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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　Cloning and functional expression of the D-glucoside 3-dehydrogenase complex from *Rhizobium* sp. S10 in Escherichia coli and its application for rare sugars production
（*Rhizobium* sp. S10由来 D-グルコシド3-デヒドロゲナーゼ遺伝子複合体のクローニングと大腸菌内での組換え酵素生産および希少糖生産への応用）

学位論文要約：
Dissertation Summary

A D-Glucoside 3-dehydrogenase (G3DH) is a FAD-related enzyme that catalyze the oxidation reaction of hydroxyl group at C-3 position of various glucosides to their corresponding 3-ketoglucosides. It transfers electron via cytochrome to terminate the oxidation reaction, however, this enzyme can use some other artificial electron acceptors, for example, 2,6-dichlorophenolindophenol (DCPIP), phenazine methosulfate (PMS), and ferric cyanide.

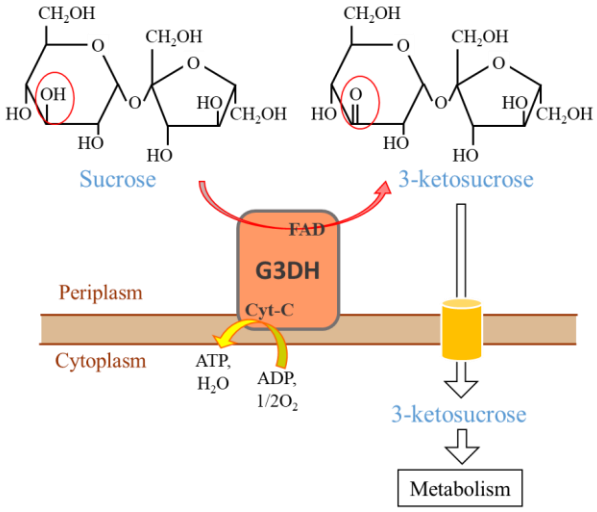

Figure 1 Oxidation reaction of sucrose by D-glucoside 3-dehydrogenase (G3DH);

G3DH has physiological importance as a common factor for glucosides and amino acids transportation in the energy-supplying system, as a membrane structure to maintain the transport reactions, and as a member of respiration system that prepares 3-ketoglucosides, the first intermediates in carbohydrate metabolic pathway. G3DH seems to play a key role in microorganisms' sugar metabolism.

G3DH has attracted interest by its broad substrate specificity and site-selective oxidation properties. The conversion product, 3-ketoglycoside, shows potential as a starting material for the chemical industry such as in polymer and surfactants since the keto group becomes a specific site for selective chemical synthesis. Several glucose oxidoreductases are known and have been used as a component of enzyme biosensors for blood glucose level measurement. Nevertheless, G3DH has practical applications over other glucose oxidoreductases because of its cofactor binding structure that would not be affected by oxygen in the reaction mixture. Another advantage is the property of reducing glycosides that lack of a hydroxyl group at the C-1 position, for example, the 1,5-anhydro-D-glucitol measurement in clinical diagnostics

G3DH producing microbe, *Agrobacterium tumefaciens*, was discovered in the early 1960s by Bernaerts and De Ley. G3DH enzyme was isolated and purified by Hayano and Fukui. Later, this enzyme was found and identified in other bacteria as well as in fungi, for example, *Flavobacterium saccharophilum*, *Cytophaga marinoflava*, *Agaricus bisporus*, *Halomonas (Deleya)* sp. α-15, and recently *Sphingobacterium faecium*.

Those G3DHs were purified and characterized, but only a few have been cloned and overexpressed in *Escherichia coli*. The recombinant G3DH from *Halomonas*)*Deleya*(sp. α-15; however, was less active than the wild type. In 1997 and 2006, a fragment containing the G3DH gene from *A. tumefaciens* was cloned and overexpressed into *E. coli*, though the outcomes were insoluble G3DH and inactive G3DH. Newly isolated *S. faecium* ZJF-D6 was cloned and functionally expressed in *E. coli*, but its activity was lower than that from the natural organism. Recently, G3DH from *R. radiobacter* (*A. tumefaciens*) was expressed in *E. coli* by a co-expression vector as a heterodimeric complex that showed dye-mediated G3DH activity toward methyl-α-D-glucoside.

From the first discovery of the G3DH, many researchers have been working on the screening for novel organisms with G3DH activity. Despite its prominent properties, only limited information of G3DH has been reported, even though it is necessary to improve G3DH utilization. This work is divided into three chapters; isolation of a novel G3DH, the cloning of a G3DH gene from *Rhizobium* sp. S10, and rare sugars production using aldo-keto conversion application of G3DH.

