Preparation of Enzymes Required for Enzymatic Quantification of 5-Keto-D-gluconate and 2-Keto-D-gluconate

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For easy measurement of 5-keto-D-gluconate (5KGA) and 2-keto D-gluconic acid (2KGA), two enzymes, 5KGA reductase (5KGR) and 2KGA reductase (2KGR) are useful. The gene for 5KGR has been reported, and a corresponding gene was found in the genome of Gluconobacter oxydans 621H and was identified as GOX2187. On the other hand, the gene for 2KGR was identified in this study as GOX0417 from the N-terminal amino acid sequence of the partially purified enzyme. Several plasmids were constructed to express GOX2187 and GOX0417, and the final constructed plasmids showed good expression of 5KGR and 2KGR in Escherichia coli. From the two E. coli transformants, large amounts of each enzyme were easily prepared after one column chromatography, and the preparation was ready to use for quantification of 5KGA or 2KGA.

Key words: 5-keto D-gluconic acid; 2-keto D-gluconic acid; 5-keto D-glucuronate reductase; 2-keto D-glucuronate reductase; Gluconobacter

Gluconobacter is a genus of acetic acid bacteria that is able to oxidize a broad range of sugars, sugar alcohols, and sugar acids, and accumulates high amounts of the corresponding oxidized products in the culture medium. Hence, it has been used for a long time to produce several valuable products, such as L-sorbose for vitamin C production from D-sorbitol. The oxidation of D-glucose to produce 2-keto-D-glucuronate (2KGA) or 5-keto-D-glucuronate (5KGA) is catalyzed by sequential enzymes located on the periplasmic face of the cytoplasmic membrane.11 Membrane-bound PQQ-glucose dehydrogenase oxidizes D-glucose to glucono-δ-lactone, and it is then converted to D-glucuronate by glucono-δ-lactonase. The formation of 2KGA and 5KGA found in Gluconobacter strains has been reported to be catalyzed by two types of membrane-bound glucuronate dehydrogenases.11 One is a FAD-containing, 2KGA-yielding enzyme (FAD-GADH), and the other is a FAD-containing, 5KGA-yielding enzyme. The former enzyme has three subunits: an FAD-containing dehydrogenase subunit, a three-heme c-containing cytochrome c subunit, and a small subunit of unknown function.21 The latter enzyme has been found to be identical to FAD-containing polyp dehydrogenase,35 which is known as P-arabitol dehydrogenase,41 D-sorbitol dehydrogenase,53 or glyceral dehydrogenase: PQQ-GLDH.6 The enzyme PQQ-GLDH has versatile substrate specificity, but at the same time it has high stereo- and regio-selectivities, obeying the so-called Bertrand-Hudson rule.33 On the other hand, the enzymes located in the cytoplasm are thought to be involved in the assimilation of 2KGA and 5KGA. Before assimilation, both 2KGA and 5KGA are normally transported into the cytoplasm by certain transporter proteins, and then they are converted to D-glucuronate by 2KGA reductase (2KGR, [EC 1.1.1.215]) and 5KGA reductase (5KGR, [EC 1.1.1.69]) respectively.11

5KGA is a useful raw material in the production of tartaric acid, xylaric acid, and 4-hydroxy-5-methyl-2,3-dehydrofuranone-3, a valuable flavor compound.7 Moreover, it has been reported to be applicable in the production of vitamin C by Gray’s method,8,9 a method distinct from Reichstein’s, the method nowadays used in industry.10 The latter method requires high temperatures and organic solvents but Gray’s method requires milder conditions. Most of the Gluconobacter strains produce both 2KGA and 5KGA from D-glucuronate, and thus production of 2KGA is a competitive reaction in

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Abbreviations: 5KGA, 5-keto-D-gluconic acid; 2KGA, 2-keto-D-gluconic acid; 5KGR, 5-keto-D-glucuronate reductase; 2KGR, 2-keto-D-glucuronate reductase; PQQ, pyrroloquinoline quinone.
vivo, and it should be a major by-product in 5KGA production. Recently, it has been reported that the FAD-GADH-defective mutant strain of *Gluconobacter oxydans* IFO12528 (genetically identical to *G. suboxydans* IFO12528) produced the 5KGA from r-glucose almost exclusively.11)

For measurement of 2KGA and 5KGA, two specific enzymes for each substrate, 2KGR and 5KGR, are useful. Both enzymes have been purified from *Gluconobacter* strains,12,13) but preparation of these enzymes from *Gluconobacter* is laborious. Therefore, overexpression of these two enzymes in *Escherichia coli* was carried out in this study.

