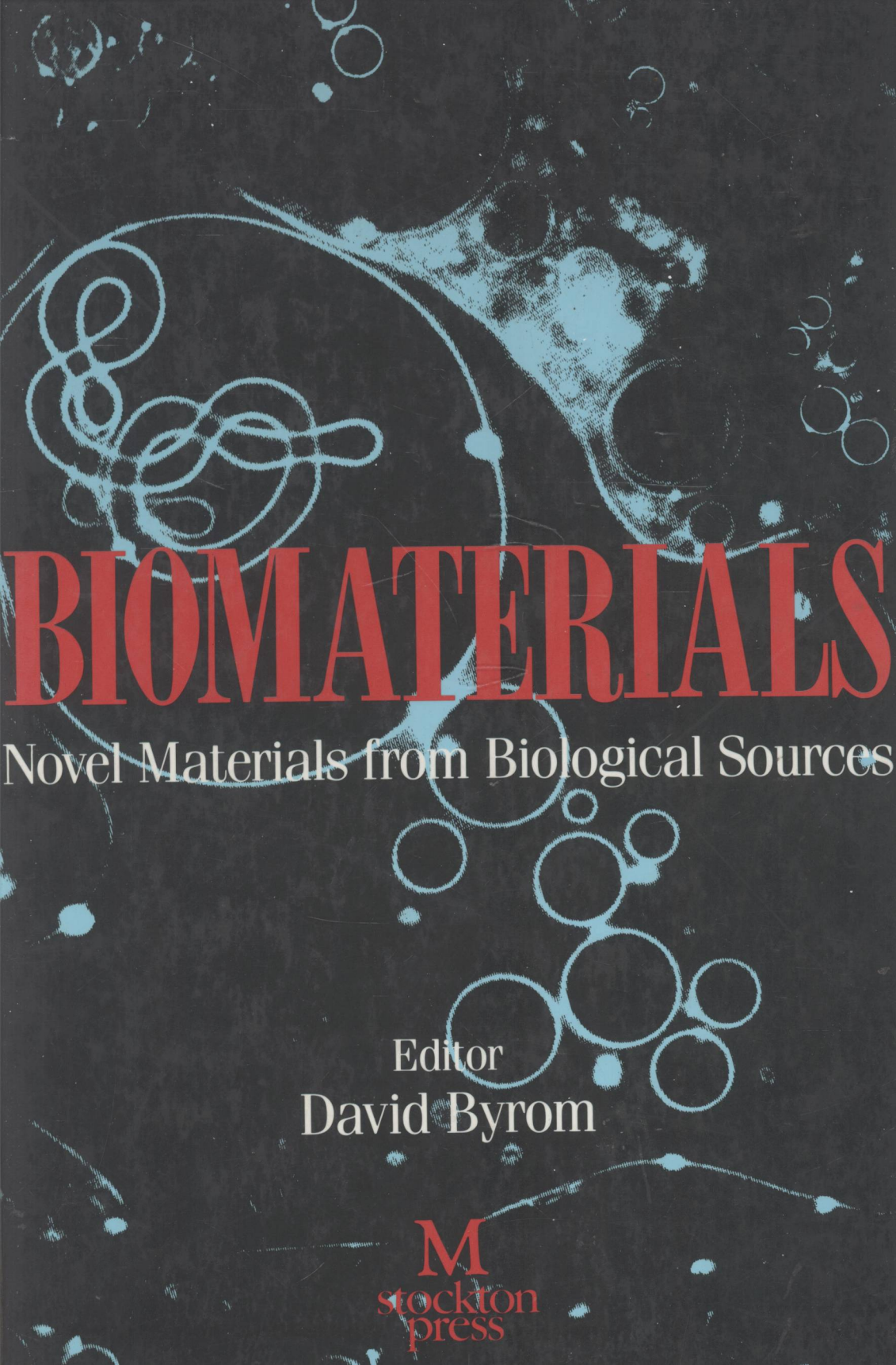
BIOMATERIALS
Novel Materials from Biological Sources
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BIOMATERIALS

Preface

Biomaterials are the very stuff of life. They provide the structure, form, function, protection and storage products of all organisms on earth. Man has just started to take advantage of the properties of some of this vast array of natural polymers and to find applications in medicine, food, packaging, consumer products and other fields.

