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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation

Studies on a novel alditol oxidase produced by *Penicillium* sp. KU-1 isolated from soil
（土壌から単離した*Penicillium* sp. KU-1株が生産する新規アルジトール酸化酵素に関する研究）

学位論文要約：
Dissertation Summary

Rare sugars are defined by the International Rare Sugar Association as "monosaccharides and their derivatives existing in nature in limited quantities". Recent studies have shown that certain rare sugars have pharmacologically beneficial functions and may be used as low-calorie sweeteners, agrochemicals, and pharmaceuticals. While these desirable properties have been identified, only a limited number of rare sugars have been intensively studied. Most rare sugars have not been studied in depth, since processes for their mass-production have not been established. The conventional enzymatic production strategy for rare sugars is based on isomerization or epimerization reactions. However, in the production of rare sugars by using this strategy, efficient mass-production has not been achieved in most cases, due to the product often has a lower yield due to chemical equilibrium. To overcome this problem, this study focused on alditol oxidases that catalyze the irreversible oxidation of alditols to produce the corresponding aldoses with an expected conversion ratio of 100%. In the process of screening for microorganisms that produce alditol oxidase, *Penicillium* sp. KU-1 was isolated from soil.

Chapter 1: Culture condition of *Penicillium* sp. KU-1 for the production of alditol oxidase

Traditionally, solid-state fermentation has been used in microbial cultures for the production of secondary metabolites or enzymes. Since the fungal growth morphology is close to their natural habitat, certain secondary metabolites and enzymes can only be produced in solid-state fermentation. As wheat-bran culturing of fungi is known to efficiently produce various fungal enzymes extracellularly, this culture method was selected, and a culture method using rice-bran was selected as a comparison target. In comparison with the specific activity (U/g medium) of each medium, the extract prepared from wheat-bran medium showed about 8-fold higher activity than that of rice-bran medium.

In general, submerged fermentation is suitable for large-scale production of metabolites and enzymes because it can control conditions such as temperature and pH during fermentation and the downstream processing is simpler than solid-state fermentation. However, when the fungus was cultured in a liquid medium, the alditol oxidase activity was not detected at all from the extracellular fraction.

In summary, the alditol oxidase from *Penicillium* sp. KU-1 was produced extracellularly only in solid-state fermentation but not in submerged fermentation.

Chapter 2: Purification and characterization of alditol oxidase

The alditol oxidase produced by *Penicillium* sp. KU-1 was purified to homogeneity with a purification fold of about 108 and an overall yield of 3.26%. The apparent molecular weight of the purified enzyme was estimated to be 143 kDa which is composed of two same subunits. The absorption spectrum of the purified enzyme showed a peak at 409 nm and a weak peak around 550 nm. This absorption spectrum was different from the typical absorption spectrum of common oxidases which has a flavin adenine dinucleotide as a prosthetic group. The enzyme showed broad substrate specificity compared to the previously reported alditol oxidases and acted on 19 kinds of alditols. Among them, the highest activity showed toward to erythritol and the highest catalytic efficiency was also observed with erythritol ($k$cat/Km: 396 $s^{-1}M^{-1}$). The optimal pH and temperature were pH 7.5 and 35 °C, respectively. The advantage of the enzyme in the production of rare sugars is its reactivity, the enzyme catalyzes the oxidation of D-sorbitol to produce L-gulose, a rare sugar (Figure, reaction a). In contrast, all previously reported alditol oxidases catalyze the oxidation of D-sorbitol to produce

D-glucose (Figure, reaction b). The enzyme has the potential to catalyze the production of several sugars including rare sugars such as (D/L-)erythrose, D-lyxose, (D/L-)ribose, (D/L-)xylose, D-mannose, L-gulose.

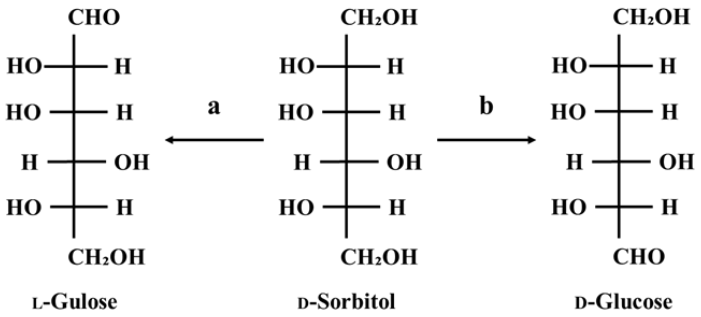

Figure. Schema of the reactions catalyzed by alditol oxidases using D-sorbitol as a substrate.

Chapter 3: Gene cloning and expression of alditol oxidase in *Escherichia coli* and *Pichia pastoris*.

The alditol oxidase has the potential to be useful in the production of rare sugars. However, the application of native alditol oxidase produced by *Penicillium* sp. KU-1 is inefficient due to its low productivity (5.23 U/g wheat-bran medium) and the need for long-term culture (total 15 days). Therefore, the construction of heterologous expression system for the enzyme is essential for its application to the production of rare sugars.

The gene encoding alditol oxidase was revealed to be composed of an open reading frame of 2,052 bp, encoding a polypeptide of 684 amino acids with a predicted molecular mass of 73.8 kDa. The sequence contains no introns and shows 73.3% identity to the galactose-binding domain-like from *Penicillium camembert*, and relatively high identity to the fungal galactose oxidases. The recombinant enzymes expressed in *E. coli* BL21 (DE3) and Rosetta-gami B (DE3) strains were obtained as insoluble inclusion bodies. The formation of inclusion bodies was not improved by using a pCold TF vector suitable for expression of solubilized proteins. For expression in *P. pastoris*, no detectable enzyme activity was observed in the extracellular fraction for 6 days.

In summary, the active-form enzyme could not be obtained in the constructed expression system.

Chapter 4: Immobilization of alditol oxidase and its application to the production of L-gulose

L-Gulose was selected as a model for the production of rare sugars using the immobilized alditol oxidase for the following reasons: 1) The efficient L-gulose production method has not been established. 2) The substrate D-sorbitol is inexpensive. 3) L-Gulose is useful as a building block for antiviral agents and anticancer agents.

Immobilized enzymes have many advantages in the production of industrial products. The insoluble immobilized enzymes are economical because they can be easily recovered from the reaction mixture and used repeatedly. In addition, immobilized enzymes are often more stable to external factors such as temperature and pH than free enzymes and can maintain their activity over a long period of time.

Three types of immobilization method such as encapsulation using alginate acid, crosslinking to the carrier with glutaraldehyde, and binding to ion exchange resin were examined. Immobilization on TOYOPEARL DEAE 650M, an anion exchange resin, was found to be the most suitable method for immobilization of the enzyme in terms of activity yield. In addition, efficient production of L-gulose was achieved by continuous reaction using the immobilized enzyme. The produced L-gulose was identified by ^{13}C NMR analysis and measurement of the specific optical rotation.

Based on the findings in this study, alditol oxidase from *Penicillium* sp. KU-1 has potential applications in the production of various aldoses including rare sugars. The production method using the enzyme irreversibly catalyzes the oxidation of alditol to produce the corresponding aldose with an expected conversion rate of 100%, so higher production yields will be achieved than conventional production method based on isomerization or epimerization reactions. In addition, efficient production of rare sugars can be expected by utilizing the immobilized enzyme. The application of alditol oxidase from *Penicillium* sp. KU-1 to the production of rare sugars will greatly contribute to further research on rare sugars.

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