

Acid hydrolysis of silk fibroins and determination of the enrichment of isotopically labeled amino acids using precolumn derivatization and HPLC-ESI-MS

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Objectives

• To develop an LC-ESI-MS method to determine the isotopical enrichment in silk proteins

Introduction

Silk proteins (fibroins) from silkworms and spiders are composed of highly repetitive Ala and Gly-rich blocks that determine their structure and function.^{1,2} Fibroins are characterized by their material strength and extensibility, leading to an extraordinary toughness.^{1,2} The analysis of these proteins is rather difficult since they are resistant to enzymatic cleavage. A common method to study such high molecular weight proteins is the implementation of stable isotope tracers followed by MS analysis.

The isotopical enrichment of the main components of solid silk proteins was determined after acid hydrolysis, derivatization of the corresponding amino acids and subsequent separation by reversed phase HPLC-MS. Enrichments of up to 58% in Gly and 31% in Ala have been observed in some silks.



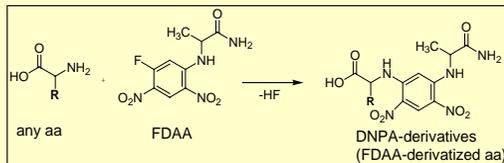
Fig. 1: *Nephila edulis* spider³



Fig. 2: *Samia cynthia ricini* moth larvae⁴

Methods

Silks from the spider *Nephila edulis* and the silk moth *Samia cynthia ricini* were collected after the animals have been fed with different isotopically labeled amino acids. Silk proteins were completely hydrolyzed with hydrochloric acid, and the resulting amino acids were derivatized with FDAA (*N*-(2,4-Dinitro-5-fluorophenyl)-L-alaninamide, Marfey's reagent) to give the responding DNPA (2,4-dinitrophenyl-L-alanine amide) derivatives. DNPA derivatives were separated by LC-MS (HP1100) and the enrichment of the stable isotopes was determined. Software was developed to aid the calculations.⁵



Scheme 1: Reaction of any aa with FDAA

Conditions of LC-MS:
Stationary phase: HAISIL HL C18, 3 μ m, 150 x 2.1 mm
Mobile phase: Gradient: 10-90% solvent B
Solvent A: 5% acetic acid
Solvent B: ACN
Flow rate: 250 μ L/min
Temperature: ambient
Instrumentation: HP1100 MSD

Results

The enrichment of stable isotopically labeled amino acids was determined by MS after complete acid hydrolysis, derivatization with FDAA and subsequent LC separation. The focus of this study was the simultaneous analysis of the major components of the fibroins, Ala and Gly, and their isotopic enrichments.

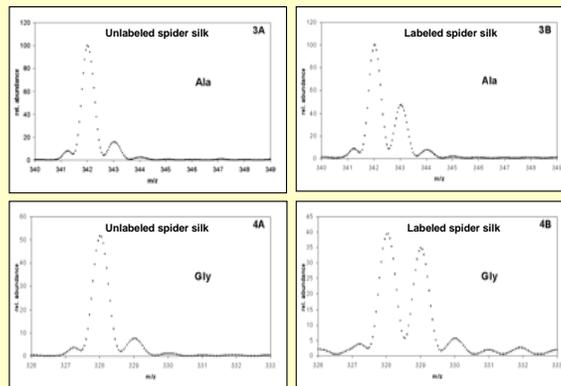


Fig. 3, 4: Isotopic enrichment determined by LC-MS.

The enrichment data of selected silk samples from *N. edulis* and from *S. cynthia ricini* are shown in Table 1. Fibroins from *N. edulis* were divided in two groups, those from spiders fed with one labeled amino acid only (group I) and those fed with a mixture of labeled and unlabeled amino acids (group II).