The first chapter is about screening for G3DH producing microbes, then purified and characterized G3DH from a selected microbe. The screening method was done in two steps; medium screening and G3DH activity assay. Lactitol was used as a carbon source for selecting glucoside-degradable microbes from soil. G3DH activity was then observed in the next step by following the reduction rate of 2,6-dichlorophenolindophenol at a wavelength of 600 nm. In the oxidized form, DCPIP is blue but when DCPIP is reduced, it would turn to colorless. From screening method, ne microorganism named ‘L35’, having the highest activity was selected. The 16s ribosomal RNA sequence of L35 was paired to the sequence database using BLAST and the result indicated that L35 showed 100% identity to *Rhizobium* sp., therefore is named ‘*Rhizobium* sp. L35’.

To purify G3DH, *Rhizobium* sp. L35 cells were cultivated in 10 L jar fermenter using YE medium containing 1.0% maltitol and the crude enzyme was prepared according to the previous described method. G3DH was purified in three steps using column chromatography. The enzyme was purified 150-fold and to 7.2% yield from crude extract. The specific activity was 7.628 U/mg.

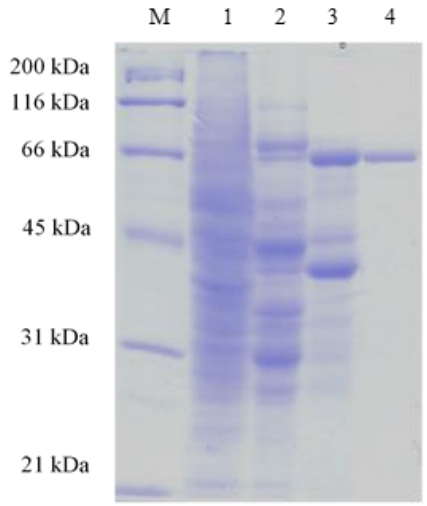

Figure 2 SDS-PAGE analysis of purified G3DH. M; molecular weight marker, Lane 2; crude enzyme, Lane 3; Hi-trap Phenyl, Lane 4; Hi-trap Q, and Lane 5; purified G3DH.

The optimal temperature for enzyme activity was determined to be 37-40°C, and the half-life of G3DH was approximately 3 h at 40°C and 50 min at 45°C. The optimum pH for enzyme activity was 7.0-7.5 when DCPIP was used as the artificial electron acceptor, and the enzyme was stable between pH 6.0-11.0 (Fig 3). Most of the investigated metal ions did not affect G3DH activity, however, the activity was slightly enhanced by adding 1 mM $FeSO_4$ to the reaction mixture)15% increase(. Activity was inhibited by the addition of $CuCl_2$ and $MnCl_2$

