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Isoforms of Glucose 6-Phosphate Dehydrogenase in *Deinococcus radiophilus*

Ji Youn Sung and Young Nam Lee*

Division of Life Sciences, College of Natural Sciences, Chungbuk National University, Cheongju 361-763, Republic of Korea

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Glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) in *Deinococcus radiophilus*, an extraordinarily UV-resistant bacterium, was investigated to gain insight into its resistance as it was shown to be involved in a scavenging system of superoxide (O_2^{-1}) and peroxide (O_2^{-2}) generated by UV and oxidative stresses. *D. radiophilus* possesses two G6PDH isoforms: G6PDH-1 and G6PDH-2, both showing dual coenzyme specificity for NAD and NADP. Both enzymes were detected throughout the growth phase; however, the substantial increase in G6PDH-1 observed at stationary phase or as the results of external oxidative stress indicates that this enzyme is inducible under stressful environmental conditions. The G6PDH-1 and G6PDH-2 were purified 122- and 44-fold (using NADP as cofactor), respectively. The purified G6PDH-1 and G6PDH-2 had the specific activity of 2,890 and 1,033 U/mg protein (using NADP as cofactor) and 3,078 and 1,076 U/mg protein (using NAD as cofactor), respectively. The isoforms also evidenced distinct structures; G6PDH-1 was a tetramer of 35 kDa subunits, whereas G6PDH-2 was a dimer of 60 kDa subunits. The pIs of G6PDH-1 and G6PDH-2 were 6.4 and 5.7, respectively. Both G6PDH-1 and G6PDH-2 were inhibited by both ATP and oleic acid, but G6PDH-1 was found to be more susceptible to oleic acid than G6PDH-2. The profound inhibition of both enzymes by β-naphthoquinone-4-sulfonic acid suggests the involvement of lysine at their active sites. Cu^{2+} was a potent inhibitor to G6PDH-2, but a lesser degree to G6PDH-1. Both G6PDH-1 and G6PDH-2 showed an optimum activity at pH 8.0 and 30°C.

Keywords: iso-G6PDH, dual coenzyme specificity, *D. radiophilus*, UV resistant bacterium

Genus *Deinococcus* is an obligate aerobic chemoorganotrophic bacterium in bright orange color due to membrane-bound carotenoids. With the exception of *D. grandis*, a Gram-negative rod, all *Deinococcus* spp. are Gram-positive cocci. Phylogeny based on 16S ribosomal RNA sequences has placed the *Deinococcus* in an independent Deinococcus/Thermus phylum (Murray, 1986; Rainey *et al.*, 1997).

The most unusual property of *Deinococcus* species is their extraordinary resistance against UV, ionizing radiation, and oxidative stress (Murray, 1986; Carroll *et al.*, 1996; Battista *et al.*, 1999; Yun and Lee, 2003). In general, UV toxicity to cells has been attributed either to thymine dimerization in DNA molecules or generation of reactive oxygen species (ROSs) including superoxide anion (O_2^{-1}), hydrogen peroxide (H_2O_2), hydroxyl radical (HO·), etc., which induce chemical modification of a variety of biological molecules such as DNA, protein, and lipids, thereby resulting in cellular toxicity (Halliwell and Gutteridge, 1999; Nordberg and Arnĕr, 2001). However, cells protect themselves against ROS toxicity via either ROS-scavenging enzymes, including superoxide dismutase (SOD), catalase, glutathione/thioredoxin peroxidase, glutathione/thioredoxin reductase, and glucose-6-phosphate dehydrogenase (G6PDH) or a number of small antioxidant molecules (Halliwell and Gutteridge, 1999).

The UV and oxidative resistance of *Deinococcus* appears to be attributable to efficiency of its ROS-scavenging system and to its ability to repair damaged cellular molecules. The ROS-scavenging enzymes of *D. radiophilus* have been extensively studied; this bacterium possesses one constitutive SOD (Yun and Lee, 2001, 2003, 2004) and three iso-catalases: one monofunctional catalase and two bifunctional catalase-peroxidases (Lee and Lee, 1995; Oh and Lee, 1998; Yun and Lee, 2000). *D. radiophilus* thioredoxin reductase was also studied (Seo and Lee, 2006). However, no investigations have been yet conducted on G6PDH linked to ROS scavenging system by supplying reducing power to oxidoreductant proteins, i.e. glutathione/thioredoxin.

