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学位論文題目	A Morphological Study of Lichen-Symbionts, Ascomycetous Fungus *Myelochroa leucotyliza* and Green Alga *Trebouxia* sp.,with Special Reference to the Mechanism of Lipid(Atranorin) Secretions
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論文内容の要旨

Lichens are symbionts of fungi and algae (or cyanobacteria). Lichen forms a unique complex called thallus and produces large amount of lipids of various kinds. These lipids were secreted from the fungal cells and deposited in the thallus to form tremendous amount of crystalline materials. The crystalline materials are considered to have roles to protect the lichen from excess light, bacterial infection, freezing, and etc. This should be the reason that lichens can exhibit a wide ability of adaptations to extreme habitats even as those of alpine, desert, and polar region. From this aspect, they considered the significance of symbiosis of lichen must depend on the unusual lipid production and secretion.

As mentioned, the fungi produce lipids and secrete them out of cells. On the other hand the algae were considered to provide precursor sugars for the fungi to produce lipids (Yamazaki et al., 1965. Mosbach, 1969). Although fungi grown in the wild secrete large amount of lipids to form crystals, those grown in the culture both alone or with algae produce only small amounts and are found unable to form crystals in the culturing medium (Honegger, 1996).

This study is focused on the mode and the mechanism of the lipid secretion as they considered that the existence of large amount of crystals is signifying the symbiosis of lichens.

To begin the study they first respectively isolated the fungi and the algae from the thallus of *Myelochroa leucotyliza.* The isolated samples were cultured both alone or with each other. The cultured samples and the lichen in the wild were examined by means of quick freezing and replicated or substituted electron microscopy. Next, it seemed essential to identify the nature of crystals. X-ray and electron microscope diffraction methods and TLC analysis on the re-crystallized lipid extracted from the wild lichen did the identification. The major component of the re-crystallized material was atranorin.

According to the observations on the morphology of wild and cultured samples with electron microscopy the following results were obtained; 1) atranorin is the predominant component of the crystalline materials in the thallus, 2) the dynamic morphological change of the plasmamembrane of the fungi occurred in parallel to the activities of lipid secretion, 3) the zymogen granules contained in the fungal cytoplasm exocytose during the lipid secretion, 4) the amount of lipid bodies contained in the fungi inversely changed with those of the crystal deposition outside of the cell.

It was considered that if we could promote the cultured fungi into actively lipid-secreting cells, and if we could find crystals around the cells, we would have an opportunity to understand the entire scheme of the way the cell secretes lipid and the significance of symbiosis of lichens. Along with this consideration they cultured fungi in the medium with higher concentration of sugar than usual. According to Hamada's report (1993) that in these condition fungi synthesize large amount of lipid. They detected neither crystal by EM or existence of atranorin by TLC. It was observed, however, that in the fungal cytoplasm cultured in this sugar fortified medium existence of multiple lipid bodies together with numbers of zymogen granules. The appearance of these accumulations of multiple organelles suggested the cellular inhibition of secretions of both lipids and proteins. In order to release the inhibition they added into the culturing medium various materials that had been reported to promote lipid secretion. Finally they soaked the fungi growing in this sugar fortified culture with a few drops of the medium in which the algae had been cultured alone. The soaking looked the fungi to release the inhibition and

made them actively exocytose zymogen granules. The plasmamembrane changed the shape with many invaginations of omega appearances. The crystals were found outside of the cells.

The amount and the size of lipid bodies decrease during the active secretion of zymogen granules. However the lipid-bodies may consist of reservoirs of sterol-derivatives and triacylglycerols. It is considered that they should represent the indirect source of atranorin. Atranorin belongs to polyketides. It has to be produced via acetate-malonate pathway by the interaction of polyketide synthase, in which two phenolic units derived from acetate join to become atranorin. The cytosolic location of polyketide synthase has been reported by showing fluorescent of GFP of the GFP-tagged enzyme. Further, they observed using a fluorescent microscope the possible cytosolic location of atranorin under UV excitation. As atranorin is extremely hydrophobic the lipid must have being bound with some associating protein in the cytoplasm. However it is not known how atranorin reaches the inner side of the plasmamembrane.

The modes of lipid secretion so far survived in the long course of histology are as follows. They are 1) the simple diffusion, 2) the exocytic secretion, and 3) the endoplasmocrine secretion. The simple diffusion is the hypothesis in which lipid in the cytoplasm diffuses freely through the lipidic plasmamembrane. The secretion by exocytosis represents the hypothesis that lipids bound by the biological membrane secrete themselves as the same mechanism to that of protein secretion. Rhodin (1971) has interpreted the endoplasmocrine secretion of lipids. The lipid-body encircled by the tubules of smooth ER makes contact with plasmamembrane by some confusing manner and in the consequence intact lipid-body is exocytosed outside.