In describing biomaterials as I have above, I have chosen to define those of commercial interest as polymeric substances produced biologically either directly, for example, by fermentation, or as products extracted from biological sources, as is the case with alginate. This book describes the state of research in a number of biomaterials of different types and with different applications.

The term biomaterials is commonly applied to chemically derived polymeric materials that are used in medical applications such as prostheses, bone plates and other implantable devices. This field of application is a vast area which has received much attention and will not be considered here except where it impinges on the use of biologically produced polymers.

The academic and industrial interest in biopolymers as materials results from a number of factors. They represent materials which have a range of specific properties which may not easily be emulated by synthetic polymers. They can be synthesised from renewable resources and are often biodegradable, which is important in some applications.

Although there is a fascination with biopolymers, there are a limited number of these materials in industrial production. This reflects their cost relative to that of chemically derived products coupled with, in some cases, limitations in mechanical and physical properties in areas where existing polymeric materials are used. It has therefore been necessary to find niche or speciality applications for biomaterials which have been developed to support their cost.

Biomaterials research is a multidisciplinary activity both in the sense that a range of expertise is necessary to produce, characterise and bring to market any novel material and in the sense that it has attracted scientists from the biological, chemical and polymer science communities. It is my intention that this book should reflect this fact and I believe that this has been illustrated in most of the examples included.

The biomaterials covered in this book also reflect the range of techniques which are used to make these biopolymers. Some are produced directly by microbial fermentation; some are extracted from natural sources and used directly; others are modified, fabricated or derived from natural polymers. Perhaps slightly paradoxically, one material included here is made by chemical synthesis using a biologically derived monomer. Some products at an early stage of development have been described in a chapter which gives an indication of the most speculative directions of biomaterials research.

Although the synthesis, modification, derivatisation and application of biomaterials may be technically sophisticated, biomaterials research is still in its infancy since most products in this class are single polymers or copolymers. Biotechnology is developing rapidly and novel methods for the synthesis of materials will arise as a result. Molecular genetics has already been used to produce a material – mollusc bioadhesive – which would be extremely difficult to manufacture were this technology not available.

Nature is adept in the synthesis of composite materials such as bone, horn, wood and shells, which have extremely interesting mechanical and physical properties. At the moment, man can only make use of this phenomenal production capability by utilisation of the materials once they are made. Gaining an understanding of the mechanism of synthesis and the fabrication of natural composites *in vivo* may allow the possibility of intervention in these processes using the techniques of molecular genetics. The capability to manufacture novel biomaterials with new properties, for example, in plants or by fermentation will be the end result of this work. Clearly the future holds exciting prospects for the field of biomaterials research.

David Byrom
November 1991

Contents

Preface vii

1 Silk 1
D. L. Kaplan, S. J. Lombardi, W. S. Muller and S. A. Fossey

2 Collagen 55
Stephen D. Gorham

3 Polyhydroxyalkanoic acids 123
Alexander Steinbüchel

4 Microbial polysaccharides 215
J. D. Linton, S. G. Ash and L. Huybrechts

5 Microbial cellulose 263
David Byrom

6 Hyaluronic acid 285
David A. Swann and Jing-wen Kuo

7 Alginates 307
Ian W. Sutherland

8 Miscellaneous biomaterials 333
David Byrom

Index 361

1 Silks

D. L. Kaplan, S. J. Lombardi, W. S. Muller & S. A. Fossey

Biotechnology Division, U.S. Army Natick Research, Development & Engineering Center, Natick, Massachusetts 01760-5020, USA

Silks: Chemistry, Properties and Genetics

Introduction

A number of reviews have been published detailing the chemical and physical properties of silkworm silks[1–6] and the genetics of silk production in the silkworm[7–9]. Here we overview some of the key chemical and physical properties of both silkworm and spider silks, and then review the genetic aspects of silk production in these organisms.