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) catalyzing the first step in the pentose-phosphate (PP) pathway, in which glucose-6-phoshate is converted to 6-phosphogluconate accompanied with the reduction of nicotinamide adenine nucleotides, is distributed ubiquitously among the prokaryotes and eukaryotes (Levy, 1979). G6PDH, largely active with NADP, but occasionally active with both NAD and NADP, contributes to the generation $NAD(P)H+H^+$, an intracellular reducing power. G6PDH gene (*zwf*)-deficient bacterial strains were shown to become more susceptible to redox recycling agents (Greenberg *et al.*, 1990; Ma *et al.*, 1998; Brita *et al.*, 1999), which indicates that G6PDH plays a role in protecting cells from oxidative damage by supplying the reducing power for antioxidant regeneration, such as thioredoxin or glutathione (Izawa *et al.*, 1998; Lundberg *et al.*, 1999).

In this study, the iso-G6PDHs detected in *D. radiophilus*

* To whom correspondence should be addressed.
(Tel) 82-43-261-2301; (Fax) 82-43-264-9600
(E-mail) ynlee@chungbuk.ac.kr

were purified and characterized in order to expand our insight into the ROS scavenging enzyme system of *D. radiophilus*.

Materials and Methods

Bacterial strains and culture condition

Deinococcus radiophilus ATCC 27603 (American Type Culture Collection, USA) was cultured in TYGM medium containing 1% tryptone, 0.5% yeast extract, 0.2% glucose, and 0.2% methionine at 30°C for 3 days with shaking at 150 rpm (Yun and Lee, 2001).

Preparation of cell - free extract

Cells were harvested by centrifugation (SUPRA 22K, Hanil, Korea) at 3,660×g for 20 min, then washed three times with 50 mM potassium phosphate buffer (pH 7.0) and re-suspended in 3.5 ml of the buffer per 1 g of wet weight (Yun and Lee, 2001). The cells were disrupted with a sonic dismembrator (Fisher Scientific Co., USA) for 60 min on ice, at 20 sec disruption interspersed with 40 sec pause interval. The cell-free extracts were acquired by centrifugation at 15,930×g for 20 min. Protein concentrations were determined either by the method of Lowry *et al.* (1951) or by absorbance measurement at 280 nm.

Electrophoretic resolution of G6PDH

Proteins were resolved electrophoretically on 8.0% polyacrylamide gel with 25 mM Tris-glycine buffer (pH 8.8) (Gersten, 1996). The G6PDH activity bands were visualized by incubating the gel for 60 min in a soaking solution containing 80 mM Tris-HCl (pH 7.5), 1.0 mM tetranitro blue tetrazolium, 40 μM phenazine methosulfate, 1.5 mM NAD(P), and 2.0 mM glucose-6-phosphate (Tian *et al.*, 1999). All procedures were carried out in dark. The proteins on the gel were stained in 0.1% Coomassie brilliant blue R-250 solution for 10 min and destained overnight with 10% methanol and 10% acetic acid.

Monitoring of G6PDH production in D. radiophilus

Cell growth in TYGM medium at 30°C was monitored by measuring $O.D._{600}$. Sonic extracts of the cells at exponential and stationary phases were prepared as described above. Proteins in the cell-free extracts were resolved electrophoretically on 8% nondenaturing polyacrylamide gel, followed by G6PDH activity staining as described above. When *D. radiophilus* cultures reached at $O.D._{600}=0.2$, the cultures were received either potassium superoxide or hydrogen peroxide at final concentrations of 5, 10, 15, and 20 mM, and allowed a further incubation for 60 min at 30°C. Proteins in the cell-free extract of each culture were subjected to PAGE resolution. After G6PDH activity staining on the gel, the densitometry of G6PDH bands was followed (Gel Doc 1000, Bio-RAD, USA).

Purification of G6PDHs

The cell-free extracts were loaded onto a column (2.5×20 cm) of DEAE-cellulose pre-equilibrated with 50 mM potassium phosphate buffer, pH 7.0 (buffer A). After sufficient washing of the materials unbound to the column with buffer A, proteins bound to the DEAE-cellulose were eluted with a step-wise NaCl gradient ranging from 0.1 M to 0.5 M in buffer A at a flow rate of 9 ml/min. The peak fractions containing the enzyme were pooled and concentrated by ultrafiltration with PM-10 (Amicon, USA), followed by dialysis in 5 L of buffer A overnight at 4°C. The dialyzed enzyme preparation was applied to a column (1×8 cm) of 2',5'-ADP Sepharose™ 4B pre-equilibrated with buffer A. After the unbound materials were sufficiently washed off, the proteins bound to 2',5'-ADP Sepharose™ 4B were eluted via a step-wise NaCl gradient ranging from 0.05 to 0.3 M in buffer A at a flow rate of 0.5 ml/min. The enzyme activity profile of the sepharose chromatography showed the existence of three peaks eluted with buffer A containing 0.05, 0.1, and 0.2 M NaCl. Each of the enzyme eluents was pooled adequately and concentrated with a PM-10 Amicon membrane filter. The enzyme preparations were then dialyzed against 5 L of buffer A and resolved on 8% polyacrylamide gel and stained with Coomassie brilliant blue R-250.

