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博士の専攻分野の名称	博士（学術）
学位記号番号	博理工甲第 1002 号
学位授与年月日	平成 27 年 9 月 18 日
学位授与の条件	学位規則第 4 条第 1 項該当
学位論文題目	Analysis of the Catabolic Pathways of Glycoconjugates （複合糖質の代謝機構の解析）
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論文の内容の要旨

Glycoconjugates are the molecules in which glycan units are covalently linked to noncarbohydrate entities by a process called glycosylation. Glycoconjugates include many different types of compounds such as glycoproteins, glycopeptides, peptidoglycans, glycolipids and lipopolysaccharides. Here we analyzed two types of glycoconjugates: (1) *N*-glycoproteins and (2) *C*-mannosyl tryptophan (CMW), which are basically related to two different types of protein glycosylation namely *N*-glycosylation and *C*-mannosylation respectively.

Study-1: Analysis of the fate of $Man_1GlcNAc_2$ in *Saccharomyces cerevisiae*

N-Glycosylation is a fundamental and evolutionarily conserved co- or post-translational protein modification that occurs in all domains of life. The biosynthetic pathways for *N*-glycosylation in mammalian cells or yeast are well clarified, while the molecular details of the catabolic pathways of the glycan parts of *N*-glycoproteins are less well understood. During *N*-glycosylation, oligosaccharyltransferase (OST) catalyzes the transfer of the fully assembled glycan, $Glc_3Man_9GlcNAc_2$, from its dolichylpyrophosphate-linked precursor to the target asparagine residue of polypeptide chains, thus forming the *N*-glycoproteins in the endoplasmic reticulum (ER). While catalyzing the *N*-glycosylation reaction, OST also liberates glycan chains in the ER lumen (referred to as free *N*-glycans or FNGs). Both the protein bound and the free *N*-glycans are processed by a series of glycosidases in the ER. First, the ER-resident de-glucosylating enzymes, glucosidase I (Gls1) and glucosidase II (Gls2) rapidly removes all of the glucose molecules from the glycans followed by ER mannosidase I (Mns1) which further removes one mannose to produce $Man_8GlcNAc_2$. The correctly folded glycoproteins are then transported out of the ER to their respective destinations. On the other hand, the glycoproteins which fail to fold properly undergo additional processing by another ER mannosidase called Htm1 which removes one more mannose molecule from the glycans on the misfolded glycoproteins. The misfolded glycoproteins and the FNGs in the ER lumen are then transported to the cytosol by unclarified mechanisms. In the cytosol of *Saccharomyces cerevisiae*, the enzyme peptide:*N*-glycanse (Png1) removes the *N*-glycans from the

misfolded glycoproteins, producing additional FNGs. The FNGs in the cytosol are finally processed by a cytosol/vacuole *α*-mannosidase, Ams1, which removes *α*-mannose molecules from the FNGs to eventually form a trisaccharide $Man_1GlcNAc_2$. This trisaccharide is believed to be the end product of FNG catabolism in *S. cerevisiae,* since no *β*-mannosidase or *β*-hexosaminidase to break down $Man_1GlcNAc_2$ is known to be produced by this yeast. In this study, we attempted to determine the fate of $Man_1GlcNAc_2$ in *S. cerevisiae* to clarify if it is really the final FNG or whether it is metabolized further by enzymatic reactions. To this end, we analysed the structures and amounts of the FNGs in the log phase (4 hour culture), post-diauxic phase (day-1 and day-3 cultures) and stationary phase (day-7 culture) in yeast cells. In the log phase, the major FNG structures detected were $Man_{6\text{-}9}GlcNAc_2$ whereas no $Man_1GlcNAc_2$ was found, indicating that this trisaccharide is not normally produced by the log phase cells. In the post-diauxic phase, in addition to the $Man_{6\text{-}9}GlcNAc_2$, smaller FNGs corresponding to $Man_{2\text{-}5}GlcNAc_2$ were also detected. These FNGs ($Man_{3\text{-}9}GlcNAc_2$), however, were barely detected in the stationary phase. Only two FNG structures were detected in the stationary phase, $Man_2GlcNAc_2$ and $Man_1GlcNAc_2$, the former however representing only 6.2 % of the total. Quantitation of total FNGs showed a steady increase from the log phase to day-7 culture. There was also a rapid increase in the amount of $Man_1GlcNAc_2$, which represented ~94% of the total FNGs at day-7. Taken all these results, it can be concluded that $Man_1GlcNAc_2$ is not enzymatically catabolised during the post-diauxic/stationary phases. Chantret *et al.* (J. Biol. Chem., 286(48), 41786-41800) reported, however, that $Man_1GlcNAc_2$ levels decline when stationary cells are re-incubated in fresh medium to resume logarithmic (log) phase, raising the possibility that there might be a log phase-specific catabolic pathway for $Man_1GlcNAc_2$. To better understand the catabolic pathway of $Man_1GlcNAc_2$ in log phase cells, stationary phase yeast cells, which contain relatively large amounts of $Man_1GlcNAc_2$, were transferred to fresh medium and the $Man_1GlcNAc_2$ levels were closely monitored at various time points. The results showed that, as the cells started to divide, the levels of $Man_1GlcNAc_2$ gradually decreased. This reduction, however, can be attributed solely to a dilution effect by the cell division, rather than by enzymatic catabolism of this trisaccharide. Our results thus imply that *S. cerevisiae* does not produce a catabolic enzyme that acts on $Man_1GlcNAc_2$ and that this trisaccharide appears to be the end product of the enzymatic catabolic pathway for FNGs in this yeast.