Sample	Intended label	Average enrichment of Gly		Average enrichment of Pro		Average enrichment of Glu	
		Ala in % \pm S.D.	in % \pm S.D.	in % \pm S.D.	in % \pm S.D.	in % \pm S.D.	in % \pm S.D.
A	unlabeled	1.4 \pm 1.4	1.2 \pm 2.3	0.1 \pm 0.1	0.2 \pm 1.6		
Group I: <i>N. edulis</i> fibroins, fed with a labeled amino acid only							
B	[¹³ C]Gly	8.9 \pm 1.1	57.8 \pm 0.6	1.5 \pm 0.5	1.9 \pm 0.7		
C	[¹³ C]Ala	26.1 \pm 1.7	2.8 \pm 1.0	0.8 \pm 0.1	2.9 \pm 0.9		
D	[¹³ C]Gly	8.9 \pm 0.7	58.3 \pm 0.2	0.5 \pm 0.8	2.4 \pm 0.2		
E	[¹³ C]Ala, [¹³ C]Gly	12.5 \pm 0.7	52.9 \pm 0.9	1.6 \pm 0.6	3.5 \pm 1.2		
Group II: <i>N. edulis</i> fibroins, fed with a labeled amino acid together with unlabeled amino acids							
F	[¹³ C]Ala	30.8 \pm 0.6	3.1 \pm 0.2	0.9 \pm 0.1	5.8 \pm 0.4		
G	[¹³ C]Gly	7.9 \pm 0.5	44.5 \pm 1.8	0.7 \pm 1.0	3.3 \pm 1.0		
H	[¹³ C]Ala	10.7 \pm 0.6	0.8 \pm 0.6	0.5 \pm 0.6	0.8 \pm 0.3		
I	[¹³ C]Gly	9.1 \pm 2.4	47.1 \pm 2.3	0.3 \pm 0.4	1.8 \pm 0.4		
J	[¹⁵ N]Gly	11.2 \pm 1.0	28.4 \pm 1.0	1.25 \pm 0.6	10.7 \pm 0.4		
Group III: <i>S. cynthia ricini</i> fibroins, fed with a labeled amino acid only							
K	[¹³ C]Gly	1.3 \pm 0.4	33.1 \pm 1.9	0.7 \pm 0.4	3.2 \pm 2.1		
L	[¹³ C]Ala, Film	14.4 \pm 0.7	0.1 \pm 0.5	0.7 \pm 1.2	1.9 \pm 0.5		

In general, the main component of silk fibroins, Gly, showed higher enrichment than Ala. This was true for the moth silks and the fibroins from both spider groups. Among the spider groups, a higher incorporation in the silk was achieved when spiders were fed only one labeled amino acid as done in group I.

A ¹³C-label is preferable over a ¹⁵N-label, since ¹⁵N-amino groups can easily be transaminated leading to cross-labeling throughout the investigated amino acids. The enrichment versus cross-labeling ratio of Gly and Ala was higher in fibroins collected from *S. cynthia ricini* moths than those from *N. edulis* spiders.

Validation

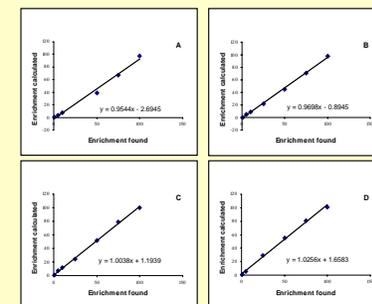


Fig. 5: Plot of observed enrichments against theoretical enrichments.

Conclusions

Our investigations helped us to choose the best samples for further NMR studies.

Although it was not the goal of this study to gain insight in the complex biosynthetic pathways of the amino acids investigated, one can conclude that the amino acids in silk fibroins are derived from both diet and de novo biosynthesis.

The present analytical method can be applied to other proteinogenic amino acids since it shows a high separation power for all DNPA-derivatives. Considering the widespread use of single quadrupole mass spectrometers, the method developed herein represents a preferred alternative for the more laborious and traditional GC-MS methods currently in use.

References

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- [4] <http://pooh.unl.edu/~scotch/samantha/moths-butterflies.html?page=cynthia-ricini>
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