Assay of glucose-6-phosphate dehydrogenase

G6PDH activity in the enzyme preparation was measured by monitoring the increase in absorbance at 340 nm as the result of the formation of $NAD(P)H+H^+$. The assay mixture for G6PDH in a total volume of 1 ml was composed of 100 mM Tris-HCl buffer (pH 8.0), 0.2 mM NAD(P), 10 mM $MgCl_2$, and 2 mM glucose-6-phosphate. After the addition of an aliquot of appropriate enzyme to the assay mixture, the $O.D._{340}$ of the reaction mixture was monitored for 5 min. One unit of enzyme activity was defined as the amount of enzyme required to yield 1 μmole of $NAD(P)H+H^+$ per min at 25°C ($\varepsilon_{340}=6.22\times10^3\ M^{-1}cm^{-1}$, Lessie and Vander Wyk, 1972; Anderson *et al.*, 1997).

Molecular weight determination

The molecular weights of the purified G6PDHs were estimated by native polyacrylamide gel electrophoresis at acrylamide concentrations ranging from 5% to 10% (Bollag and Edelstein, 1996) or gel filtration in a Sepharcyl S-200 column (Ibraheem *et al.*, 2005). Bovine lactalbumin (14.2 kD), bovine erythrocyte carbonic anhydrase (29 kD), bovine serum albumin (66 kD: monomer, 132 kD: dimer), and jack bean urease (272 kD: trimer, 545 kD: hexamer) were employed as size markers. The molecular weights of the G6PDHs subunits were determined by SDS-polyacrylamide gel electrophoresis (Bollag and Edelstein, 1996). Electrophoresis was carried out using 12% polyacrylamide gel containing 0.1% SDS with the appropriate size markers, including aprotinin (6.5 kD), bovine lactalbumin (14.2 kD), soybean trypsin inhibitor (20 kD), bovine trypsinogen (24 kD), bovine erythrocyte carbonic anhydrase (29 kD), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kD), chicken egg albumin (45 kD), and bovine serum albumin (66 kD) (Sigma, USA).

Isoelectric focusing of G6PDHs

The isoelectric points of the G6PDHs were determined by isoelectric focusing on 4% polyacryamide gel containing 2.5% ampholytes of which pH span is 3 to 10, with the pI markers of amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.2), myoglobin (pI 6.8, 7.2), and lentil lectin (pI 8.2, 8.6, 8.8) (Sigma)

Effect of pH and temperature on G6PDHs and stability

The effect of pH was measured under the standard assay conditions described above with the purified G6PDHs incubated in the various buffer systems: 0.1 M glycine-HCl (pH 2.0-3.0), 0.1 M acetate (pH 4.0-5.0), 0.1 M potassium phosphate (pH 6.0-7.0), 0.1 M Tris-Cl (pH 7.0-9.0), and 0.1 M glycine-NaOH (pH 9.0-11.0). The pH stability of the enzymes was determined following the preincubation of the enzymes at different pHs (pH 2.0-11.0) for 30 min at 25°C. The optimum temperatures for the G6PDHs were determined by carrying out in a temperature range of 10-70°C. The thermostabilities of G6PDHs were evaluated via assays of residual activity after 30 min post-incubation at various temperatures.

Effect of various chemicals on G6PDHs

The enzyme activity was assayed under standard assay conditions in the presence of either EDTA or group-specific reagents (1 mM and 5 mM) including glyoxal, ρ-diazobenzene sulfonic acid, β-naphtoquinone-4-sulfonic acid, N-ethylmaleimide, and iodine. The effects of a number of cationic metals on the enzyme were investigated by measuring activity in a standard assay system containing 1 mM of cation.