Silks represent a broad class of polymers which can be loosely defined as externally spun fibrous protein secretions[10]. Of all the natural fibres, silks are the only spun ones. Fibrous proteins, including silks, collagen, elastin and keratin, are distinctive owing to their repetitive primary structure, encoded by repetitive gene sequences under tight regulation. These protein products, containing repetitive domains that influence higher-order conformations, result in fibres and materials with unusual physical and mechanical properties[11]. In addition to the unusual mechanical properties, silks exhibit interesting interference patterns within the electromagnetic spectrum[12], unusual viscometric patterns related to processing[13], and piezoelectric properties[12,14,15]. Silks differ in composition, properties and morphology depending on their source. Differences are found between species as well as between different silks produced by the same organism, as is the case for many orb-weaving spiders. In general, silks are formed in sets of specialized glands by insects in the class Insecta and spiders in the class Arachnida. Silks are also produced by pseudoscorpions, mites[16] and the aquatic larvae of the midge, *Chironomus tentans* (Diptera), which produce a family of secretory proteins with unusual repeats including periodic cysteine residues[17]. There are thousands of silk-spinning insects and spiders, yet only a few have been investigated in detail.

Sericulture involves the growth of the domesticated silkworm *Bombyx mori* and related species for silk production for textiles. This art has been practised for thousands of years, originating in China. World silk production has declined since the 1930s because of competition from less expensive synthetic fibres[2]. Silkworms produce silk during one stage in their lifecycle. Silkworm silk (*B. mori* unless otherwise specified) consists of two fibroin protein filaments adhered together

with a sericin protein gum resulting in a single thread about 10 to 25 μm in diameter[2]. Threads of silks from wild silkworms often have larger diameters up to 65 μm[6]. Silk threads from different organisms can have different morphologies in cross-section, including triangular, crescent, rectangular and wedge.

The life cycle of *B. mori* lasts 55 to 60 days and passes through a series of developmental stages or molts. Cocoon formation occurs at around 26 days in the cycle, during the later stages of the fifth instar[18]. This stage is characterized by the extrusion of silk from a spinneret located in the head and drawing of the fibre by a characteristic figure-of-eight head movement. The silk is extruded into air and a change in conformation to a β-sheet occurs owing to shear, tension due to the movement of the head of the silkworm, and loss of water[6]. Rheology experiments with the soluble silk illustrate that crystallinity is positively correlated with shear rate and rate of drawing, and negatively correlated with the diameter of the spinneret. A critical extrusion rate of 500 mm per min was found by Magoshi *et al.*[13] to induce the conformational change to a β-sheet. During this process the sericin does not undergo a conformational change owing to its higher water content (86%); a shear rate several orders of magnitude higher than for the fibroin is required for the change to the β-sheet conformation. Commercially, silk fibres are harvested from silkworm cocoons, which are exposed to boiling-hot soapy water to remove the sericin before reeling the silk. About 300 to 1200 metres of usable thread are recovered per cocoon[3].

The sericin component of silkworm silk is represented by a family of proteins. Komatsu[19] distinguished sericin I, II, III and IV on the basis of solubility in hot water, and each of these proteins has a different amino-acid composition. Sericin exhibits primarily a random-coil conformation with some β-sheet structure. Conversion to the β-sheet occurs with moisture, heat or mechanical stretching, but the conversion is to a crystalline but not fully oriented form.

Spiders represent some of the most diverse and abundant terrestrial organisms on earth, with over 35,000 species having been identified[20,21]. Despite their presence for over 300 million years, spiders have never been commercialized like silkworms, although they have been of interest for medical reasons for hundreds of years[5]. The diversity of lifestyles among spiders is so great as to preclude further description here. In general, spiders are carnivorous and can be classified as either web-builders or hunters; the silks under review here are

produced by web-builders. Unlike the silkworm, some orb-spinning spiders produce silks throughout their adult life and some spiders produce a variety of different silks, each synthesized in separate sets of specialized glands[22]. Female spiders are responsible for orb-web construction, and web recycling by partial resolubilization has been demonstrated for *Araneus cavaticus* using radioactive tracers[23].

