Molecular and Functional Study of Monalysin, a Pore-Forming Toxin from Pseudomonas entomophila

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学位論文要旨

Pseudomonas entomophila is an entomophatogenic bacterium that infects and kills *Drosophila* and other insects upon ingestion. It is a suitable model to study the interaction between pathogen and *Drosophila's* innate immunity. Monalysin, a β-barrel pore-forming toxin from *P. entomophila,* impairs *Drosophila's* tissues leading to the necrotic cell death.

In this study, I present the first purification and characterization of endogenous Monalysin. Monalysin is successfully purified as a pro-from, which is cleaved by trypsin treatment into its active form that is able to kill *Drosophila* cell lines and adult flies. Electrophysiological analysis of Monalysin in a lipid membrane with an on-chip device proves that active Monalysin forms a pore. Using current amplitude for a single pore, this analysis also provides a pore-size estimate of Monalysin and suggests its lipid insertion preferences. Atomic Force Microscope (AFM) analysis in a solution demonstrates that active-Monalysin is stable and composed of an 8-mer complex; which is consistently supported with mass spectrometry data. AFM analysis also proves 8-mer structure of active-Monalysin in a lipid bilayer, and real-time imaging shows dynamic insertion of Monalysin into the lipid membrane. Together, these results suggest that endogenous Monalysin is a pore-forming toxin which has a rigid structure before pore formation in the lipid membrane. This study provides a sophisticated tool to analyze the mechanism of host innate immunity in response to the invasion of entomopathogenic bacteria that produce pore-forming toxin.

Pseudomonas entomophila is an entomophatogenic bacterium that infects and kills *Drosophila* and other insects upon ingestion. This bacterium was isolated from a single *Drosophila melanogaster* female collected in Calvaire (Guadeloupe) as part of a *Drosophila* bacterial pathogen screen. It is the only *Pseudomonas* species that can naturally infects and kills insects after being ingested. This Gram-negative bacterium has become one of the best model to investigate the interaction of insect and microbe. This bacterium induces systemic immune response in both *Drosophila* larvae and adults after ingestion. Oral infection with large doses of this bacterium is particularly pathogenic to *Drosophila* and causes massive disruption of the *Drosophila* gut epithelium. The virulence factor of this bacterium is mainly derived from Monalysin, a β-barrel pore-forming toxin, that causes the damage of *Drosophila* gut epithelial cells. This toxin is synthesized as a soluble 30.2 kDa pro-form. Upon release, alkaline protease A cleave N-terminus part of pro-Monalysin resulting to the transformation of this protein to 26.5 kDa mature form. Well-characterized of Monalysin might be beneficial in studying the interaction between the host and entomopathogenic bacteria that produce damage-inducing toxins. Furthermore, pore-forming proteins such as Monalysin might be employed as biological control agents against insects, as well as biological "nanopores" that are used as single-molecule detectors. In that sense, utilizing endogenous Monalysin isolated from *P. entomophila* rather than the recombinant protein produced by *E. coli*, which may have a different intracellular environment than *P. entomophila*, might give a more detailed understanding of its protein function. The difference in intracellular environment may result in an altered protein subunit composition, which might affect the structural and functional properties of the toxin. A detailed study on Monalysin structure and dynamics in solution and in the lipid membrane would also provide useful knowledge for a variety of applications.

In this study, I present for the first time, the purification and characterization of endogenous Monalysin. This protein was isolated from *P. entomophila* extract as a pro-form using Ammonium Sulphate precipitation, followed by Anion Exchange Chromatography and Gel Filtration Chromatography method. SDS-PAGE analysis showed a single band of 30 kDa, which is the

estimate size of Monalysin. The single band was subjected to Mass Spectroscopy analysis and the result indicates that its amino acid sequences correspond to Monalysin. Then, pro-Monalysin was subjected to trypsin treatment to transform it to its mature form. Toxicity study of Monalysin mature form on *Drosophila* S2 cells and indicates that this protein is toxic to the cells. Moreover, survival study indicates that this mature protein is toxic to *Drosophila* adult. I found that this toxin disrupted the organization of *Drosophila* gut epithelial cells.

Electrophysiological analysis of active-Monalysin was performed to investigate whether this protein forms a pore. The results indicate that active-Monalysin form pores on artificial lipid bilayer membrane. Using this technique, the pore size of active-Monalysin is estimated to be around 0.7-1 nm. Electrophysiology analysis revealed that active-Monalysin forms nanopore.

Next, we investigated the structure of active-Monalysin using High-Speed Atomic Forces Microscope (HS-AFM). Unexpectedly, the result indicates that the structure of endogenous active-Monalysin is 8-mer which is different to that of the 9-mer structure of recombinant Monalysin as previously reported. We also studied the real-time dynamic of active-Monalysin using HS-AFM. We successfully recorded the insertion moment of active-Monalysin onto lipid membrane. HS-AFM analysis indicates that active-Monalysin is preferentially inserted into high-curved part of the membrane.

Together, these results suggest that endogenous active-Monalysin is a pore-forming toxin which has 8-mer structure with preference to be inserted in the high-curve area of lipid membrane. This study provides a sophisticated tool to analyze the mechanism of host innate immunity in response to the invasion of entomopathogenic bacteria that produce pore-forming toxin.

審査結果の要旨

Emil Salim 氏から提出された学位論文について、上記 5 名の審査委員による査読後、2021 年 7 月 26 日に口頭発表会が行われた。同日の最終審査委員会で審議した結果、以下のとおり判定した。孔形成毒素 Monalysin は昆虫に感染する細菌 *Pseudomonas entomophila* の病原性に強く関与する重要な毒素である。しかし、*Pseudomonas entomophila* が産生する内在性の Monalysin を単離・精製してその機能と構造を解析する研究は行われていなかった。本研究では、内在性の Monalysin を菌抽出液から精製する手法を世界に先駆けて確立し、その機能と構造を解析することに成功した。内在性の Monalysin はプロテアーゼ処理により活性型になり、活性型 Monalysin は昆虫細胞に細胞死を誘導し、ショウジョウバエ個体を傷害することを明らかにした。また、脂質二重層に孔を形成することを電気生理学的実験に示し、孔のサイズを見積もった。加えて、高速原子間力顕微鏡を用いた解析から、活性型 Monalysin は 8 量体であることを初めて明らかにし、脂質膜に Monalysin が挿入する様子をリアルタイムで観察することに成功した。本研究は昆虫病原体毒素 Monalysin の機能と構造を明らかにし、その成果は昆虫の病原体応答の基礎を形成する成果であると評価され、博士（創薬科学）の学位に値すると判定した。