Study-2: Analysis of FNGs in the *Saccharomyces cerevisiae* glucosidase mutants: Looking for novel glucosidase in yeast.

The two *α*-glucosidases in the endoplasmic reticulum, Glucosidase 1 (Gls1) and Glucosidase 2 (Gls2/Gtb1), are responsible for the removal of all the glucose molecules from the *N*-glycans ($Glc_3Man_9GlcNAc_2$) of glycoproteins. Removal of the glucose molecules is known to be critical for the efficient catabolism of the FNGs so that Ams1 gets access to all the *α*-mannose molecules on the *N*-glycans leading to the formation of $Man_1GlcNAc_2$. Interestingly, however, Chantret *et al.* . (J. Biol. Chem., 286(48), 41786-41800) previously detected $Man_1GlcNAc_2$ in the glucosidase deletion mutants of *S. cerevisiae*. This observation may imply that one or more additional catabolic *α*-glucosidases working on the *N*-glycans may exist in this yeast. Therefore, in the second study, in order to investigate the occurrence of any novel *α*-glucosidases in *S. cerevisiae*, we carried out precise structure analysis of the FNGs at different growth stages in three glucosidase deletion mutants of this yeast, *i.e.*, *gls1Δ*, *gls2Δ*, and *gls1Δgls2Δ*. We found the FNGs $Glc_3Man_{9\text{-}6}GlcNAc_2$ and $Glc_3Man_{8\text{-}4}GlcNAc_2$ in the log and stationary phases respectively in the *gls1Δ*and *gls1Δgls2Δ*cells. Whereas corresponding two-glucose containing FNGs were detected in the *gls2Δ*cells. All the major structural isomers of the FNGs along with their respective quantities have also been determined. To our surprise, however, no $Man_1GlcNAc_2$ or other deglucosylation products of the FNGs were detected in any of these cells under the

all conditions examined. Our results thus indicate that no additional catabolic *α*-glucosidase acting on the *N*-glycans exist in *S. cerevisiae*.

Study-3: Study on the degradation of *C*-mannosyl tryptophan (CMW) by bacteria.