Lucas *et al.*[5] referenced work from the nineteenth century where silk from the spider *Nephila madagascariensis* was spun into thread. This spider spins webs that, with a diameter of over a metre, can trap small birds. Historically, spider silks have also been used in eyepieces of optical instruments[5]. Devices for the controlled silking of spiders have been described[24,25]. Spider silks are diverse and serve in prey capture, reproduction and as vibration receptors. Emerging young spiders use silks for dispersion by wind and also to build miniature webs. Unusual visual displays on orb webs are characteristic of some spiders, such as the stabilimenta or zigzag pattern displayed on orb webs produced by the Argiope spider[10]. The function of this pattern is unknown, although it has been speculated that it stabilizes and strengthens the web, disguises the spider, absorbs water and warns off birds[10].

Gosline *et al.*[10] have studied structure–function relationships with fibrous natural polymers including spider silks. Spiders produce a variety of silks for different functions and are therefore useful organisms for the study of molecular design of natural structural polymers. The orb-weaving Aranaeid spider produces at least seven different silks, each of which is synthesized, processed and spun in a different set of glands[10,22,26–28]. Other species of spider synthesize only one type of silk[29]. The different glands and their associated silks include: major ampullate gland which produces structural silk for the orb frame, radii and dragline; flagelliform gland which produces the viscid silk for prey capture; aggregate gland which produces a glue-like silk; minor ampullate gland which produces support fibres for the frame and dragline silks; cylindrical gland for cocoon silk; aciniform gland for wrapping captured prey; and piriform gland for attachment silks which couple the frame and dragline silks to environmental substrates[10]. Each of these silks has a different characteristic amino-acid composition which reflects their functions. Primary DNA, RNA and amino-acid sequence data are beginning to be determined for these different silks to permit the detailed characterization of these relationships and of the genetic regulation involved in the synthesis of the various silks.

Silks play a major structural role in the survival of orb web-spinning

spiders throughout the adult life cycle, unlike silkworm silk which functions in cocoon formation at one stage of the life cycle. Spider silk web fibres generally vary in diameter from 1 μm to 5 μm or larger, depending on the source and type of silk[10,22,25,30,31]. The diameters of the web fibres from *Araneus diadematus*, *A. sericatus* and *A. gemma* averaged 3 μm and ranged between 1.03 μm and 3.75 μm (ref. 31). Gosline *et al.*[10], in studies on *A. sericatus*, reported that a 75-mg spider uses 180 μg of protein to spin a web up to 100 cm^2, representing very efficient engineering and use of this material.

Anderson and Tillinghast[32] studied *Argiope aurantia* and *Argiope trifasciata* and identified soluble chemical compounds present on web fibres that may function in species identification, prey capture or in water retention to maintain the adhesive and elastomeric properties of the silk. Salts and neurotransmitters such as γ-aminobutyric acid were indentified on the web fibres. Tillinghast and Christenson[32] identified similar water-soluble compounds on the orb web of *N. clavipes*. Vollrath *et al.*[34] studied *A. diadematus*, an orb weaver, and also found webs covered with aqueous droplets containing salts and neurotransmitters.

Structure

Silks adopt a variety of secondary structures including α helices, β-sheets and cross-β-sheets. The α-helical conformations are characteristic of silks from bees, wasps and ants[35,36], β-sheets are characteristic of many silkworm silks and spider silks, and cross-β-sheets are characteristic of many insects, such as the green lace-wing fly *Chrysopa flava*[37]. The β-sheets incorporate the silk polymer chains parallel to the fibre axis, while in the cross-β conformation the polymer chains are positioned at right angles to the fibre axis and are often characterized by a higher content of serine residues. The secondary and tertiary structures of the silk β-sheet have been described[2,6,38]. The polymer chains run antiparallel, with hydrogen-bonds, roughly perpendicular to the chain axis, being formed between the carbonyl and amine groups from the peptide bonds that hold the chains together in sheets. The sheets assemble through hydrophobic interactions owing to their close packing density, which is attributable to the preponderance of short side-chain amino acids in the polymer chain[5].