They chose two out of above mentioned three in regard to the mechanisms of lipid secretion of our fungi for the following reasons. 1) Upon incubation with ethanol, Kabakibi et al. (1998) demonstrated the release of fatty acid ethyl ester of Hep-G2 cell into the culturing medium. The secretion stopped when BFA was added to the medium. However, the secretion resumed when lipoprotein or albumin was introduced into the medium that contained BFA. They concluded that the release of the lipid had occurred via an independent pathway of vesicular transport. That is, regardless of whether or not the protein secretion was inhibited by BFA, the release of lipid actually occurred due to the added protein operating outside of the cell. 2) They also did the experiment along the line shown by these authors. To determine whether albumin can pull atranorin out of lipid membrane they prepared atranorin-containing phosphatidyl choline (PC) liposome. The obtained atranorin-PC-liposome was added with BSA-albumin and after extensive shaking the mixture was centrifuged. The pellet consisted of lipid-bileaflet membrane. The supernatant was examined using a spectrofluorometer to detect whether it contains atranorin. Added albumin actually pulled atranorin out of the lipid membrane. In order to try to isolate "effective" protein/or proteins that pull atranorin out of fungal cells they employed anionic columns. The columns separated plenty of proteins from the fungal colony. A few of them showed the extraction ability of atranorin from the atranorin-PC-liposome. However, many of them had no ability to pull atranorin out. The elution of the homogenate of the fungal colony by the "albumin column" showed the most effective pulling ability of atranorin out of the atranorin containing lipid artificial membrane.

Regarding the mode of atranorin secretion, they concluded as follows. 1) When humoral signals come from the symbiotic algae, the zymogen granules in the fungal cytoplasm exocytose the contents into the extracellular space. 2) The contents of the zymogen granules once secreted elicit atranorin locating on the other side of the membrane. Atranorin goes through the plasmamembrane in the mode of simple diffusion providing if there exists the force of proteins outside.

論文の審査結果の要旨

地衣類は菌類と緑藻類またはシアノバクテリアとの共生体である。地衣体は、共生により大量の脂質を細胞外に分泌することが可能となり、この脂質は紫外線防御、抗菌作用などを持ち、地衣類の生存に必須である。

脂質の合成は菌類が行い、藻類は脂質の炭素源として光合成により合成した糖を供給していると考えられてきたが、その細胞、分子基盤はよくわかっていなかった。この大きな理由は従来、地衣類には実験に適した培養系が無かった事である。申請者は、まず *Myelochroa leucotyliza* の地衣体から藻類と菌類を単独培養、および、両者を共培養する系を確立した。さらに、地衣体に含まれる脂質がアトラノリンであることを薄層クロマトグラフィーおよびX線回折法によって明らかにした。

次に確立した培養系より得られた地衣体、および野生の地衣体を生鮮状態において急速凍結レプリカ法と置換超薄切片法を用いて透過電子顕微鏡法によって観察した。そして、1)地衣体の主要な脂質結晶はアトラノリンである、2)脂質が分泌されるとともに菌の細胞膜にダイナミックな形態変化が生じる、3)脂質分泌の過程で菌細胞内の分泌顆粒がエキソサイトーシスされる、4)菌細胞外の脂質結晶の蓄積に伴い菌細胞内の脂肪滴が減ることを明らかにした。これらのことと、従来の脂質分泌に関する研究結果より、藻類からの誘導物質によりタンパク質分泌顆粒が菌類細胞からエキソサイトーシスされ、その分泌顆粒中のタンパク質が菌細胞からの脂質分泌を誘導しているのではないかという作業仮説をたてた。

まず、藻類からの誘導物質の有無と性質を調べた。従来、培地の糖濃度増加に伴い菌類の脂質合成量が上昇するという報告があった。*M. leucotyliza* 菌を高糖濃度培地で培養すると、細胞中に分泌顆粒と脂肪滴が蓄積したがアトラノリンは細胞外に分泌されなかった。一方、藻類培養液を加えて菌を培養すると分泌顆粒のエキソサイトーシスが誘発され、細胞外にアトラノリン結晶が析出した。このことから、藻類からの何らかの物質が分泌顆粒とアトラノリンの分泌を誘導しているらしいことがわかった。

次に、分泌顆粒によって菌細胞外に分泌されたタンパク質によって、菌細胞からアトラノリンが分泌されることを間接的に調べるために、アトラノリンを含んだフォスファチジルコリンリポソームを作成し、外生的に与えたアルブミンがアトラノリンの分泌を引き起こすかどうかを調べた。その結果、アトラノリンがリポソーム外に分泌された。分泌顆粒に含まれアトラノリンを誘導するタンパク質を同定することを目指し、菌抽出液を陰イオンカラムによって分画し菌細胞からのアトラノリン誘導能を調べた。その結果、特定の分画に活性があることがわかった。これらの実験より、分泌顆粒により菌細胞外に分泌された未同定のタンパク質が菌細胞からのアトラノリン分泌を促進している可能性が示唆できた。

以上より本研究は、地衣類の共生の分子機構解明に必須の培養系を確立するとともに、地衣類共生の意義を考える上で重要である脂質の分泌について新しい作業仮説を提唱した点で、審査委員会は学位論文として十分な内容を持つものと判定した。

学位論文として提出された研究結果について申請者による口頭発表後、審査員が論文の内容、次いで関連研究分野の一般的知識とその背景となる基礎的知識について口頭試問により審査を行った。これらの試問に対し申請者は的確な応答を行った。これらの結果をもとに、審査委員会は申請者が学位取得に足りうる学識と研究遂行能力を持つものと判定した。

英語の能力に関しても、英文で書かれた学位論文及び、その内容の一部が国際専門誌に受理されていることなどから博士の学位に足る十分な能力を有するものと判定した。