様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

# 科学研究費助成事業　　研究成果報告書

令和　５ 年　 ５ 月 ２４ 日現在

機関番号：８２４０１
研究種目：若手研究
研究期間：2020～2022
課題番号：２０Ｋ１５７３８

研究課題名（和文）Elucidation of effect of phase separation on conformation and dynamics of spider silk proteins

研究課題名（英文）Elucidation of effect of phase separation on conformation and dynamics of spider silk proteins

研究代表者

Ｏｋｔａｖｉａｎｉ　ＮＡ（Oktaviani, Nur Alia）

国立研究開発法人理化学研究所・環境資源科学研究センター・基礎科学特別研究員

研究者番号：７０７５３８３８

交付決定額（研究期間全体）：（直接経費）　　3,100,000円

研究成果の概要（和文）：本年度は、反復ドメインと全長ミニスピドロインNR6C、NR12Cのコンフォメーションとダイナミクスに及ぼす相分離の影響について研究しました。その結果、繰り返しドメインのチロシン残基がLLPS現象の影響を受けることを発見しました。そこで、繰り返しドメインのチロシン残基をセリン残基に置き換えた変異体を作製しました。この変異体は、kPi濃度が高くなるとオリゴマー化が劇的に抑制され、LLPS現象におけるオリゴマー化を促進するチロシン残基の役割が示唆された。以上のことから、C末端ドメイン（CTD）と反復ドメインのチロシンは、LLPS形成時のオリゴマー化を媒介する重要なステッカーであることがわかった。

研究成果の学術的意義や社会的意義
In natural spinning process, spidroins undergo the liquid-liquid phase separation (LLPS). Since LLPS is one of the emerging topics in biology, thus this study is useful not only to understand the mechanism of natural spinning process, but also to obtain the insight into the molecular basis of LLPS.

研究成果の概要（英文）：In this fiscal year, I investigated the effect of phase separation on conformation and dynamics of the repetitive domain and full length minispidroin NR6C and NR12C. The tyrosine residue of the repetitive domain was found to be affected upon LLPS phenomenon. Then, we made a mutant on the repetitive domain which substituted the tyrosine with serine residue. The mutant does not have an effect on the conformattion and dynamics of minispidroin. However, we found a drastic effect on the mutant, where the oligomerization was suppressed at higher kPi concentration, suggesting the important role of tyrosine residues in promoting oligomerization upon LLPS phenomenon. Overall, we found that C-terminal domain (CTD) and tyrosine from the repetitive domain are important as stickers which mediate the oligomerization on the LLPS formation.

研究分野：protein NMR

キーワード：NMR　spider silk　protein　structure　mechanism　LLPS

１．研究開始当初の背景

Spider dragline silks have attracted great interest due to their superior mechanical properties and biocompatibility, which will be useful for many industrial and biomedical applications. Spider dragline silks are high molecular weight proteins (250-350 kDa), which is composed of N-terminal domain (NTD) and C-terminal domain (CTD) (16.5 kDa and 10.5 kDa, respectively) separated by a long repetitive domain. The repetitive domain mainly consist of polyalanine stretches (number of alanine residues = 4-12), which form ß-sheet structure in crystalline region and glycine-rich sequences (GGX-), which are found in amorphous region (Simmons, A.H, Science,1996; van Beek, J.D, PNAS, 2002).

Prior to spinning, spider silk proteins (spidroin) are stored in the spinning dope at high concentrations (up to 50% w/v). These proteins are transformed into insoluble silk fibers after experiencing pH and ions-gradient. The pH in the spinning dope is slightly basic and it contains a high concentration of chaotropic ions ($Na^+$, $Cl^-$, $Mg^{2+}$). In contrast, closer to spinning duct, the pH is getting more acidic, the concentration of chaotropic ions decrease and the concentration of kosmotropic ion ($PO_4^{3-}$). The structure of NTD and CTD of spidroin display strong pH dependence and these domains are essential for controlling the pH-dependent of fiber assembly. The CTD forms a folded dimer at slightly basic pH (pH > 7) and becomes unfolded at acidic pH (pH 5), while the NTD is in monomeric form at basic pH and high concentration of chaotropic ions and forms a homodimer with an antiparallel orientation at acidic pH (Hagn F et al, Nature 2010; Hagn F et al, Angew. Chem. Int. Ed Engl., 2011) (**Figure 1**). In contrast, the isolated repetitive domain is pH independent, but the conformation and dynamics of this domain is affected by the ion compositions and concentrations (Oktaviani, N.A et al, Nat. Commun, 2018 and Oktaviani, N.A et al, Chem. Commun 2019).