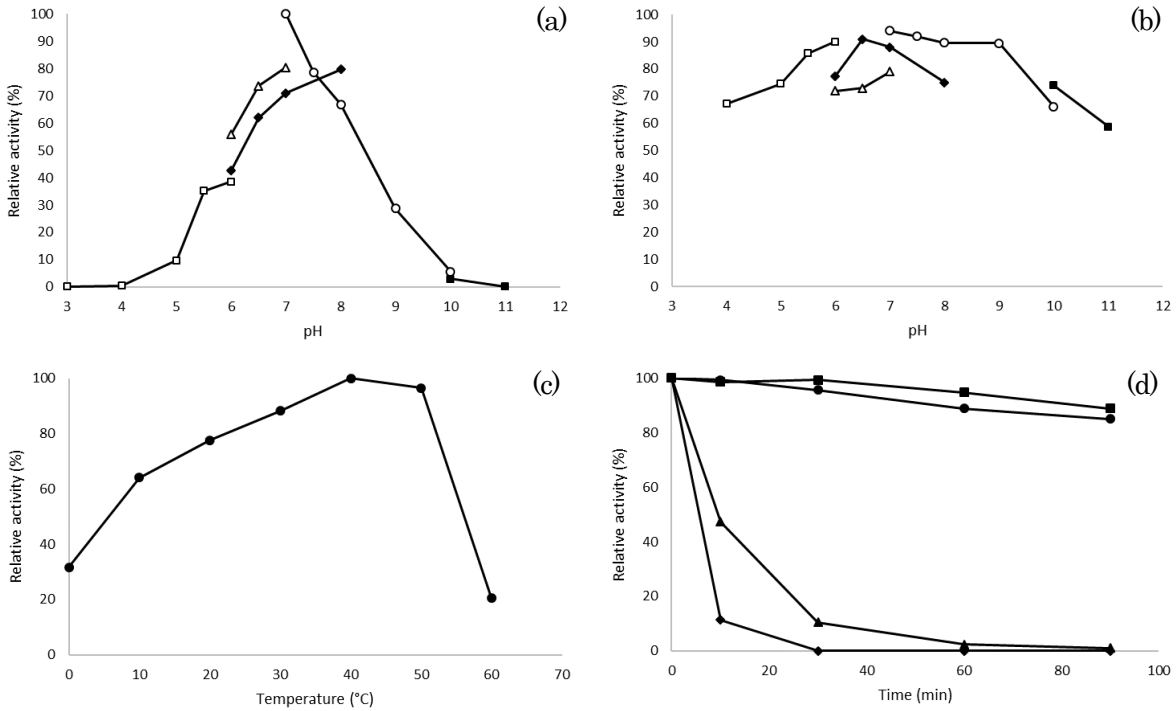

Figure 3 Effects of temperature on G3DH activity (a(and stability)b(. Effects of pH on G3DH activity)c(and stability)d(. Citrate buffer)pH 3.0–4.0; ●(, acetate buffer)pH 4.0–6.0; ▲(, sodium phosphate buffer (pH 6.0–8.0; □), Tris-HCl buffer)pH 7.5–9.0; ◆(, glycine-NaOH buffer)pH 9.0–11.0; ○(

In terms of substrates, the enzyme showed its highest activity toward cellobiose, followed by salicine (91.5%), lactose (84.7%), maltose (82.8%), gentiobiose (78.6%), maltotriose (75.3%), and D-glucose (63.3%). This enzyme exhibited slight activity towards D-xylobiose)7%(and D-mannobiose)5.1%). While G3DH showed almost no activity towards D-xylose and D-mannose. A disaccharide substrate is likely to be recognized and bound more easily by the active site because of the increased interactions between the pyranose rings and amino acid residues compared with a monosaccharide.

The second chapter, G3DH gene cluster from newly isolated *Rhizobium* sp. S10 was extracted and analyzed for its nucleotide sequence. The result revealed that this fragment contains 1,686 bp of a complete open reading frame)ORF(, which is encoded for a catalytic subunit of G3DH. This sequence was deposited in the Genbank database under accession number **LC348386**. The translated amino acid sequence of the catalytic subunit showed the FAD binding motif (GXGXXG), which could be found in FAD-harboring enzymes. Another two shorter ORFs were also pointed out. The first nucleotide of a 558-bp ORF, named as small subunit, has one nucleotide overlapping with the catalytic subunit. While the other 396 bp ORF, named as cytochrome C subunit, is located downstream of the former ORFs.

To create a gene fragment with restriction sites for cloning, specific primers were designed. Then, the amplified gene was ligated into pQE30 and pQE3060 expression vectors, before transformed into *E. coli*. The recombinant *E. coli* JM109 was cultured in SB medium and incubated at 37°C, 180 rpm until the culture reached OD600 of 0.4-0.6. The protein expression was then induced by adding 1 mM IPTG and was continuously incubated at 23°C for 16 h. The recombinant enzyme, identified as rG3DH, was overexpressed in a soluble fraction. The rG3DH from pQE30 expression vector was purified by 2-step column chromatography with a relatively high specific activity of 38.54 U/mg. While the rG3DH from pQE30 expression vector was purified by 4-step column chromatography.

Table 1 Purification of rG3DH

Expression vector	Purification step	Total protein)mg(	Activity)U(	Specific activity)U/mg(	Recovery)%(	Purification)fold(
pQE30	Crude enzyme	549.5	186.2	0.34	100	1
	His trap	16.74	67.23	4.02	36.12	11.85
	Hi-trap Q	1.10	42.51	38.54	22.84	9.59
pQE60	Crude enzyme	464	299	0.65	100	1
	Hi-trap phenyl	8.40	20.17	2.40	6.75	3.69
	Hi-trap Q	0.52	9.52	9.52	3.19	3.97
	Resource Q	0.14	1.65	11.87	0.52	1.25
	Gel filtration	0.09	1.62	20.61	0.54	1.74

The purified pQE30-rG3DH showed a two- band with MW of 66 kDa and 18 kDa, which represented the catalytic subunit and the small subunit, respectively (Fig. 4). The purified pQE60-rG3DH showed a single band of 66 kDa after four steps of purification; however, the 18 kDa band could not be detected (Fig. 5). The reason is that the small subunit might be separated throughout the purification steps. Although the MW of the purified rG3DH catalytic subunit was estimated to be 66 kDa in SDS-PAGE, it was analyzed to be 63303 Da by MALDI-TOF mass spectrometry, which is consistent with the calculated MW based on the predicted protein sequence (63350 Da).