**Materials and Methods**

**Materials.** 2-Ketogluconic acid hemicalcium salt and 5-ketogluconic acid potassium salt were purchased from Sigma (St. Louis, MO). Yeast extract was a kind gift from Oriental Yeast (Tokyo, Japan). The oligonucleotides used for PCR in this study are listed in Table 1.

**Bacterial strains and growth conditions.** *Gluconobacter suboxydans* IFO12528 (now unified as *G. oxydans* NBRC3172 in culture collection at the NITE Biological Resource Center) was maintained on a Potato agar slant (20 g glycerol, 5 g glucose, 10 g yeast extract, 10 g polypeptide, and 100 ml potato extract filled to 1 liter with tap water) supplemented with 5 g CaCO3. The *Escherichia coli* strains used in cloning and expression experiments were maintained on a Luria-Bertani (LB) plate (10 g polypeptide, 10 g yeast extract, 5 g NaCl, and 15 g agar, filled to 1 liter) supplemented with appropriate antibiotics at concentrations as follows: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 25 μg/ml. The growth of all bacterial strains was measured with a Klett Sumerson photometer with a red filter.

**2KGR, 5KGR, and aldehyde dehydrogenase assay.** 2KGR12) and 5KGR13) activities were measured by following the decrease in absorbance at 340 nm at 25 °C. The reaction mixture (1 ml) contained 10 mM of 2KGA or 5KGA, 100 μM of NADPH, and an appropriate amount of the enzyme solution, and 50 mM potassium phosphate buffer (KPB), pH 6.0. The aldehyde dehydrogenase activity was measured by following the increase in absorbance at 340 nm at 25 °C. The reaction mixture (1 ml) contained 10 mM of acetaldehyde, 100 μM of NAD+ or NADP+, and an appropriate amount of enzyme solution, and 50 mM Tris–HCl, pH 9.0. One unit of enzyme activity was defined as the amount of enzyme reacting 1 μmol of 2KGA, 5KGA, or acetaldehyde per min under these assay conditions.

**Purification of 2KGR from Gluconobacter suboxydans IFO12528.** All purification steps were performed at 4 °C. 2KGR was purified from the soluble fraction of *G. suboxydans* IFO12528 grown on Potato medium for 24 h. After it was harvested and resuspended in 10 mM sodium acetate buffer (AcB), pH 5.0, the cell suspension was passed twice through a French pressure cell press, and the intact cells and cell debris were removed by centrifugation at 9,000 rpm for 10 min. The membrane fraction was then removed by ultracentrifugation at 40,000 rpm for 90 min. (NH4)2SO4 was added to the soluble fraction obtained to achieve a final concentration of 30% saturation, and this was allowed to stand overnight. The proteins precipitated were removed by centrifugation at 9,000 rpm at 4 °C for 30 min. Then (NH4)2SO4 was added to achieve a final concentration of 80% saturation, and this was allowed to stand overnight. The precipitated proteins were collected by centrifugation at 9,000 rpm at 4 °C for 30 min. The protein pellets were then dissolved and dialyzed twice against 10 mM KPB, pH 6.0, containing 1 mM 2-mercaptoethanol (buffer A) for at least 3 h each. The enzyme solution was then applied to a DEAE-cellulose column equilibrated with buffer A and eluted with a linear gradient of NaCl from 0 to 300 mM in buffer A. The active fractions were pooled and dialyzed against the same dialysis buffer. Then the dialyzed fraction was applied to a ceramic hydroxyapatite column equilibrated with buffer A. The active fractions were pooled and dialyzed against 10 mM AcB, pH 5.0, containing 5 mM 2-mercaptoethanol (buffer B) overnight. The precipitates formed were removed by centrifugation at 9,000 rpm for 10 min, and the supernatant was then applied to a CM-cellulose column.

