Microbial Synthesis of Oxo Cyclic Amino Acids

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Abstract

In order to obtain microorganisms that can synthesize of oxo cyclic amino acids from hydroxy cyclic amino acids, we performed screening of microorganisms that are able to do so from 10 microbial samples collected from the Kyoto University Botanical Garden. Isolated microorganism colonies were shake-incubated in a liquid nutrient medium containing hydroxy cyclic amino acids as the only source of carbon, and the resulting suspension was subjected to TLC analysis with ninhydrin detection. According to the analysis, hydroxy cyclic amino acids in the liquid medium were decomposed by the microorganisms; however, oxo cyclic amino acids could not be detected. The resting microbial reaction of the isolated colonies was also performed, but the same results were obtained. Therefore, we isolated microorganisms that utilize hydroxy cyclic amino acids from the environment during single carbon source–medium cultivation, but were unable to isolate any microorganisms able to produce oxo cyclic amino acids from hydroxy cyclic amino acids.

Key words: Oxo cyclic amino acid, Hydroxy cyclic amino acid, Microorganism

Introduction

In the coming post-petrochemical period, more reasonable synthesis methods for industrial products will be required to save energy and reduce environmental damage. In this sense, enzymatic reactions are now widely recognized as practical alternatives to conventional chemical reactions [(1, 2)]. There exists a vast array of microorganisms on our planet, many of which support human life. For example, lactic acid bacteria contribute to our health in our intestines, and nitrifying bacteria are used to decompose organic substances in waste water. Microorganisms thus play an essential role in our lives.

At present, there is an effort to identify new reactions undertaken by microorganisms. These efforts are driven by a desire to improve our quality of life and reduce human impact on the environment, for example, by slowing the rate of global climate change or by curing human disease. Human health and disease is the chief concern of this study. Oxo cyclic amino acids are starting materials for synthesizing pharmaceutical products but there are certain problems concerning these compounds. One such problem is that it is difficult to produce oxo cyclic amino acids by chemical means. Microorganisms can possibly convert hydroxy cyclic amino acids into oxo cyclic amino acids and greatly facilitate the production of oxo cyclic amino acids. Hence, we were motivated to try and identify microorganisms that produce oxo cyclic amino acids from hydroxy cyclic amino acids.

Materials & Methods

Isolation of microorganisms from the environment

To facilitate the bio-production of oxo cyclic amino acids from hydroxy cyclic amino acids, we attempted to identify microorganisms that can react with hydroxy cyclic amino acids, the precursor of oxo cyclic amino acids. For isolation of microorganisms, we collected 10 different samples, including bean flour, mushrooms (*Trametes versicolor*), soil under dead leaves, fallen leaves of mold, Kawara mushroom, wood ear, the soil near a bamboo grove, moss, and tree nuts from the Kyoto University Botanical Garden (Table 1). These samples were suspended in 2 ml of a liquid medium containing 0.10% hydroxy cyclic amino acids, 0.10% KH_2PO_4, 0.10% K_2HPO_4, 0.01% yeast extract, 0.03% $MgSO_4 \cdot 7H_2O$ and 0.10% NH_4Cl (pH 7.0), and incubated at 20˚C for 2 weeks with shaking at 300 rpm. After incubation, 20 µl of each culture broth was streaked on to a 2.0% agar medium plate containing 0.10% hydroxy cyclic amino acids, 0.10% KH_2PO_4, 0.10% K_2HPO_4, 0.01% yeast extract, 0.03% $MgSO_4 \cdot 7H_2O$ and 0.10% NH_4Cl (pH 7.0), and incubated at 20˚C for 5 d. After incubation, colonies grown on agar plates were distinguished morphologically and restreaked onto new agar plates to generate pure cultures.

Table 1. Samples.

1	beans powder
2	mushroom
3	soil and leaf
4	leaf's mold
5	*Trametes versicolor*
6	Jew's-ear
7	soil near the bamboo
8	moss
9	nuts (inside)
10	nuts (outside)

Assay for microbial activity towards hydroxy cyclic amino acids

Isolated microorganisms from agar plate mediums were inoculated in the same liquid medium again and incubated for a week at 20˚C with shaking at 300 rpm. After incubation, 2 µl of the supernatant of each culture broth was subjected to thin-layer chromatography analysis (TLC) and developed with a solvent system of n-butanol/methanol/water (5/3/3, v/v/v) on a silica gel plate (Kieselgel 60 F 254, Merck Co.). Amino acids in the sample were detected by ninhydrin detection in daylight. For the resting microbial reaction, colonies of microorganism isolated from the agar plate were also suspended in a 200 µl reaction mixture containing 10 mM hydroxy cyclic amino acids, 5 mM

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NAD^+, 5 mM $NADP^+$, 100 mM sodium pyruvate, 100 mM KPB (pH 7.0), and 10 µl lactate dehydrogenase solution. The microbial suspensions were incubated at 28˚C for 24 h with shaking at 300 rpm.