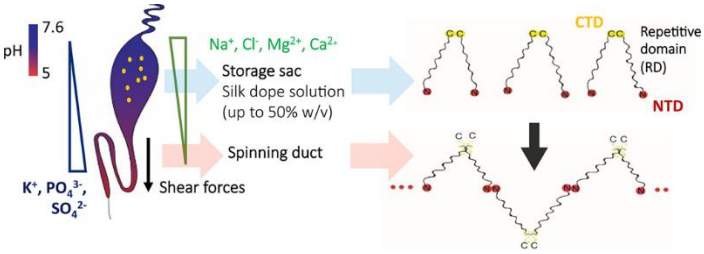


Figure 1. Spider gland and ions and pH-dependent conformation on NTD and CTD (Malay A. D et al, Biomacromolecules, 2022).

Production of artificial spider silk protein (spidroin) is required because spiders cannot be farmed due to their cannibalistic behavior. Biomimetic spinning is an essential task to achieve strong and elastic artificial spider dragline silk, which perfectly mimic the mechanical properties of natural spider silk. Comprehensive knowledge of natural spinning process is required to perform and develop biomimetic spinning of artificial spider dragline silks. For a long time, techniques that have been applied to form fibers from silk solution have been based on solvent extrusion, especially using organic solvents (Scheibel, T. Microb. Cell. Fact, 2004). However, this process did not result in strong and elastic fibers.

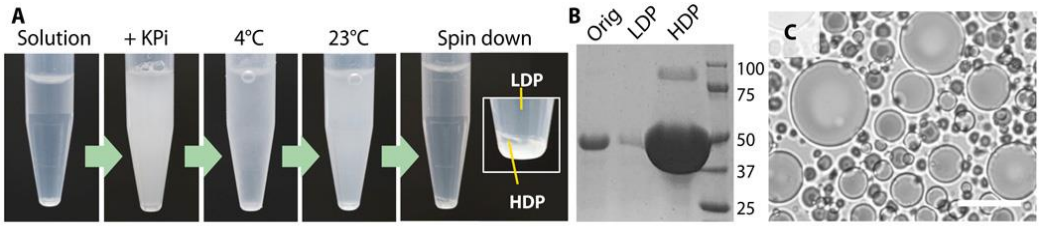


Figure 2. LLPS in Spidroin MaSp2. (**A**) Purified MaSp2 became turbid upon adding KPi, pH 7.3 (to 0.4 M), with the turbidity varying with temperature. (**B**) SDS-PAGE of 1-µl aliquots from LDP and HDP in (A) shows the partitioning of MaSp2 into the HDP, with HDP:LDP ratio above 100:1. (**C**) Microscopic droplets formed immediately in a mixture of N-R6-C (4.5 mg/ml) and 1.0 M KPi (pH 7); scale bar, 20 µm (Malay, A.D, Sci, Adv, 2020).

One of the important process during natural spinning mechanism is that spidroins undergo liquid-liquid phase separation (Exler, J.H et al Angew. Chem Int. Ed. 2007; Malay, A.D, Sci. Adv, 2020). The LLPS formation of spidroin is stimulated by higher concentration on kosmotropic ions, especially, phosphate ions (Malay, A.D, Sci. Adv, 2020). However, the conformation and dynamics of spidroin upon LLPS formation remains unclear. The molecular basis which drives the LLPS formation of spidroin is still poorly understood.

２．研究の目的

The purpose of this study is to elucidate the effect of phase separation on conformation and dynamics of spidroin. Furthermore, the molecular basis which mediates the LLPS formation will be determined as well.

