Molecular Design and Production of Recombinant New Silk-like Material with High Strength on the Basis of NMR Structural Characterization of an African Wild Silkworm, *Anaphe*

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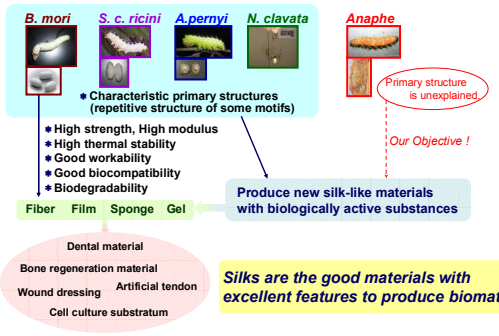
Objective

To Ascertain Primary Structure of *Anaphe* Silk Fibroin and To Produce Novel Silk-like Biomaterial with the characteristic motifs.

Why Silks ?



What's Anaphe ?

Wild Silkworm belong to the *Thaumetopoeidae*

- They live in equatorial and southern Africa (Uganda, Nigeria, Togo, Zaire, Congo)
- Their food is leaves of the *Brideria*
- They oviposit on the *Brideria*
- The silk nests are formed on the *Brideria*
- The offspring larvae of a mother moth make a group and move in a line. (When they move to new leaves, descend to the ground and ascend again to the branches for molt)

Cocoon made by *Anaphe*

- The offset larvae of a mother moth make a large silk nest communally. Like a rugby ball !
- Common silk shell is made by all the larvae and then individual cocoons are made.
  - Common silk shell is divided into three parts; membranous outer layer, soft middle layer, hard inner layer
- Individual cocoons are thin enough to see inside and the major axis is 3-4 cm, minor axis is 1-2 cm.

Reference: Int. *J. Wild Silkmoth & Silk* 4, 7-12(1999)

Why Anaphe Silk Fibroin ?

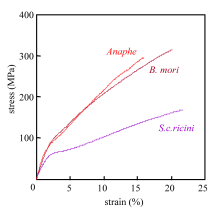
Amino acid composition

Amino Acid	Composition (in mol %)		
	Anaphe	*B.mori*	*S.c.ricini*
Ala	59.1	30.0	48.4
Gly	32.3	42.9	33.2
Ser	2.4	12.2	5.5
Asp*1	1.7	1.9	2.7
Glu*1	0.9	1.4	0.7
Tyr	0.8	4.8	4.5
Pro	0.6	0.5	0.4
Leu	0.6	0.6	0.3
His	0.5	0.2	1.0
Val	0.4	2.5	0.4
Thr	0.3	0.9	0.5
Ile	0.2	0.6	0.4
Phe	0.1	0.7	0.2
Lys	0.1	0.4	0.2
Met	0.02	0.1	0.01
Arg	-	0.5	1.7
Cys	-	0.03	0.01
Trp*2	-	-	0.3

*1 Asn and Gln are converted to Asp and Glu, respectively.
*2 Trp is at least paritially destroyed.

- The Ala content is very high for silks.
- The sum of Ala and Gly content accounts for more than 90 mole %.
- The molar ratio of Ala to Gly is about 2:1.

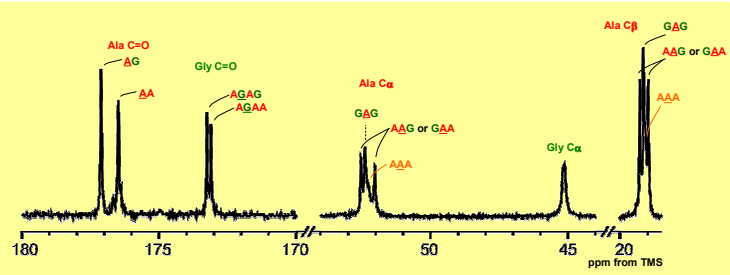
Stress – strain curve



- S-S curve of *Anaphe* silk fibroin is as same as that of *B.mori*.

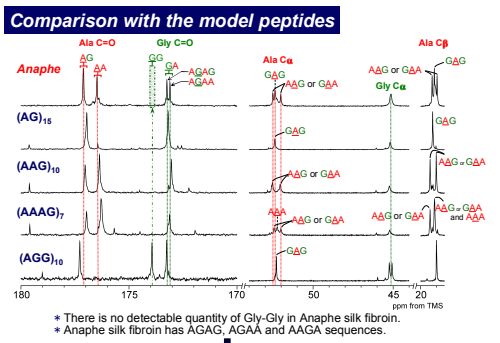
(1) Amino acid composition is very simple.
(2) High strength and high modulus.
These are very good character to design and produce novel silk-like materials !