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**Table 1. Oligonucleotide Primers Used in This Study**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOX2187-1</td>
<td>TTCAGGCTGCTAGGAAAGAAC</td>
</tr>
<tr>
<td>GOX2187-2</td>
<td>CAGAAAGGCTACAGGCTAGATAC</td>
</tr>
<tr>
<td>F-gno-Nco</td>
<td>CTAgcttgagcgaacaga</td>
</tr>
<tr>
<td>R-gno-Bam</td>
<td>TGATCCAGAAAGGCTACAGGCTAGATAC</td>
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<tr>
<td>2KGR-1-Xba</td>
<td>TTTACGCTACGATCAACCCTT</td>
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<tr>
<td>2KGR-2-Sal</td>
<td>GCCGAACAGTTCGTGGCATA</td>
</tr>
<tr>
<td>F-FOX1122-Ncol</td>
<td>TGCTAGCGCAGCGACAGTTCGTGGCATA</td>
</tr>
<tr>
<td>R-FOX1122-Bam</td>
<td>TGCATCGCTTACGCTACGATCAACCCTT</td>
</tr>
<tr>
<td>RBS-GOX0417</td>
<td>GGGATACATGAAACTCTCC</td>
</tr>
<tr>
<td>RBS-GOX0417</td>
<td>TCTTACGCTACGATCAACCCTT</td>
</tr>
<tr>
<td>RBS-GOX0417</td>
<td>GCCGAACAGTTCGTGGCATA</td>
</tr>
</tbody>
</table>

Underlined sequences indicate artificial restriction enzyme sites and exchanged bases.
equilibrated with buffer B. Then the active fractions were pooled and dialyzed against 10 mm KPB, pH 6.0, containing 5 mm 2-mercaptoethanol (buffer C). After the precipitate formed was removed by centrifugation, the enzyme solution was applied to a Superdex 200 column equilibrated with buffer C, containing 100 mm NaCl, with the flow rate at 40 ml/h.

Electroblotting for N-terminal amino acid sequencing. After resolution by SDS-Page, the proteins in the polyacrylamide gel were then transferred electrophoretically to a polyvinylidene difluoride membrane in N-cyclohexyl-3-aminopropanesulfonic acid buffer, pH 11.0, at 100 mA for 4 h. The membrane was stained with Coomassie Brilliant Blue G-250 and washed with 50% methanol. The desired band was cut and subjected to N-terminal amino acid sequencing by with a Model PPSQ-21 protein sequencer (Shimadzu, Kyoto, Japan).

DNA techniques. Restriction enzyme digestion, DNA ligation, and other DNA modification and general molecular biological techniques were performed as described by Sambrook et al. Polymerase chain reaction (PCR) was carried out in 25 μl of reaction volume (PuReTaq Ready-To-Go PCR beads Kit, Amersham Biosciences, Uppsala, Sweden) using a GeneAmpPCR System 2400 (Perkin Elmer, Waltham, MA) or a MyCycler Thermal Cycler (BioRad, Hercules, California). DNA fragment separated in agarose gel was purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) or a MagExtracter DNA fragment purification kit (Toyobo, Tokyo, Japan).

Cloning of the 2KGR, 5KGR, and GOX1122 encoding genes. All three genes were amplified by PCR with a PuReTaq Ready-To-Go PCR beads Kit (Amersham Biosciences, Uppsala, Sweden) with the genomic DNA of G. suboxydans IFO12528 as a template. The PCR fragments were cloned into pGEM-T Easy Vector (Promega, Madison, WI), and then subcloned into pET-28a(+) vector (Novagen, Madison, WI) for overexpression, and the identity of the nucleotide sequence was confirmed by DNA sequencing using an ABI PRISM310 DNA sequencer (PE Biosystems, Foster City, CA). The nucleotide sequence data were analyzed by GENETYX-MAC (Software Development, Tokyo, Japan) or Clone Manager (Scientific and Educational Software, Cary, NC). Homology search and alignment analyses were performed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and CLUSTAL W (www.ebi.ac.uk/clustalw) respectively.