The structure of cocoon silk fibroin from *B. mori* consists of antiparallel β-sheets first described by Marsh *et al.*[39]. The fibroin consists

of both crystalline and amorphous domains with the amorphous regions characterized by the presence of amino acids with bulkier side chains. The crystalline domains are characterized by a high percentage of glycine, alanine and serine, in a 3:2:1 ratio, which contain short side chains to permit the close packing densities for overlying sheets.

The statistical coil form, β-sheet form (also called silk II or β-silk) and a second crystalline form called silk I have been reported for silkworm fibroin (Fig. 1). The nomenclature silk I and silk II was first proposed by Kratky *et al.*[40,41]. Silk I has also been called α-silk or water-soluble silk[42]. The use of the term α-silk should probably be avoided since it may lead to confusion with the α-helix of Pauling and Corey. The two crystalline forms of *B. mori* silk fibroin have been shown by Lotz *et al.*[4] to be isomorphous with the two crystalline forms of poly(L-alanine-glycine). Poly(L-alanine-glycine) has often been used as a model for the crystalline regions of silk, both experimentally[1,43] and computationally[44,45]. Unfortunately, the crystalline forms of silk and poly(L-alanine-glycine) were named independently of one another and the AGII form of poly(L-alanine-glycine) is isomorphous to silk I, and AGI isomorphous to silk II, which may lead to some confusion.

Warwicker[46] studied spider silks and silkworm silks with X-ray diffraction, including cocoon silk from the spider *Nephila senegalensis* and reeled silk from *N. madagascariensis*. Both silks exhibited antiparallel β-sheet structures but had different intersheet distances. Five different classes of fibroins were identified. The *a*-axis (dimension of the unit cell, or direction of the side chains or intersheet distance) went from 0.93 to 1.57 nm depending on the classification of the fibroin, while the *b*-axis (hydrogen-bonds of the antiparallel β-pleated sheets, or interchain distance) was 0.944 nm and the *c*-axis (fibre axis) was 0.695 nm. The 0.695-nm distance is characteristic of an antiparallel β-sheet. X-ray diffraction data on spider silk indicate 55% to 60% of the crystallinity of silkworm silk[10].

Infrared and X-ray diffraction data indicate a degree of cystallinity of between 62% and 66% for *B. mori* cocoon silk (silk II) fibroin, while comparable values for cocoon silk from wild-type silkworms ranged slightly lower, from 50 to 63%[47]. The crystallite size of silk II based on X-ray diffraction data for silkworm silks varies from 1.5 to 2.0 nm perpendicular to the plane on the sheets in the domesticated silk and from 4.7 to 6.8 nm in wild silks (summarized in ref. 47). Marsh *et al.*[39] reported that the unit cell for the crystalline domains included