３．研究の方法

Recombinant spider dragline silk proteins from *Triconephila clavipes* were employed as model for this study. Solution-state NMR spectroscopy was mainly used to investigate the conformation and dynamics of spidroin upon LLPS formation. To do this, spidroins were isotopically ($^{13}C$, $^{15}N$) labeled. The spidroins were encoded in the pET15b plasmid vector and transformed into *E. coli* BL21(DE3). The bacteria were initially grown in 5 mL of LB media at 37°C, 160 rpm overnight. Then, the culture was transferred to 100 mL of M9 minimal medium containing $^{13}C$-glucose and $^{15}N$ ammonium chloride. The bacteria were continuously growing at 37°C, 160 rpm overnight. The culture was transferred to big M9 culture (900 mL) containing $^{13}C$-glucose and $^{15}N$ ammonium chloride at 37°C, 160 rpm until $OD_{600}$ reached ~1. Protein expression was induced by adding 0.4 mM IPTG (Wako) with overnight shaking, and the temperature was decreased to 20 °C. The spidroin was purified using His-trap purification. Several constructs, which are NTD, Repetitive domain which contain six repeat regions (R6), CTD, NTD-R6 (NTD with 6 repeat regions), R6-CTD (6 repeat regions with CTD) and full length minispidroin NTD-R6-CTD (NR6C) and full length mini spidroin NTD-R12-CTD (NR12C) were used in this study. In addition to the wild type constructs, site directed mutagenesis on the repetitive domain, in which tyrosine residue was substituted by serine was also created. The mutant was expressed and purified in the same way with wildtypes. NMR samples of spidroins were prepared at different kPi concentrations at pH 7 (10 mM, 310 mM, 510 mM kPi) in the presence of 300 mM NaCl, supplemented by 10% D20 and 0.1 mM DSS.

The NMR spectra of each domain at pH 7 in the presence of 300 mM NaCl were recorded at temperature 35°C. The backbone and side chain chemical shifts were assigned based on 2D and 3D NMR experiments, as following :2D $^{1}H$-$^{15}N$ HSQC, 2D $^{1}H$-$^{13}C$ HSQC Aliphatic, 2D $^{1}H$-$^{13}C$ HSQC Aromatic, 3D HNCA, 3D HN(CO)CA, 3D HNCO, 3D HN(CA)CO, 3D CBCA(CO)NH, 3D HNCACB, 3D HBHA(CO)NH, 3D H(CCO)NH, 3D (H)C(CO)NH. All spectra were processed using NMRPipe and analyzed using NMRFAM-SPARKY. The structural propensities of the monomer, dimer, trimer, hexamer, and 15-mer were calculated using the neighbor corrected structural propensity calculator (ncSPC) with all assigned backbone (Cα, Hα, C′, Cβ, and $N^{H}$) chemical shifts. Dynamics of spidroin were determined by recording $^{15}NT_1$, $^{15}NT_2$ relaxation and $\{^{1}H\}$-$^{15}N$ Heteronuclear NOE experiments. Additionally, microscopy images of each domain at different kPi concentrations were also recorded.

４．研究成果

Expression, purification, and assignment of each domain were successfully established. The NMR assignment of NTD MaSp2 and the structure of NTD MaSp1 and MaSp2 of *Triconephila clavipes* as well as the sequential NTD dimerization mechanism were elucidated presented in the papers and conferences (Oktaviani, N.A et al Biomol NMR assign, 2020; Oktaviani, N.A et al, Biomacromolecules, 2023).

In the third fiscal year, the effect of phase separation on conformation and dynamics of spidroin has been successfully elucidated. Adding higher kPi concentrations leads to significant line broadening, suggesting the spidroins form oligomer upon LLPS phenomenon. This process was mediated by the C-terminal domain and the glycine-rich region of the repetitive domain. The amino acid sequence of CTD which is affected by kPi concentration

was also determined. Furthermore, the tyrosine residue of the repetitive domain was affected by the kPi concentration. Drastic effect was found on the mutant, where the oligomerization was suppressed at higher kPi concentration, suggesting the important role of tyrosine residues in promoting oligomerization upon LLPS phenomenon. The results of this study are still **in preparation**. One of the result related to the conformation of spidroin MaSp1 is now **under review**.

In the fiscal year 2022, I published two papers, which are:
(1). Kazuharu Arakawa, Nobuaki Kono, Ali D. Malay, Ayaka Tateishi, Nao Ifuku, Hiroyasu Masunaga, Ryota Sato, Kousuke Tsuchiya, Rintaro Ohtoshi, Daniel Pedrazzoli, Asaka Shinohara, Yusuke Ito, Hiroyuki Nakamura, Akio Tanikawa, Yuya Suzuki, Takeaki Ichikawa, Shohei Fujita, Masayuki Fujiwara, Masaru Tomita, Sean J. Blamires, Jo-Ann Chuah, Hamish Craig, Choon P. Foong, Gabriele Greco, Juan Guan, Chris Holland, David L. Kaplan, Kumar Sudesh, Biman B. Mandal, Y. Norma-Rashid, Nur Alia Oktaviani, Rucsanda C. Preda, Nicola M. Pugno, Rangam Rajkhowa, Xiaoqin Wang, Kenjiro Yazawa, Zhaozhu Zheng, Keiji Numata. 1000 spider silkomes: Linking sequences to silk physical properties. Science Advances, 8, 41, 2022.
(2). Nur Alia Oktaviani, Ali. D. Malay, Akimasa Matsugami, Fumiaki Hayashi, and Keiji Numata. Unusual p$K_a$ Values Mediate the Self-Assembly of Spider Dragline Silk Proteins.Biomacromolecules, 24, 4, 1604-1616, 2023.