Site-directed mutagenesis of the ribosome-binding site of GOX0417. To improve the expression level of GOX0417, the ribosome binding sequence of this gene was modified by DpnI-mediated site-directed mutagenesis. The sequence, ΔATTGGGA, 7 bases upstream of the start codon of GOX0417 was mutated to GAAGGA. Plasmid pET-GOX0417, prepared in E. coli DH5α as a methylated DNA at adenine bases, was used as a template for PCR using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). The PCR mixture in the total volume of 50 μl contained 10 ng of pET-GOX0417, Pfu Turbo buffer, 200 nm each of F-RBS-GOX0417 and R-RBS-GOX0417 primers, 250 μM of each dNTP, and 2.5 units of Pfu Turbo DNA polymerase. The temperature cycles were as follows: 1 cycle of 95°C for 30 s, 16 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 6 min and 30 s. The temperature was held at 37°C after finishing all cycles. The original methylated plasmid was then digested by the addition of 20 units of DpnI to the PCR mixture, which was incubated at 37°C for 1 h. Then 10 μl of DpnI-treated PCR mixture was transformed to E. coli XL1-Blue by the heat-shock method. The mutation was confirmed by DNA sequencing. The desired mutated plasmid was designated pET-GOX0417 M6.

Purification of overexpressed 5KGR from E. coli. E. coli BL21 (DE3) transformant with pET-gno-NB was grown in 1 liter of LB medium supplemented with 50 μg/ml kanamycin in 3-liter flasks at 30°C until growth reached about 250 Klett units. Then IPTG was added to achieve a final concentration of 1 mM, and this was incubated for 5 h. The cells were then harvested and washed twice. The soluble fraction obtained as described above was applied to a DEAE-cellulose column equilibrated with buffer C, and then eluted with 0–300 mm KCl. The active fractions were pooled, concentrated, and dialyzed against buffer C.

Purification of overexpressed 2KGR from E. coli. E. coli BL21 (DE3) transformant with pET-GOX0417 M6 was grown in 1 liter of LB medium supplemented with 50 μg/ml kanamycin in 3-liter flasks at 37°C until the growth reached about 100 Klett units. Then IPTG was added to achieve a final concentration of 1 mM and incubated until growth reached 350 Klett units. The cells were then harvested and washed twice. The soluble fraction obtained as described above was applied to a DEAE-cellulose column equilibrated with buffer C, and then eluted with 0–300 mm KCl. The active fractions were pooled, concentrated, and dialyzed against the same buffer.

Results and Discussion

Cloning and expression of the gno gene encoding 5KGR

The gno gene encoding 5KGR has been reported from Gluconobacter oxydans DSM 3503. Recently, the genome of G. oxydans 621H, the genotype of which is identical to that of G. suboxydans IFO12528, has been published, and the locus GOX2187 on the genome is annotated as gno. Thus a pair of PCR primers
(GOX2187-1 and GOX2187-2) covering the locus of GOX2187, including the probable Shine-Dalgarno sequence (SD) of this gene, was designed, and the 827 bp-PCR product was obtained with the genomic DNA of *G. suboxydans* IFO12528 as a template. The PCR product was cloned into pGEM-T Easy Vector and sequenced, and then cloned into pUC119 at the *Sph*I and *Pst*I sites (Fig. 1). The resulting plasmid, pUC-gno, had the *gno* gene inserted under the *lac* promoter on pUC119. Very low activity (0.02–0.05 units/mg) was observed in 5KGR in strains JM109 and TG1 grown at 37°C, whereas no activity was observed in these strains with the empty vector pUC119 and in *E. coli* DH5α carrying pUC-gno. The activity detected was still at a very low level in the JM109 and TG1 strains after induction with IPTG at 37°C, and it became undetectable when the expression condition was shifted to lower temperatures (30 and 25°C). The protein patterns of the whole cell on SDS–PAGE also showed no intent band (data not shown), suggesting that the low activity was due to the low amount of 5KGR produced.

