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学 位 論 文 名	Metabolic engineering approaches for glutamate production in *Corynebacterium glutamicum* （代謝工学的アプローチによるコリネ型細菌のグルタミン酸生産に関する研究）
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論 文 内 容 の 要 旨

Abstract

In this study, metabolic engineering approaches were applied to understand the regulatory mechanism of glutamate production and to improve glutamate production in *Corynebacterium glutamicum*. To identify the protein(s) responsible for glutamate production in *C. glutamicum*, proteomics analysis was performed using two-dimensional gel electrophoresis. Because OdhI protein was the most upregulated protein during glutamate production, the *odhI*-overexpressing strain was constructed, resulting in glutamate production without any triggers. To investigate the effect of modulation of 2-oxoglutarate dehydrogenase complex (ODHC) specific activity on glutamate production in *C. glutamicum*, the additional decrease of ODHC specific activity by the *odhA* antisense RNA expression was also conducted, resulting in the enhanced glutamate production triggered by Tween 40 addition. Moreover, the protein expression changes during glutamate production in *C. glutamicum* were also investigated, resulting in the increased unphosphorylated OdhI expression.

Chapter 1. General introduction

Metabolic engineering is an approach to understanding and using metabolic processes in order to achieve a particular desired goal. *C. glutamicum*, a glutamate-producing bacterium, is one of the most important model microorganisms. It has been used for the industrial production of amino acids such as glutamate, lysine, and so forth. Glutamate production by *C. glutamicum* is induced by some triggers such as biotin limitation, Tween 40 addition, and penicillin addition. It was also reported that metabolic change at the branching point of 2-oxoglutarate in the TCA cycle is important for glutamate production by decrease in ODHC specific activity. Moreover, the novel protein OdhI, an inhibitory protein for ODHC, was also identified, regulating ODHC specific activity. However, the regulatory mechanism of glutamate production by *C. glutamicum* is still not completely understood. Therefore, metabolic engineering approaches for glutamate production in *C. glutamicum* was considered in order to understand the regulatory mechanism of glutamate overproduction in *C. glutamicum* and to improve glutamate production. Moreover, for a better understanding of a cellular state in *C. glutamicum*, the protein expression change was also comprehensively analyzed during glutamate production in *C. glutamicum* in this study.

Chapter 2. Requirement of *de novo* synthesis of the OdhI protein on glutamate production by *C. glutamicum*

For a better understanding of a cellular state, the protein production change was investigated in *C. glutamicum* during glutamate production. Firstly, the effect of chloramphenicol, an inhibitor of *de novo* protein synthesis, on penicillin-induced glutamate production was examined, showing that the *de novo* protein synthesis after penicillin addition is absolutely required for glutamate production. To identify the *de novo* protein(s) responsible for penicillin-induced glutamate production in *C. glutamicum*, proteomics analysis was performed using two-dimensional gel electrophoresis. Of more than 500 proteins detected, 13 proteins including OdhI protein were significantly increased by penicillin addition. Therefore, the most upregulated OdhI protein was targeted to determine whether *de novo* synthesis of OdhI protein is necessary for penicillin-induced glutamate production in *C. glutamicum*. As results, the *odhI*-overexpressing strain as a metabolic engineering approach was constructed and examined, resulting in glutamate production without any triggers with the decreased ODHC specific activity. Moreover, continuous glutamate production was also achieved by the *odhI*-overexpressing strain. In these respects, the overexpression of *odhI* gene as metabolic engineering in *C. glutamicum* is useful for efficient glutamate production.

Chapter 3. Effect of *odhA* overexpression and *odhA* antisense RNA expression on glutamate production by *C. glutamicum*

To investigate the effect of modulation of ODHC specific activity on glutamate production in *C. glutamicum*, the expression of the *odhA* gene and its product, encoding the E1o subunit OdhA of ODHC and adjacent to the OdhI protein in the regulatory mechanism in glutamate production by *C. glutamicum*, was controlled by using the *odhA* overexpression or *odhA* antisense RNA expression, respectively. As results, the *odhA* overexpression led to the increased ODHC specific activity, resulting in dramatically reduced glutamate production despite Tween 40 addition, indicating that a decrease in ODHC specific activity is required for glutamate production. Moreover, the *odhA* antisense RNA expression alone as a metabolic engineering approach did not result in glutamate production in spite of the decrease in ODHC specific activity. Rather, the *odhA* antisense RNA expression as a metabolic engineering approach achieved the enhanced glutamate production triggered by Tween 40 addition due to the additional decrease in ODHC specific activity, suggesting that the *odhA* antisense RNA expression as metabolic engineering is effective in enhancing Tween 40-triggered glutamate production.

Chapter 4. Expression changes of OdhA and OdhI proteins during glutamate production in *C. glutamicum*

The changes of protein expression and its phosphorylation status play important roles in prokaryotic signaling and regulation. However, the dynamic of protein expression and its phosphorylation status during glutamate production in *C. glutamicum* is not yet fully reported. Therefore, the expression changes of OdhA and OdhI proteins to understand a cellular state during glutamate production in *C. glutamicum* were investigated to correlate between protein expression changes and glutamate production by Western blotting. As results, the overexpression of *odhI* gene showed the strongly increased expression of unphosphorylated and phosphorylated OdhI proteins with the almost constant OdhA expression, resulting in the enhanced glutamate production. Moreover, Tween 40 addition resulted in the increased unphosphorylated OdhI expression with the constant OdhA expression, leading to glutamate production. In these respects, the increased expression of unphosphorylated OdhI is functionally involved in glutamate production in *C. glutamicum*.