Determination of Primary Structure of Anaphe Silk Fibroin



The main motifs of Anaphe silk fibroin are $(AG)_m$ and $(AAG)_n$.
The $(A)_l$ (where $l > 2$) sequence is also in Anaphe fibroin.

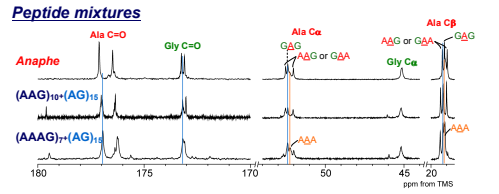
Comparison with the model peptides



- There is no detectable quantity of Gly-Gly in Anaphe silk fibroin.
- Anaphe silk fibroin has AGAG, AGAA and AAGA sequences.

The mixture of $(AG)_{15}$/$(AAG)_{10}$ & $(AG)_{15}$/$(AAAG)_7$ are the candidate for the model of Anaphe silk fibroin.

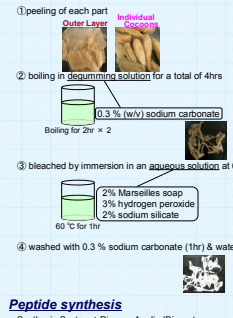
Peptide mixtures



- The mixture of $(AAG)_{10}$ & $(AG)_{15}$ is markedly similar to the spectrum of *Anaphe* silk fibroin .
- The shoulders of Ala Cα and Cβ central peaks of *Anaphe* silk are assigned to $(A)_l$ sequence.

Experimental

Degum & Bleach



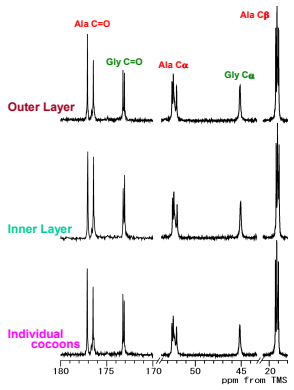
Peptide synthesis

Synthesis System ; Pioneer;AppliedBiosystems
Cycle : Capping
Cleavage cocktail : TFA/Triisopropylsilane/H_2O/Phenol=88/2/5/5
After cleavage : dissolved in aqueous 60% w/v LiSCN solution and dialyzed against distilled water for four days using cellulose tubes (MWCO; 1000). Thereafter the contents of the dialysis tubes were freeze-dried.

Solution ^{13}C-NMR

Spectrometer : Bruker DMX 500 MHz
Samples : 40 mg of each silk fibroin or 10 mg of each peptides
mixture ratio of $(AG)_{15}$/$(AAG)_{10}$: 2/3 in molar ratio
mixture ratio of $(AG)_{15}$/$(AAAG)_7$: 7/15 in molar ratio
Solvent : 960 µl of 60 % aqueous LiSCN solution + 40µl D_2O
* To avoid the large peak derived from the thiocyanate carbon from the LiSCN, the ^{13}C NMR spectra were measured by dividing into two regions

^{13}C NMR spectra of Anaphe

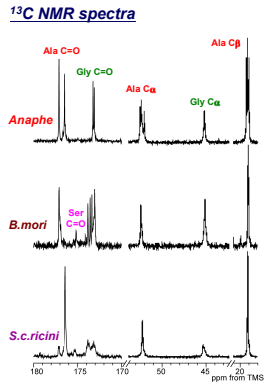


- Spectra for each part of the silk nest were closely similar.
- Only Ala and Gly peaks could be observed.

Amino acid composition and primary structure in each part of the silk nest is almost same.

Comparison with the other silk fibroins

^{13}C NMR spectra



- The spectrum for Anaphe is markedly different from the other silks.
  - Gly C=O peaks are only two. (In others, it split into many peaks.)
  - Ala C=O peaks are also two. (In others, it is only one.)
  - Ala Cα & Cβ peaks split into roughly three peaks.

The amino acid sequence of Anaphe silk fibroin is different from other silk fibroins.