C-mannosylation is a unique type of protein glycosylation that involves attachment of single mannose molecule to selected tryptophan residues in proteins, catalyzed by *C*-mannosyl transferase. This type of protein modification was reported to be present in *C. elegans*, amphibians, birds, and mammals, but not in plants, yeasts, and *E. coli*. So far twenty three proteins are known to receive *C*-mannosylation. Previously, isolation of *C*-mannosyl tryptophan (CMW) as free amino acid from human and rat urine has been reported. This indicates that *C*-mannosyl proteins in the body undergo proteolytic degradation to produce CMW which is then released outside with urine. Despite its regular excretion into the environment, however, CMW has not been reported to be accumulated in the environment suggesting that at least microbes can degrade this compound. In this study, we aim to find out if there are any bacteria which can degrade CMW and to clarify the degradation mechanism. To this end, we searched for the microbes in soil and could successfully isolate a mixed bacterial culture which can grow in a minimal media containing CMW as the only carbon source. Utilization of CMW was confirmed by HPLC which showed much reduction of CMW in the media after two days of incubation. We then attempted to isolate pure culture, i.e., the single species of bacteria degrading CMW. Because the amount of CMW was limited, we used rich media, e.g., LB to get the single colony isolates. First, single colonies from the CMW plate were streaked on LB plate and after repeated sub-culturing, a few single colonies were isolated. But these colonies isolated from the LB plate did not grow on CMW anymore, though originally the bacteria came from the CMW plate. By 16S rRNA gene sequence analysis, we found that the single colony on the CMW plate actually contained several different bacterial species. So it was possible that when the colony was transferred from the CMW plate to LB, the CMW-degrading bacteria are either not growing or growing poorly on LB, thereby outgrown by other bacteria. We then attempted to isolate each of the different bacteria present in the single but mixed colony by using rich media. We used different conditions and isolated 73 colonies of bacteria which belong to six different genera of, or similar to, *Microbacterium*, *Ochrobactrum*, *Achromobacter*, *Shinella*, *Bacterium DP-2*, and Uncultured clone H-2-16. But none of these six bacteria grew in CMW media indicating that the one degrading CMW could not be isolated. To determine which bacteria are actually involved in the catabolism of CMW, we performed next generation sequencing analysis which revealed that abundance of one bacterial species from the Sphingomonadaceae family increased concomitant with the decrease in the amount of CMW in the media whereas the abundance of the other bacteria remained more or less constant regardless of the amount of CMW in the media, implying that the Sphingomonadaceae bacterium might be the one responsible for utilizing CMW.

論文の審査結果の要旨

Tanim Jabid Hossain の博士後期課程論文発表会は 2015 年 8 月 11 日（火）に理化学研究所で実施された。“Analysis of the catabolic pathway of glycoconjugates”（邦題：複合糖質の代謝機構の解析）という題で発表が行われ、Hossain 氏がこれまで行ってきた 3 つの研究、1. Analysis of the fate of Man1GlcNAc2 in *Saccharomyces cerevisiae*（博士論文第 2 章）, 2. Analysis of FNGs in the *Saccharomyces cerevisiae* glucosidase mutants: Looking for a novel glucosidase in yeast（博士論文第 3 章）, および 3. Study on the degradation of C-mannosyl tryptophan（CMW）by bacteria（博士論文第 4 章）について英語で口頭発表が行われた。これに加えて博士論文は序論（第 1 章）と引用論文（第 5 章）の 5 章で構成されている。

1. Analysis of the fate of $Man_1GlcNAc_2$ in *Saccharomyces cerevisiae*（第 2 章）

タンパク質の N 型（アスパラギン結合型）糖鎖は真核生物において最も普遍的な共翻訳 / 翻訳後修飾反応のひとつであり、タンパク質の可溶性や熱安定性といった物理化学的性質に加え、生理活性や局在性など生理学的性質にも大きな影響を与える。出芽酵母において N 型糖鎖の生合成過程はそのほとんどが明らかにされているものの、分解においては未解決の問題が数多く残されている。例えば小胞体で合成された N 型糖鎖を持つタンパク質のうち、うまく高次構造をとれないタンパク質は細胞質に逆輸送されてプロテアソームによる分解を受けるが、その際脱糖鎖酵素であるペプチド :*N*- グリカナーゼ（PNGase; 出芽酵母では Png1）による作用によって糖鎖が脱離され、結果遊離 N 型糖鎖（Free *N*-glycan; FNGs）が生成される。その後 FNG は α- マンノシダーゼである Ams1 によって分解され、最終産物として $Man_1GlcNAc_2$（MGn2）という 3 糖を生成する。出芽酵母においては Png1 と Ams1 以外に N 型糖鎖の代謝に関わる酵素は知られておらず、この MGn2 という 3 糖が酵母において更に酵素的な代謝を受けるのかどうかについてはこれまで不明であった。本章では MGn2 の生成、および分解について詳細な解析を行った。まず増殖の状態と MGn2 の蓄積の関係であるが、増殖期には MGn2 はほとんど検出されないのに対し、定常期に入ると蓄積が始まり、その量は培養時間に伴って増え続け、最後はほぼ MGn2 のみという状態になった。これらの結果から定常期に MGn2 の分解は起こらないと判断できた。一方定常期から増殖期に細胞を移すと細胞あたりの量は減少したが、その割合は分裂による希釈効果のみの減少と仮定したとき算出される計算値との間で有意な差が観察できなかった。これらの結果は増殖期においても MGn2 の分解が検出できないことを意味し、これらの結果から出芽酵母においては遊離 N 型糖鎖の代謝最終産物は MGn2 であることが強く示唆された。