To improve the expression level, the *gno* gene was subcloned into pET-28a(+), designated pET-gno (Fig. 1). Very low activity (0.01–0.02 units/mg) was detected in the control strain carrying pET-28a(+), whereas more than 10-fold activity (about 0.3 units/mg) was observed in the strain carrying pET-gno grown at 25, 30, and 37°C. Although the activity observed in the *E. coli* BL21 (DE3) strain carrying pET-gno was much higher than in the strain with pUC-gno, the level of activity was still low, and it was not much different from the 5KGR activity observed in the *Gluconobacter* strains.

Next, a pair of PCR primers was designed to cover the *gno* gene from the start to the stop codon with an additional *Nco*I site at the 5′ end and an additional *Bam*HI site at the 3′ end of the gene. To create a new *Nco*I site at the start codon of *gno*, a base change is required, and this resulted in a change in the second amino acid residue from Ser to Ala. The PCR product was then subcloned into pET-28a(+) at the *Nco*I and *Bam*HI sites and the resulting plasmid was named pET-gno-NB. This plasmid was transformed into *E. coli* BL21 (DE3) and checked for *gno* expression. A high level of 5KGR expression, estimated to be 30% or more in cytoplasmic protein, was observed by enzyme assay (50–90 units/mg) and SDS–PAGE analysis, although the activity and protein expression level varied from colony to colony (Fig. 2). The cells showing higher 5KGR activity tended to show less growth. The 5KGR-overexpressed strains were unstable, since the expression level of 5KGR decreased after it was subcultured for several times or kept on an agar plate for more than 1 week. Using freshly transformed cells from freshly prepared competent cells gave a higher expression level than the old ones. The reason for this instability is not certain, but high 5KGR activity might disturb the metabolism of D-gluconate or NADP in *E. coli*, and thus suppression of 5KGR expression might occur.

The soluble fraction of 5KGR-overexpressed *E. coli*
was prepared in 10 mM KPi, pH 6.0, and then it was applied to a DEAE-cellulose column. After it was washed with 100 mM KCl, the enzyme was eluted with a linear gradient of 100–300 mM KCl. Purified 5KGR was obtained (Fig. 3) and it was ready to use for measurement of 5KGA. The enzyme obtained appeared more stable than the original 5KGR from *G. oxydans* strain G. suboxydan (IFO12528), partially purified 2KGR was obtained with a yield of 11% by Superdex S-200 column chromatography, and the final specific activity was 13.9 units/mg. The partially purified enzyme contained three major protein bands (52, 33, and 28 kDa) on SDS–PAGE (data not shown). In Superdex S-200 column chromatography, the 33-kDa protein band appeared to correspond to 2KGR because the profiles of the enzyme activity and of the intensity of the band detected by SDS–PAGE corresponded well, whereas the 52-kDa band was the most intense band in each fraction analyzed (data not shown). The 33-kDa protein showed the N-terminal amino acid sequence as SSXPDLAI, which almost matched the sequence MSSKPDILTID encoded by the locus GOX0417 in the genome of *G. oxydans* 621H. This locus was annotated as a putative 2-hydroxycacid dehydrogenase. On the other hand, the N-terminal amino acid sequence of the 52-kDa protein was AYATTNPYTGETXTXEAT, almost identical to the sequence MAYATNPYTGETXXTFXEAT encoded by the locus GOX1122, a putative NAD-dependent aldehyde dehydrogenase gene.

**Fig. 2.** SDS–PAGE Analysis of Crude Extract from *E. coli* Transforms with pET-gno-NB.

*E. coli* BL21 (DE3) transforms were grown in LB medium supplemented with 50 μg/ml kanamycin at 30 °C until growth reached about 250 Klett units. Then IPTG was added to achieve a final concentration of 1 mM and this was incubated for 5 h. Crude extracts (30 μg of protein) from *E. coli* BL21 (DE3) transforms with pET-gno-NB from four independent colonies (lanes 1–4) or pET-28a(+) (lane 5) were analyzed by SDS–PAGE. Lane M, marker proteins.