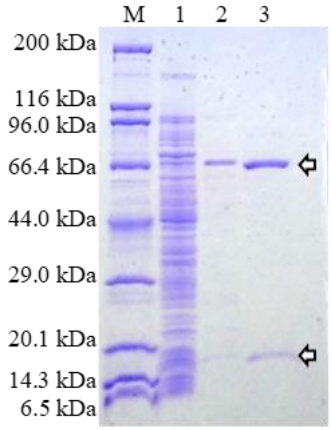

Figure 4 SDS-PAGE of pQE30-rG3DH purification. Lane 1, crude extract; lanes 2–3, sample of the enzyme from His-trap HP and Hi trap Q column (purified enzyme). Arrows indicate catalytic subunit (66 kDa) and small subunit (18 kDa).

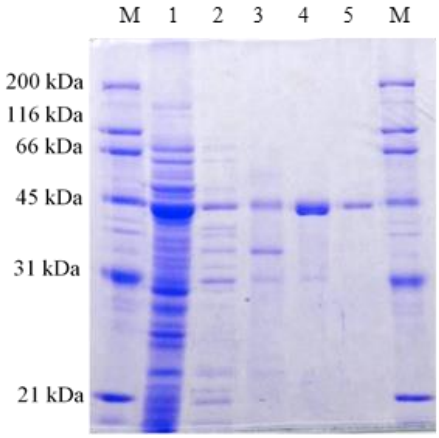

Figure 5 SDS-PAGE of pQE60-rG3DH purification. Lane 1, crude extract; lanes 2–5, sample of the enzyme from Hi trap Phenyl HP, Hi trap Q, Resource Q, and Gel filtration (purified enzyme).

Remark: Even though the specific activity of the crude extract of rG3DH from pQE60 is higher than that of pQE30, the purification of a non-Histidine tag protein is quite difficult and takes time. Therefore, rG3DH form pQE30 expression vector was used in the following experiments.

To investigate the effect of pH and temperature on activity and stability, G3DH activity was assayed in a pH range of 5.0–10.0 and the temperature range of 0–60°C. The results showed that rG3DH exhibited the highest activity in Tris-HCl buffer pH 7.0, 40°C with DCPIP as an electron acceptor. The rG3DH should be stored in the weakly-basic condition and low temperature.