2. Analysis of FNGs in the *Saccharomyces cerevisiae* glucosidase mutants: Looking for a novel glucosidase in yeast（第 3 章）

出芽酵母の N 型糖鎖修飾ではまず Glc3Man9GlcNAc2 という 14 糖が小胞体膜のドリコール 2 リン酸上に合成され、オリゴ糖転移酵素によって Asn-Xaa-Ser/Thr（Xaa=any amino acids except Pro）という N 型糖鎖付加配列上の Asn に糖鎖が転移される。その後 Glc は速やかに小胞体内のグルコシダーゼ I（Gls1）および II（Gls2）の作用によってトリミングされる。一方で、この小胞体におけるプロセシングが阻害されたときに、他に代謝を担う α- グルコシダーゼが出芽酵母に存在するかは不明であった。そこで本章では、Gls1、Gls2 の欠損株、および Gls1/Gls2 の 2 重欠損株における増殖期、および定常期の遊離 N 型糖鎖の詳細な構造を解析した。その結果、すべての増殖状態において Glc を持つ遊離糖鎖のみが観察されたことから、出芽酵

母は小胞体のプロセシング酵素（Gls1/2）以外にN型糖鎖上のGlcを分解する酵素を持たないことが明らかとなった。

3. Study on the degradation of C-mannosyl tryptophan（CMW）by bacteria（第4章）

タンパク質の*C*-マンノシル化は特異的な配列中のトリプトファン残基にMannoseがC-C結合で結合する、比較的最近発見された糖鎖修飾である。*C*-マンノシル化されたタンパク質は体内でC-マンノシルトリプトファン（C-Man Trp; CMW）まで分解され、その後尿として体外に排出されると考えられている。しかしながらCMWは通常土壌などには観察されないことから、少なくとも微生物はこの化合物を分解出来ると考えられる。一方、CMWの化学構造、特にManとTrpの間のC-C結合は化学的に安定であると考えられ、CMWがどのように生体内で分解を受けるのかの分子機構はまったく不明である。本章では、CMWを唯一の炭素源として生育できるバクテリアを単離し、その分解機構の解明を目指した。土壌からバクテリアを単離し、そのシングルコロニーの単離を試みたところ、固体培地のシングルコロニー、および液体培地の限界希釈からも複数のバクテリアが観察された。すなわち、CMWの代謝は複数のバクテリアの共生によって行われている可能性が示唆された。様々な培地を用いてこのバクテリア群から様々なバクテリアを単離したが、それぞれ単独、あるいは混ぜ合わせてもCMWを唯一の炭素源とする培地での増殖は再現できなかった。そこで単離出来ていないバクテリアがCMWの資化に重要であると考え、CMW液体培地の増殖において経時的なCMWの消費量およびポピュレーションの変化を次世代シークエンスによって解析したところ、単離されていない*Sphingomonadaceae*（スフィンゴモナデシエ）に属するバクテリアがCMWの消費にともないその割合を増していることが確かめられた。これらの結果から、可能性のひとつとして、この*Sphingomonadaceae*がCMWの代謝に重要な役割を果たしていることが考えられる。

第2章の研究はHossain氏が第一著者として*Biosci. Biotechnol. Biochem.*誌に受理されており、第3章の研究はHossain氏が第一著者として近日中に投稿する予定である。また、第4章の研究についても投稿論文を準備中である。さらに出芽酵母を用いてカビの脱糖鎖酵素のひとつ、ENGaseの機能解析を行った研究については氏が実験の一部を担当し、共著者（8人中3番目）として*Biochem. Biophys. Res. Commun.*誌に2014年に発表されている。

結論としてHossain氏の研究では　長年その機構が不明だった出芽酵母におけるN型糖鎖代謝の分子機構の一端を明らかにするとともに、*C*-Man-Trpという化合物がバクテリアによって分解を受けること、またその分解が複数のバクテリア種の協調作用によって行われる興味深い可能性を提示するに至った。即ち本研究は未だ不明な点の多い糖タンパク質の分解機構の解明に大きく寄与するものと考えられ､学位論文として「合格」と判定した。