Amino acid sequence

Design & Production of Anaphe Silk Fibroin like Cell Adhesive Proteins

Design

AAG6 & AG9
Derived from *Anaphe* silk fibroin

Expected to be a good performance as a material

- High strength, High modulus
- High thermal stability
- Good workability
- Good biocompatibility
- Biodegradability

TS(AAGAAGAAGAAGAAGAAGASTGRGDSPAAS)$_n$
TS(AGAGAGAGAGAGAGAGAGASTGRGDSPAAS)$_n$

FN (TGRGDSPA)
Derived from Fibronectin

High cell adhesive activity is expected

- Fibronectin is one of the constituent of extracellular matrix.
- This region contains most famous cell adhesive motif, RGD.

Construction of the genes

Design of oligo nucleotides

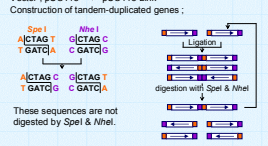
For Target Sequence
Spe I – Target Sequence - *Nhe* I
Encoding $(AAG)_n$, $(AG)_n$ and TGRGDSPA

For Linker
*Bam*HI–*Xba*I-Met-*Spe*I–*Apa*I-*Nhe*I–Met-*Xba*I-*Bam*HI

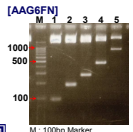
*Bam*HI, *Xba*I, *Spe*I, *Nhe*I; restriction enzyme digestion site for cloning
Met; encoding methionine for cleaving by cyanogen bromide

Cloning of the genes

Bacterial strain ; *E.coli* DH5α
Vector ; pUC118 → pUC118-Link
Construction of tandem-duplicated genes ;

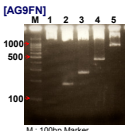


[AAG6FN]



M ; 100bp Marker
1 ; monomer / *Spe* I & *Nhe* I
2 ; dimer / *Spe* I & *Nhe* I
3 ; Trimer / *Spe* I & *Nhe* I
4 ; Pentamer / *Spe* I & *Nhe* I
5 ; decamer / *Spe* I & *Nhe* I

[AG9FN]

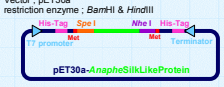


M ; 100bp Marker
1 ; monomer / *Spe* I & *Nhe* I
2 ; dimer / *Spe* I & *Nhe* I
3 ; Trimer / *Spe* I & *Nhe* I
4 ; Pentamer / *Spe* I & *Nhe* I
5 ; decamer / *Spe* I & *Nhe* I

Production of the proteins

Construction of expression vectors

Bacterial strain ; *E.coli* DH5α
Vector ; pET30a
restriction enzyme ; *Bam*HI & *Hind*III



Expression of the protein

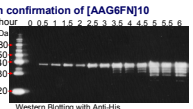
Bacterial strain ; *E.coli* BLR(DE3) pLysS
Vector ; pET30a-AAG6FN-10mer
Expression medium ; TB (1.2L)
Cultivation temperature ; 30 ℃
Induction of expression ; 1mM IPTG
Induction hour ; 1 hr

Purification of the protein

Affinity chromatography;
Chromatography carrier ; Ni^{2+}-NTA
Buffer ; 8M Urea-Tris pH 8.0 for lysis
pH 6.3 for wash
pH 4.5 for elution

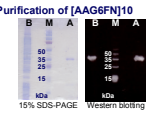
Expression confirmation of [AAG6FN]10

Induction hour 0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6



Western Blotting with Anti-His after 15% polyacrylamide gel electrophoresis

Purification of [AAG6FN]10



A: After purification
B: Before purification
M: MW Marker

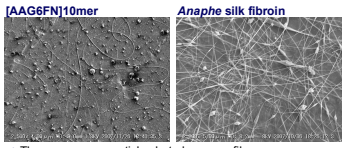
15% SDS-PAGE Western blotting

Electrospinning of AAG6FN-10mer

Conditions

Sample condition ; 10mg protein / 100µL HFIP
Solution sending rate ; 1mL / hour
Power voltage ; 10kV
Distance ; 12 cm
SEM observation ; KEYENCE VE-7800

[AAG6FN]10mer *Anaphe* silk fibroin



- There are many particles but also some fibers.

- **[AAG6FN] & [AG9FN] were designed.**
- **Tandem-duplicated genes (max. 10mer) were constructed.**
- **Purified [AAG6FN]10 was obtained.**
- **[AAG6FN]10 was spun by electrospinning.**

Anaphe silk like proteins were designed and produced. The proteins are expected to be a excellent biomaterial, while it needs some condition adjustment.

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