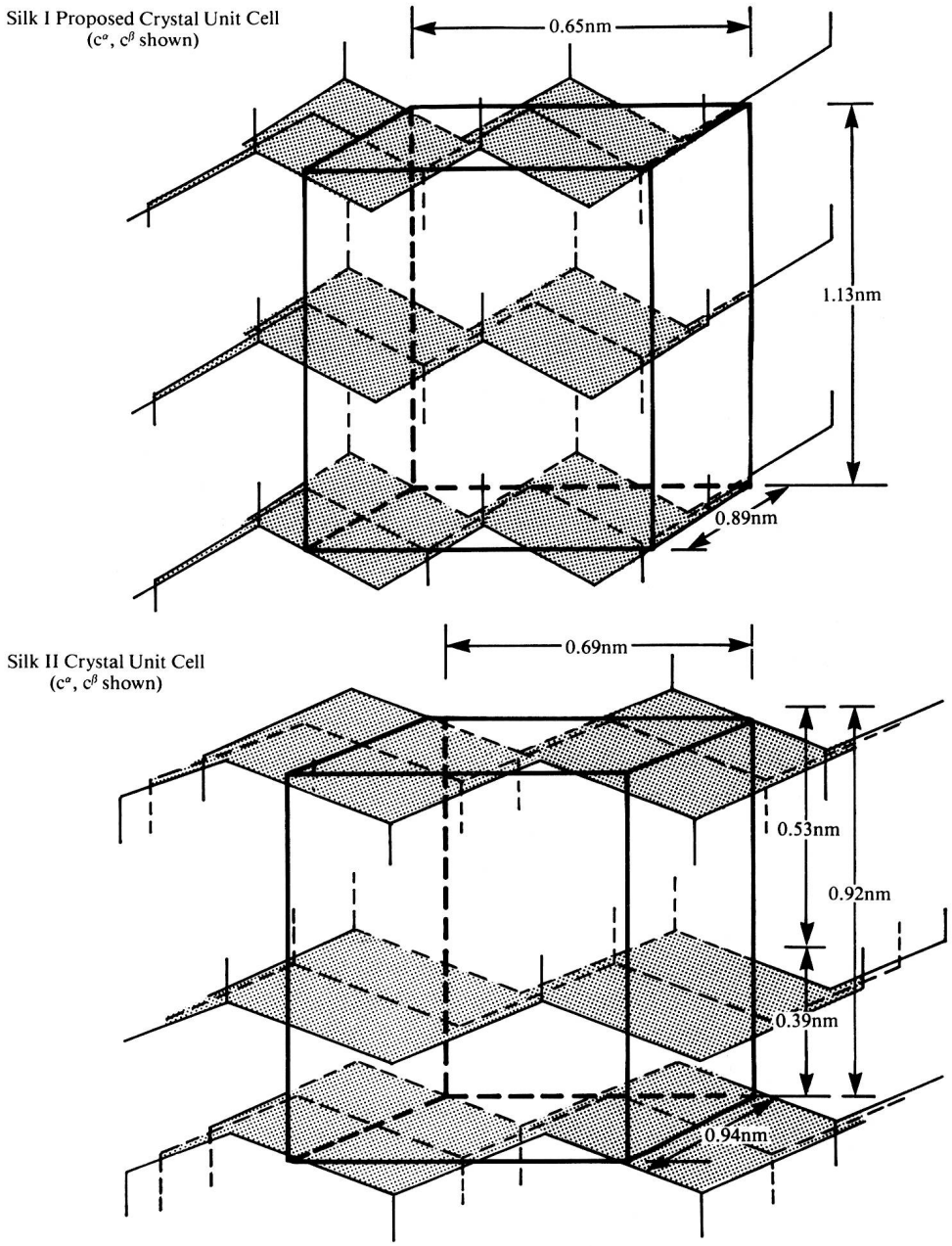

Fig. 1 Model structure of silk I and silk II, end view with hydrogen-bonds shown, illustrating orientation of amino-acid side chains.

an interchain distance of 0.94 nm (*a*), a fibre axis distance of 0.697 nm (*b*) and an intersheet distance of 0.92 nm (*c*). The smaller glycine side chains alternate with serine or alanine side chains. The *c* dimension of a unit cell has been related to the glycine content[2] and ranges from 21 glycine per 1000 monomers to 450 glycine per 1000 monomers, corresponding to *c* dimensions of 1.57 nm in *N. senegalensis* spider silk and 0.92 nm in *B. mori* silk, respectively[1,36]. In *B. mori* fibroin (silk II), the long glycine-X repeat within the crystal results in a structure in which the glycine side chains all project from the same side of the sheet and the alanine and serine side chains from the other side of the sheet. The sheets then stack with the glycine faces together and the alanine/serine faces together.