In the fiscal year 2022, I presented my research results in the conferences, as follows:
(1). Nur Alia Oktaviani, Ali D. Malay, Akimasa Matsugami, Fumiaki Hayashi and Keiji Numata. Rapid self-assembly of spider dragline silk is governed by unusual pKa value of conserved acidic residues on N-terminal domain". CSRS progress report. November 4$^{th}$, 2022. Invited
(2). Nur Alia Oktaviani. Unraveling the mysteries behind the spider silk formation by NMR. Guest lecture at department chemistry, Institut Teknologi Bandung. October 22$^{nd}$, 2022. Invited
(3). Nur Alia Oktaviani, Ali D. Malay, Akimasa Matsugami, Fumiaki Hayashi and Keiji Numata. Unusual p$K_a$ values of conserved residues governs the pH response dimerization of N-terminal domain of spider dragline silk protein. Presented at 71$^{st}$ Symposium on Macromolecules, Sep 7, 2022.

５．主な発表論文等

〔雑誌論文〕　計5件（うち査読付論文　5件／うち国際共著　1件／うちオープンアクセス　1件）

| | |
|---|---|
| １．著者名<br>Ali D Malay, Hamish C. Craig, Jianming Chen, Nur Alia Oktaviani, Keiji Numata | ４．巻<br>23 |
| ２．論文標題<br>Complexity of Spider Dragline silk | ５．発行年<br>2022年 |
| ３．雑誌名<br>Biomacromolecules | ６．最初と最後の頁<br>1827-1840 |
| 掲載論文のDOI（デジタルオブジェクト識別子）<br>10.1021/acs.biomac.1c01682 | 査読の有無<br>有 |
| オープンアクセス<br>オープンアクセスとしている（また、その予定である） | 国際共著<br>該当する |

| | |
|---|---|
| １．著者名<br>Nur Alia Oktaviani, Ali D. Malay, Akimasa Matsugami, Fumiaki Hayashi, Keiji Numata | ４．巻<br>14 |
| ２．論文標題<br>Nearly complete 1 H, 13 C and 15 N chemical shift assignment of monomeric form of N-terminal domain of Nephila clavipes major ampullate spidroin 2 | ５．発行年<br>2020年 |
| ３．雑誌名<br>Biomolecular NMR assignments | ６．最初と最後の頁<br>335-338 |
| 掲載論文のDOI（デジタルオブジェクト識別子）<br>10.1007/s12104-020-09972-5 | 査読の有無<br>有 |
| オープンアクセス<br>オープンアクセスではない、又はオープンアクセスが困難 | 国際共著<br>－ |

| | |
|---|---|
| １．著者名<br>Choon Ping Foong, Mieko Higuchi-Takeuchi, Ali D. Malay, Nur Alia Oktaviani, Chonprakun Thagun, Keiji Numata | ４．巻<br>3 |
| ２．論文標題<br>A marine photosynthetic microbial cell factory as a platform for spider silk production | ５．発行年<br>2020年 |
| ３．雑誌名<br>Communications Biology | ６．最初と最後の頁<br>357 |
| 掲載論文のDOI（デジタルオブジェクト識別子）<br>10.1038/s42003-020-1099-6 | 査読の有無<br>有 |
| オープンアクセス<br>オープンアクセスではない、又はオープンアクセスが困難 | 国際共著<br>－ |

| | |
|---|---|
| １．著者名<br>K Arakawa, N Kono, AD Malay, A Tateishi, N Ifuku, H Masunaga, R Sato, K Tsuchiya ,.., NA Oktaviani, RC Preda, NM Pugno, R Rajkhowa, X Wang, K Yazawa, Z Zheng, K Numata | ４．巻<br>8 |
| ２．論文標題<br>1000 spider silkomes: Linking sequences to silk physical properties | ５．発行年<br>2022年 |
| ３．雑誌名<br>Science Advances | ６．最初と最後の頁<br>6043 |
| 掲載論文のDOI（デジタルオブジェクト識別子）<br>10.1126/sciadv.abo6043 | 査読の有無<br>有 |
| オープンアクセス<br>オープンアクセスではない、又はオープンアクセスが困難 | 国際共著<br>－ |