**Fig. 3.** SDS–PAGE Analysis of 5KGR Purified from *E. coli* BL21 (DE3) with pET-gno-NB.

Lane 1, soluble fraction of *E. coli* BL21 (DE3) with pET-gno-NB. Lane 2, Purified 5KGR after DEAE-cellulose column chromatography. Thirty micrograms of protein was applied.

**Purification of 2KGR from G. suboxydans IFO12528**

The gene for 2KGR had not been identified yet, and hence purification of 2KGR was attempted first. The soluble fraction of *G. suboxydans* IFO12528 was subjected to several steps of purification, as described in “Materials and Methods.” Since 2KGR presented in a very low amount in the soluble fraction (about 0.2 units/mg) of *G. suboxydans* IFO12528, partially purified 2KGR was obtained with a yield of 11% by Superdex S-200 column chromatography, and the final specific activity was 13.9 units/mg. The partially purified enzyme contained three major protein bands (52, 33, and 28 kDa) on SDS–PAGE (data not shown). In Superdex S-200 column chromatography, the 33-kDa protein band appeared to correspond to 2KGR because the profiles of the enzyme activity and of the intensity of the band detected by SDS–PAGE corresponded well, whereas the 52-kDa band was the most intense band in each fraction analyzed (data not shown). The 33-kDa protein showed the N-terminal amino acid sequence as SSXPDLAI, which almost matched the sequence MSSKPDILTID encoded by the locus GOX0417 in the genome of *G. oxydans* 621H. This locus was annotated as a putative 2-hydroxycacid dehydrogenase. On the other hand, the N-terminal amino acid sequence of the 52-kDa protein was AYATTNPYTGETXTXEAT, almost identical to the sequence MAYATNPYTGETXXTFXEAT encoded by the locus GOX1122, a putative NAD-dependent aldehyde dehydrogenase gene.

**Cloning and expression of GOX0417 and GOX1122**

A pair of PCR primers, 2KGR-1-Xba and 2KGR-2-Sal, was designed to cover locus GOX0417 from the start to the stop codon, including the SD sequence of this gene. Using these primers, a PCR product of 1,066 bps was obtained and then cloned into pGEM-T Easy Vector. 2KGR activity was observed in *E. coli* DH5α carrying this plasmid, pGEM-GOX0417, at a level about 20 times higher than in the soluble fraction of *Gluconobacter* strains. To obtain a higher expression level, the inserted fragment on pGEM-GOX0417 was then subcloned into pET-28a(+) at the XbaI and SalI sites, resulting in the pET-GOX0417 plasmid (Fig. 4). This plasmid was transformed to *E. coli* BL21 (DE3) and checked for expression. It was found that 2KGR activity was slightly increased as compared with *E. coli* DH5α carrying pGEM-GOX0417. Thus the gene GOX0417 was identified as the gene for 2KGR. GOX0417 showed up to 46% identity to various putative N-isomer specific 2-hydroxyacid dehydrogenases from the genome database, and 30% and 24% identity to N-glycerate dehydrogenase from *Hyphomicrobium methylivorans* (accession no. BAA06662) and L-lactate dehydrogenase from *Lactobacillus bulgaricus* (accession no. CAA42781) respectively. The sequence alignment of GOX0417 and other related enzymes is shown in Fig. 5. In the genome sequence around GOX0417, no genes related to this enzyme activity were found, but this gene was only 40 bp downstream of GOX0417, a putative protein-tyrosine phosphatase (data not shown). Locus GOX1122 was also expressed to identify its gene product. The open reading frame was amplified from the start to the stop codon from *G. suboxydans*
PCR product

Digested with Xba I & Sal

Cloned into pET-28a(+)

SD: AATGGA

*  

SD: GAAGGA

**

Fig. 4. Construction of Plasmids for 2KGR Production.

Fig. 5. Sequence Alignment of GOX0417 with Related Sequences.