Chou *et al.*[48–50] have carried out energy-minimization studies on silk II structural motifs, including homopolymers of alanine, valine, isoleucine, serine, threonine, phenylalanine and tyrosine in parallel and antiparallel configurations for neighbouring polymer chains. They found a higher-energy less-stable structure for the parallel versus the antiparallel chain configuration in the case of the glycine, alanine, leucine and aminobutyric acid homopolymers, and the reverse for valine, isoleucine, serine, threonine, phenylalanine and tyrosine. Tsukada *et al.*[51] studied poly(Gly-L-Ala) and copolymers of (L-Ala-Gly and L-Ser-Gly) in 2:1 ratios using X-ray diffraction, infrared spectroscopy and circular dichroism. Introduction of the serine into the polymer backbone resulted in better crystallization into the antiparallel or cross-β forms. Colona-Cessari *et al.*[44] modelled the silk II form with conformational energy calculations on several sheets of poly(L-alanine-glycine). Oka *et al.*[52] have recently used Ala-Gly energy-minimization calculations of single strands to predict right-handed and left-handed helical configurations as models for silk I.

Either silk I or silk II forms can be obtained from the solvated form depending on crystallization conditions. The silk II form is, however, more stable than the silk I form and any mechanical agitation during drying will result in the conversion to the silk II form. Transformation from silk I to silk II in the solid state has also been effected by exposure to hydrophilic organic solvents such as methanol or acetone by an electric field, changes in temperature and by mechanical drawing[53–60]. Similar thermal transition will occur above 180 °C and on exposure to steam.

Because of the instability of silk I to mechanical strain, attempts to induce orientation in a sample for X-ray diffraction, NMR or other analytical methods cause a transition to the silk II form. Experimental

studies of silk I have been only on samples of low orientation. Elucidation of the structure of silk I must therefore follow from model building and comparison of the model with experimental evidence. A number of models have been proposed for silk I on the basis of computer-generated predictions usually tied to experimental data. For example, Lotz and Keith[61] proposed a crankshaft model using poly(L-Ala-Gly) lamaellar crystals with resulting unit-cell dimensions of a = 0.472nm (interchain), b = 1.44nm (intersheet) and c = 0.96nm, the fibre axis repeat being a multiple of 0.32nm. Ilzuka[2] reported that fibroin contains three subunit chains, each of which folds back on itself many times to form rod-like structures with adjacent segments of each chain interacting through hydrogen-bonds; it was reported that the silk I form is a meta-stable crystalline structure in an orthorhombic cell containing two polymer chains. Recent work by Fossey *et al.*[45] based on conformational energy calculations with homopolymers of glycine or alanine and copolymers of Gly-Ala repeats, has resulted in a new proposed model for silk I (fig. 1). The unit cell structure proposed has a fibre axis repeat of 0.646nm and an interchain spacing of 8.94nm and an intersheet spacing of 1.13nm. The structure is an alternating left-hand and right-hand 3_1 helix with alanine residues configured in a left-handed helix and glycine residues in a right-handed helix.

Physical properties

Silk fibroin represents one of the largest polypeptides in nature with reported relative molecular masses of 350K to 415K (refs 62,63). Initially, fibroin molecular masses from 33K up to 400K were reported using a variety of methods, until the use of denaturants such as urea or guanidine hydrochloride produced more consistent results. The difficulty in obtaining homogeneous preparations of the fibroin and complete solubilization without degradation is primarily responsible for these early discrepancies. Tashiro and Otsuki[64] and Tashiro *et al.*[65] had previously reported molecular masses of 300K to 420K for fibroin extracted from the posterior region of the silk gland using ultracentrifugation and sedimentation equilibrium analyses. Molecular masses reported for sericin protein have ranged from 20K to 310K (refs 66,67). Candelas and Cintron[68] reported a molecular mass of 320K for silk collected from the major ampullate gland from the spider *N. clavipes*. Lombardi and Kaplan[69] have reported a mass of 350K from this species.