| | |
|---|---|
| 1．著者名<br>Nur Alia Oktaviani, Ali D. Malay, Akimasa Matsugami, Fumiaki Hayashi, Keiji Numata | 4．巻<br>24 |
| 2．論文標題<br>Unusual pKa Values Mediate the Self-Assembly of Spider Dragline Silk Proteins | 5．発行年<br>2023年 |
| 3．雑誌名<br>Biomacromolecules | 6．最初と最後の頁<br>1604-1616 |
| 掲載論文のDOI（デジタルオブジェクト識別子）<br>10.1021/acs.biomac.2c01344 | 査読の有無<br>有 |
| オープンアクセス<br>オープンアクセスではない、又はオープンアクセスが困難 | 国際共著<br>－ |

〔学会発表〕　計9件（うち招待講演　5件／うち国際学会　2件）

| |
|---|
| 1．発表者名<br>Nur Alia Oktaviani, Ali D. Malay, Akimasa Matsugami, Fumiaki Hayashi and Keiji Numata |
| 2．発表標題<br>Unraveling dimerization mechanism of N-terminal domain of spider dragline silk proteins |
| 3．学会等名<br>70th symposium on macromolecules（招待講演） |
| 4．発表年<br>2021年 |

| |
|---|
| 1．発表者名<br>Nur Alia Oktaviani,  Akimasa Matsugami, Fumiaki Hayashi and Keiji Numata |
| 2．発表標題<br>Elucidation of ions effect on conformation and dynamics of repetitive domain of spider dragline silk protein: Implication on solubility and self-assembly |
| 3．学会等名<br>2021 Fiber Annual Meeting |
| 4．発表年<br>2021年 |

| |
|---|
| 1．発表者名<br>Nur Alia Oktaviani, Ruud M. Scheek, Frans A.A Mulder |
| 2．発表標題<br>Electrostatic interactions of protein elucidated by solution state NMR spectroscopy: an application to photoactive yellow protein |
| 3．学会等名<br>International Seminar on New Paradigm and Innovation on Natural Sciences and its Application (ISNPINSA)（招待講演）（国際学会） |
| 4．発表年<br>2021年 |

１．発表者名
Nur Alia Oktaviani

２．発表標題
Understanding Electrostatic Interactions in Photoactive Yellow Protein

３．学会等名
International summer course in marine natural products (ISCMNP)（招待講演）（国際学会）

４．発表年
2021年

１．発表者名
Nur Alia Oktaviani, Ali D. Malay, Akimasa Matsugami, Fumiaki Hayashi and Keiji Numata

２．発表標題
Molecular basis underlying the sequential dimerization mechanism of N-terminal domain of spider dragline silk proteins

３．学会等名
69th symposium on macromolecules

４．発表年
2020年～2021年

１．発表者名
Nur Alia Oktaviani, Akimasa Matsugami, Fumiaki Hayashi and Keiji Numata

２．発表標題
Roles of ion on solubility and self-assembly of repetitive domain of spider dragline silk protein

３．学会等名
RIKEN BDR symposium

４．発表年
2020年～2021年

１．発表者名
Nur Alia Oktaviani, Ali D. Malay, Akimasa Matsugami, Fumiaki Hayashi and Keiji Numata

２．発表標題
Rapid self-assembly of spider dragline silk is governed by unusual pKa value of conserved acidic residues on N-terminal domain

３．学会等名
CSRS progress report（招待講演）

４．発表年
2022年

| |
|---|
| １．発表者名<br>Nur Alia Oktaviani |
| ２．発表標題<br>Unraveling the mysteries behind the spider silk formation by NMR |
| ３．学会等名<br>Guest lecture at department chemistry, Institut Teknologi Bandung, Indonesia（招待講演） |
| ４．発表年<br>2022年 |

| |
|---|
| １．発表者名<br>Nur Alia Oktaviani, Ali D. Malay, Akimasa Matsugami, Fumiaki Hayashi and Keiji Numata |
| ２．発表標題<br>Unusual pKa values of conserved residues governs the pH response dimerization of N-terminal domain of spider dragline silk protein |
| ３．学会等名<br>Symposium on Macromolecules |
| ４．発表年<br>2022年 |

〔図書〕　計0件

〔産業財産権〕

〔その他〕

―

６．研究組織

| | 氏名<br>（ローマ字氏名）<br>（研究者番号） | 所属研究機関・部局・職<br>（機関番号） | 備考 |
|---|---|---|---|

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

| 共同研究相手国 | 相手方研究機関 |
|---|---|