2KGR from G. oxydans 621H (Gox-2KGR) is aligned with two putative D-isomer specific 2-hydroxyacid dehydrogenases of Burkholderia sp. YP_367972, Bk-2HADH, and Roseobacter denitrificans OCh 114 (YP_681071), Rd-2HADH, and with two enzymes of known tertiary structure, D-glycerate dehydrogenase of Hyphomicrobium methylovorum (BAA06662), Hme-GADH, and D-lactate dehydrogenase of Lactobacillus bulgaricus (CAA42781), Lde-DLDH. The identities in amino acid sequence to GOX0417 were 45, 44, 30, and 24% respectively. The amino acid residues conserved in all sequences are marked with asterisks at the bottom of the alignment. Colons and dots mean that conserved and semi-conserved residues respectively are to be observed in that column. The box shows the motif GXGXXG, which appears in many NAD-binding enzymes.
IFO12528 using F-GOX1122-NcoI and R-GOX1122-BamHI primers. The PCR product of 1,422 bp was then ligated with pGEM-T Easy Vector and subcloned into pET-28a(+) at the NcoI and NotI sites. The resulting plasmid, pET-GOX1122, was then transformed into E. coli BL21 (DE3). SDS–PAGE analysis of the cell-free extract showed an overexpressed protein band (data not shown). In the crude extract, the activity with 100 mM acetaldehyde and 10 mM NADP\(^+\) was 67.7 units/mg, whereas the activity with 10 mM NAD\(^+\) was 0.0479 units/mg. The cell-free extract from E. coli BL21 (DE3) with empty vector pET-28a(+) showed no acetaldehyde dehydrogenase activity with either NAD\(^+\) or NADP\(^+\).

These results confirm that GOX1122 is the gene for NADP\(^+\)-aldehyde dehydrogenase. The N-terminal sequence of the previously purified aldehyde dehydrogenase from G. melanogenus\(^20\) was identified as AYATINPLTGEVLKTFPNAT, also almost identical to that of GOX1122 (unpublished result). The alignment of GOX1122 with other NAD(P)\(^+\)-aldehyde dehydrogenases is shown in Fig. 6. The Cys and Glu residues are conserved in GOX1122 (C265 and E231). The catalytic Cys residue is thought to form a thiohemiketal covalent intermediate with aldehyde,\(^21\) and recently such a covalent intermediate was solved in the crystal structure of glyceraldehyde 3-phosphate dehydrogenase from Streptococcus mutans.\(^22\) The Glu residue involved in the hydrolysis step.\(^22\) In the genome sequence around GOX1122, no genes related to this enzyme activity were found, and this gene appeared to be monocistronic (data not shown).
Fig. 7. Improvement of 2KGR Production Due to Changing the Ribosomal Binding Sequence.

Crude extract (30 µg protein) from *E. coli* BL21 (DE3) harboring pET-GOX0417 (A) or pET-GOX0417 M6 (B) was analyzed by SDS–PAGE. Lanes 1–4 are samples from independent transformant colonies. Lane 28a is the result due to the vector plasmid, pET28a(+). M, marker proteins.

Fig. 8. Induction of 2KGR Production.

*E. coli* BL21 (DE3) with pET-GOX0417-M6 was cultured in 100 ml of LB medium to mid-exponential phase (Klett about 100 units) at 37 °C. IPTG was added to a final concentration of 1 mM, and then cultivation continued for induction. After the cells were harvested and disrupted, the extract was centrifuged at 9,000 rpm. Supernatant (30 µg of proteins) was analyzed by SDS–PAGE. Lane M, marker proteins. Lane 1, pET-28a(+) after induction for 5 h. Lanes 2–6, pET-GOX0417-M6 after induction for 0, 2, 3, 4, and 5 h respectively.

Fig. 9. SDS–PAGE Analysis of 2KGR Purified from *E. coli* BL21 (DE3) with pET-GOX0417 M6.

Thirty micrograms of proteins was analyzed by SDS–PAGE. Lane M, marker proteins. Lane 1, soluble fraction of *E. coli* BL21 (DE3) with pET-GOX0417-M6. Lane 2, purified 2KGR after DEAE-cellulose column chromatography.

(Fig. 9, specific activity of about 60 units/mg) was ready to use for measurement of 2KGA. The enzyme was stable and could be kept for more than 3 months at 4 °C, like the original enzyme from *Gluconobacter*. A range from 100 to 500 n mol was appropriate for 2KGA quantification.

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