Results

After inoculating the microbial samples and subsequent incubation, the color of all the liquid mediums changed from colorless and transparent to yellow. This indicated that a potentially large number of microorganisms can live in minimum nutrient media containing hydroxy cyclic amino acids as the sole carbon source. Based on our TLC analysis, all microorganisms cultivated in the liquid medium were able to decompose the hydroxy cyclic amino acids (Fig. 1). The position of each sample corresponding to oxo cyclic amino acids were colorized; however, their colors differed from that of standard of oxo cyclic amino acids. It appeared that some other products were produced by the microorganisms from hydroxy cyclic amino acids. In the resting microbial reaction, decomposition of hydroxy cyclic amino acids was observed in all sample isolates; however, the production of oxo cyclic amino acids was not detected as in the liquid medium (Fig. 2).

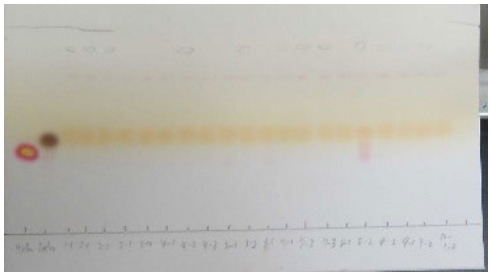

Fig. 1. TLC analysis of liquid medium.

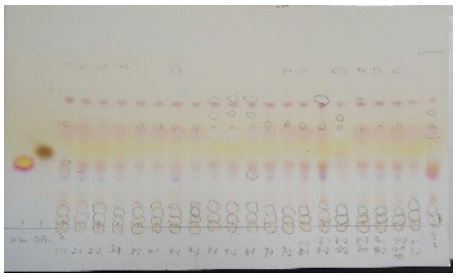

Fig. 2. TLC analysis of resting microbial reaction.

Discussion

We isolated various microorganisms using a minimum nutrient medium containing hydroxy cyclic amino acids as the sole carbon source from the Kyoto University Botanical Gardens. Microorganisms that produce oxo cyclic amino acids from hydroxy amino acids were attempted to be isolated in this study. Using TLC analysis, we verified that such microorganisms can utilize hydroxy cyclic amino acids as their sole carbon source under restricted nutrient conditions and decompose them into other substances. By using appropriate methods and procedures, we have successfully isolated special microorganisms from unknown environmental microbial sources.

For oxo cyclic amino acids production, it is also important to produce hydroxy cyclic amino acids as starting materials. It has been found that a filamentous fungus, *Fusarium oxysporum* c8D has a novel hydroxylating enzyme with activity toward L-pipecolic acid (L-Pip)[3]. Amino acid sequence analysis revealed several fungal enzymes homologous with FoPip4H, and these also had L-Pip *trans*-4-hydroxylation activity. An L-proline hydroxylase was identified to be the first Fe/ KG-DO from a fungus, *Glarea lozoyensis*. They have reported six novel fungal Fe/ KG-DOs, Pip4Hs, which had hydroxylation activities toward L-Pip and were widely distributed among several classes of filamentous ascomycetes. More importantly, FoPip4H and AnPip4H preferentially reacted with the substrate L-Pip rather than L-Pro, in contrast to bacterial proline hydroxylases, which favor L-Pro over L-Pip. Furthermore, they found that FoPip4H is an inducible enzyme strictly regulated by L-Pip but not by L-Pro. It suggests that Pip4Hs primarily catalyze the hydroxylation of L-Pip. Under natural circumstances, it is presumed that *trans*-4-LHyPip is formed as a secondary metabolite of filamentous ascomycetes possessing Pip4Hs. Thus, *F. oxysporum* c8D and other fungi might produce *trans*-4-L-HyPip as a metabolite, though its role in fungi is still unknown. The preparative-scale production of optically pure *trans*-4-L-HyPip was possible using FoPip4H as the biocatalyst. Some reports said that *trans*-4-L-HyPip has physiological functions. Thus, there may be an increasing demand for *trans*-4-L-HyPip, and the production method is promising for its high efficiency and regio- and stereoselectivity.

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オキソ環状アミノ酸の微生物による合成

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要旨

医薬品の出発素材であるオキソ環状アミノ酸の化学合成には

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コストがかかり，環境負荷も大きい．そこで，水酸化環状アミノ酸を基質とする温和かつ安価なオキソ環状アミノ酸の微生物変換を目指し，環境中の有用微生物の探索を行った．まず，水酸化環状アミノ酸資化性菌の入手を目的として，京大理学部植物園から 10 個の微生物分離源を採取，水酸化環状アミノ酸を単一炭素源とする液体培地で 20℃，2 週間の振とう培養を行った．黄濁が認められた培養液を TLC 分析に供したところ，基質となる水酸化環状アミノ酸の消失が観察された．そこで，培養液中の微生物を同一組成の寒天培地上で分離し，得られた各水酸化環状アミノ酸資化性菌を水酸化環状アミノ酸を基質とする最小栄養液体培地での振とう培養と休止菌体反応に供した．TLC 分析の結果，両溶液中で水酸化環状アミノ酸の消費が認められたものの，目的物であるオキソ環状アミノ酸は検出されなかった．

重要語句 : オキソ環状アミノ酸，水酸化環状アミノ酸，微生物