Chapter 5. Conclusion

Metabolic engineering techniques based on the better understanding of the cellular state by comprehensive analysis for glutamate production in *C. glutamicum* were applied to understand the regulatory mechanism of glutamate production in *C. glutamicum* and to improve glutamate production. As results, the *de novo* synthesis of the OdhI protein is required for glutamate production in *C. glutamicum*. Therefore, the overexpression of *odhI* gene alone as a metabolic engineering approach can produce glutamate without any triggers, possessing the increased unphosphorylated OdhI protein and the decreased ODHC specific activity. Moreover, the additional decrease of ODHC specific activity by the *odhA* antisense RNA expression as a metabolic engineering approach is effective for the enhancement of glutamate production triggered by Tween 40 addition. In these respects, the increased expression of unphosphorylated OdhI and the decreased ODHC specific activity is functionally involved in glutamate production in *C. glutamicum*. As a result, glutamate production in *C. glutamicum* was successively improved by metabolic engineering using the *odhI* overexpression and *odhA* antisense RNA expression in this study, respectively. Therefore, metabolic engineering approaches used in this study can be applied as a promising tool for production of targeted materials.

論文審査の結果の要旨

本論文は、代謝工学によるコリネ型細菌のグルタミン酸生産の研究に関するものである。コリネ型細菌はグルタミン酸をはじめとして多くのアミノ酸生産に用いられてきた産業有用微生物の代表的な微生物である。最近では、さらに、有機酸やバイオ燃料などに生産物質の領域は広まりつつあり、その工学的重要性は高く認識されている。コリネ型細菌においては、従来、ペニシリン添加、界面活性剤である Tween40 添加によってグルタミン酸が生産誘導されることが明らかとなっているが、これらの生産誘導が細胞内でどのようにして代謝経路におけるフラックス変化をもたらすのかについては未だ解明が十分でない。本論文においては、コリネ型細菌を用いたグルタミン酸において、代謝工学により、グルタミン酸の生産メカニズムを解明し、その解明されたメカニズムから導かれる原理に基づき分子育種を行って、グルタミン酸の生産性の向上が達成された。本論文は序章と結論を含め５章からなっている。

１章においては緒言として、代謝工学による微生物育種と物質生産、コリネ型細菌を用いた物質生産、グルタミン酸生産のメカニズム解明に関する研究背景と意義、および既存の研究成果について述べ、本研究の目的および構成について詳述している。

２章においては、２次元電気泳動と質量分析を基盤とする網羅的なタンパク質発現解析において、ペニシリン添加によって誘導されるグルタミン酸生産に必要なタンパク質の同定とその過剰発現によるグルタミン酸生産への影響の解析を行っている。OdhI タンパク質が同定され、*odhI* 遺伝子の過剰発現を行ったところ、グルタミン酸合成経路に競合する TCA 回路の代謝経路を触媒する ODHC 比活性が減少し、グルタミン酸生産フラックスが上昇することが明らかとなった。また、この *odhI* 過剰発現株は連続的なグルコースの供給によりグルタミン酸を持続的に生産することが明らかとなった。

３章においては、ODHC のサブユニットをコードしている *odhA* 遺伝子の発現を人為的に制御することが可能な株の構築を目的として、*odhA* 遺伝子過剰発現株、*odhA* アンチセンス RNA 過剰発現株を構築し、グルタミン酸生産に関する影響を解析している。その結果、*odhA* 遺伝子過剰発現株においては ODHC の比活性が上昇するとともにグルタミン酸生産の減少が確認された。また、Tween40 を添加した生産誘導において、*odhA* アンチセンス RNA 過剰発現株は ODHC の比活性が減少するとともにグルタミン酸生産を向上させることが確認された。これらの結果から、ODHC の比活性の制御、特に、生産期におけるアンチセンス RNA 法による比活性減少がグルタミン酸生産フラックス向上にとって有効であることが明らかにされた。

４章では、ODHC タンパク質の比活性が減少と OdhI タンパク質発現およびリン酸化・脱リン酸化の関係、さらにこれらの因子がグルタミン酸生産に及ぼす影響について明らかにするために、OdhA および OdhI タンパク質の抗体を作成し、Western 解析を行っている。その結果、増殖期および生産期を通じて OdhA タンパク質の発現量は全体を通じて大きく変化しないが、グルタミン酸生産時においては OdhI タンパク質の発現量が上昇し、これが ODHC 比活性の減少をもたらすことで代謝フラックスがグルタミン酸生産に向かうことが明らかとなった。また、Tween40 を添加した生産誘導においては、脱リン酸化された OdhI 発現量が顕著に増加し、脱リン酸化された OdhI が ODHC 比活性減少に有効に働くことが明らかとなった。

５章では結言として本研究で得られた知見をまとめ、コリネ型細菌による物質生産のための代謝工学に関して今後の展望について述べている。

以上のように、本論文は、産業上重要なコリネ型細菌のグルタミン酸生産に関する分子メカニズム解明のみならず、網羅的な細胞情報を基盤として合理的に物質生産のための分子育種、細胞改良を行う代謝工学の発展に大きく貢献することが期待される。

よって本論文は博士論文として価値あるものと認める。