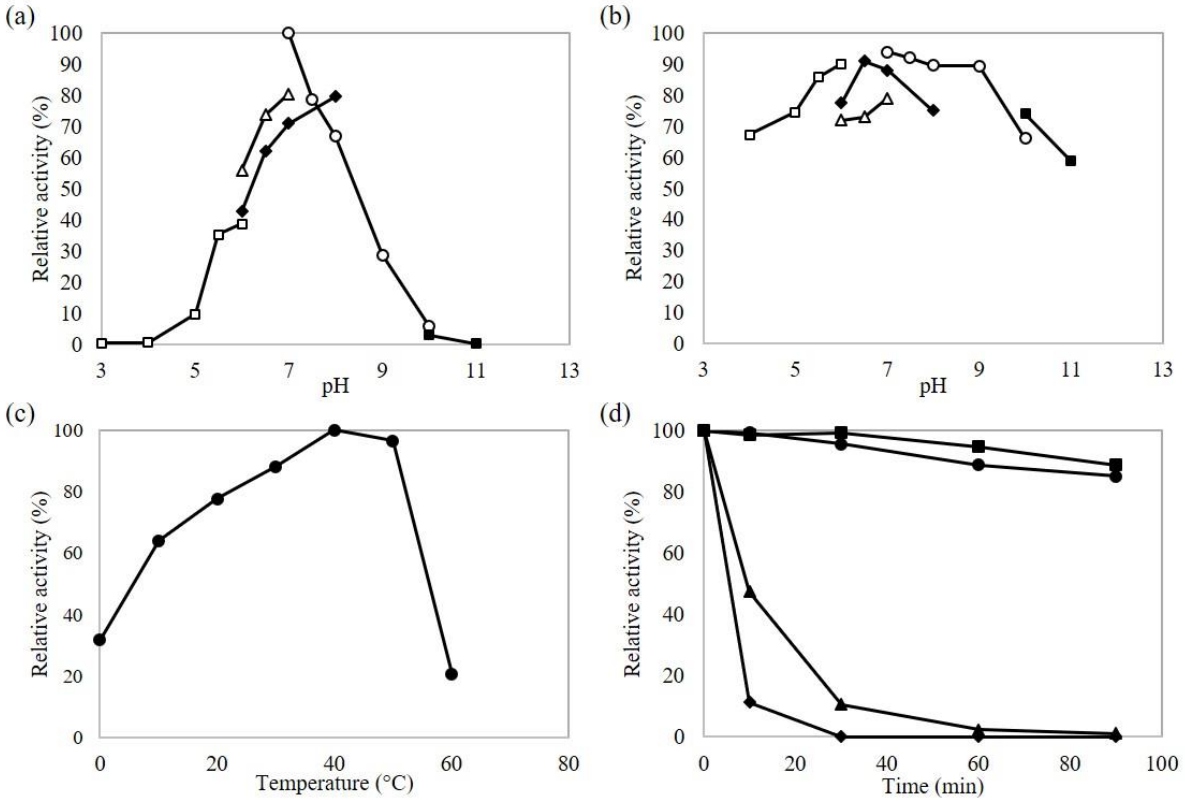

Figure 6 (a) Optimum pH for rG3DH. **(b)** Effect of pH on rG3DH activity. The effect of pH on rG3DH activity was examined with acetate buffer)pH 3.0–6.0; open square(, sodium phosphate buffer)pH 6.0–8.0; open triangle(, Tris-HCl buffer)pH 7.0–10.0; open circle(, MES buffer)pH 6.0–7.0; close diamond(, and sodium bicarbonate buffer)pH 10.0–11.0; close square(. **(c)** Optimum temperature for rG3D. **(d)** Effect of temperature on rG3DH activity. Thermostability of rG3DH was examined at 30°C (square), 40°C (circle), 50°C (triangle), and 60°C (diamond).

A wide range of disaccharides such as cellobiose, gentiobiose, and lactose served as good substrates for rG3DH. Among the monosaccharides used in this study, rG3DH showed high activity toward glucose and its derivatives harboring pyranose rings but low activity toward monosaccharides with furanose rings. Toward various metal ions, rG3DH activity was not affected much. Except Co^{2-}, Mn^{2-}, Cu^{2+} and Ag^{+} that could inhibit rG3DH activity. On the other hand, $FeSO_4$ slightly increased the activity by 12%.

Based on the deduced amino acid sequence of the catalytic subunit of rG3DH, we found sequence features that are highly conserved in FAD-harboring dehydrogenase. Interestingly, several dehydrogenases share similarity of

having hetero-oligomeric structure composed of a catalytic subunit containing FAD, a multiheme cytochrome complex subunit, and a chaperon-like subunit. From the primary structure resemblance between G3DHs and sorbitol dehydrogenase from *Gluconobacter oxidans* and 2-keto-D-gluconate dehydrogenases from *Erwinia herbicola* and *Pantoea citrea*, which are oligomeric enzymes made up of three subunits. The nucleotide sequencing of the gene fragment from *Rhizobium* sp. S10 also revealed three ORFs, therefore, we predict that G3DH is a three-component enzyme.

In the third chapter, the partial purified G3DH from *Rhizobium* sp. L35 was utilized to produce D-allose from cellobiose by a three-step process of enzymatic-dehydrogenation, chemical reduction, and acid-hydrolysis. The final yield of D-allose was approximately 30%, which was three-time higher than the conventional method. The recombinant *E. coli* harboring pQE60-G3DH expression vector also exhibited an outstanding activity, therefore, the resting cell reaction was performed. The glycosides oxidizing activity combined with chemical reaction, could produce D-gulose from lactitol via 3-ketolactitol with roughly 8% yield.

Over decades, many researchers have been exploring novel G3DH or more deeply analyzing those G3DHs discovered in different organisms but only a limited number of studies about gene cloning and overexpression of this enzyme have been reported. To conclude this research, a novel G3DH producing microbe was screened. Additionally, G3DH gene from a newly isolated *Rhizobium* sp. S10 was cloned in *E. coli* and the recombinant protein was successfully expressed in the soluble fraction with an outstanding G3DH activity. Finally, this new process of microbiological reaction together with chemical reaction can be applied not just for D-allose and D-gulose, but also various rare sugars production. Despite the importance of, the secondary-tertiary structure and the mechanism of G3DH are still unknow. Further research on genetic information is necessary to clarify the native structure of G3DH and its substrate binding mechanism. So that we could make use of the enzyme more efficiently.