Results and Discussion

D. radiophilus G6PDHs during growth and under oxidative stress

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), which occurs ubiquitously in bacterial, archaeal, and eucaryotic cells, is a key enzyme of the pentose phosphate pathway, which catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconate. This reaction is accompanied with the reduction of either NADP or NAD, thereby resulting in the generation of $NAD(P)H+H^+$, the major reducing power required for the proper operation of a variety of synthetic pathways, as well as the scavenging reactive oxygen species (ROS) generated by internal or external environmental stresses (Ursini *et al.*, 1997; Wright *et al.*, 1997; Brita *et al.*, 1999; Tian *et al.*, 1999). In *Escherichia coli*, substantial increases of G6PDH activity was reported upon the exposure of cells to paraquat, a redox cycling drug that generates superoxide radicals (Greenberg *et al.*, 1990). Similarly, *Pseudomonas aeruginosa* lacking the G6PDH gene (*zwf*) was shown to be more susceptible to paraquat (Ma *et al.*, 1998). The transcription of *E. coli zwf* is governed by the SoxRS (superoxide radical response) regulon in response to oxidative stress (Demple, 1996). Moreover, the role of the reduced form of nicotinamide dinucleotides, $NAD(P)H+H^+$, as an electron source to thioredoxin or glutathione, which repair oxidative damage and regenerate antioxidants, has been extensively evaluated in living cells (Izawa *et al.*, 1998; Lundberg *et al.*, 1999). Unlike eukaryotic G6PDH, which usually operates with only NADP as a cofactor (Tian *et al.*, 1999; Heise and Opperdoes, 1999; Esposito *et al.*, 2001; Honjoh *et al.*, 2003; Ibraheem *et al.*, 2005), many bacterial G6PDHs, including those from *Acetobacter xylinum* (Benziman and Mazover, 1973), *Bacillus licheniformis* (Opheim and Bernlohr, 1973), *Pseudomonas multivorans* (Lessie and Vander Wyk, 1972), *Azotobacter vinelandii* (Anderson and Anderson, 1995), and *Thermatoga maritima* (Hansen *et al.*, 2002), are active with both NAD and NADP. In *D. radiophilus* grown in a medium containing glucose, two G6PDH isoforms were observed, and evidenced dual specificity with both NADP and NAD (Fig. 1). The isoform migrating more slowly on the gel was designated as G6PDH-1, and the faster isoform as G6PDH-2. Both G6PDH-1 and G6PDH-2 activity bands were observed at log and stationary phases, but the intensity of the G6PDH-1 band was increased substantially at stationary phase as compared with that at log phase. The increase in production of *D. radiophilus* G6PDHs occurred in the cells that were treated with potassium superoxide, but the increase in G6PDH-1 was more profound than that in G6PDH-2 (Fig. 2). The hydrogen peroxide treated cells revealed slight increases in G6PDH-1 production, but lesser increases in the production of G6PDH-2 (data not shown). The substantial increase of G6PDH-1 in the stationary phase and upon ex-

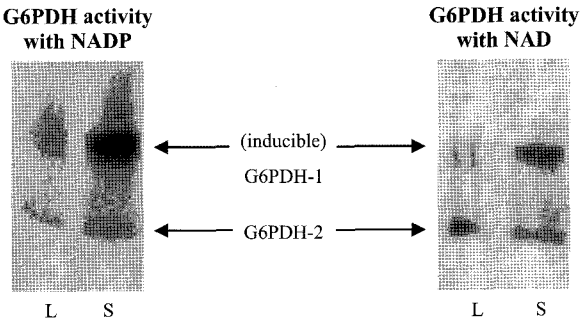
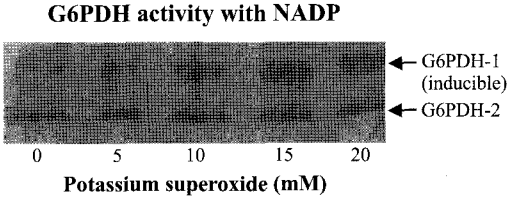


Fig. 1. Electrophoretic resolution of G6PDHs from *D. radiophilus*. Bacteria were grown in TYGM medium contained 10 mM glucose for 30 h (log phase, L) and 80 h (stationary phase, S). Each well was loaded with 100 μg of protein.



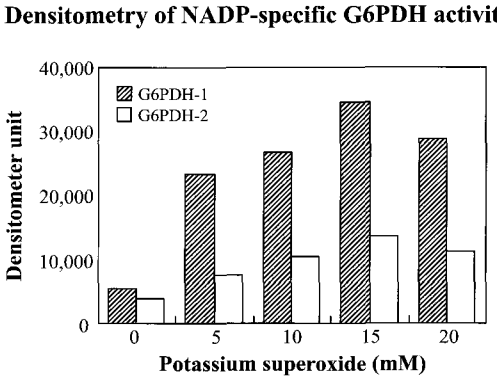


Fig. 2. The production of G6PDHs in *D. radiophilus* treated with potassium superoxide. Each well was loaded with 100 μg of protein.

posure to external oxidative stresses suggests that G6PDH-1 is an oxidative-inducible enzyme. Oxidative-inducible G6PDHs have also been detected in other cells, including yeast (Miki *et al.*, 1996) and human cells (Ursini *et al.*, 1997).

Purification of iso-G6PDHs

Two G6PDH isoforms were purified from the cell-free extract of *D. radiophilus* by DEAE-cellulose ion-exchange and 2',5'-ADP Sepharose™ 4B affinity chromatography. In the DEAE-cellulose chromatography step, two activity peaks were obtained-a minor one eluted with buffer A containing 0.1 M NaCl, and the major one with buffer A containing 0.3 M NaCl. The electrophoretic resolution of proteins in each enzyme preparation on gel followed by G6PDH activity staining showed that the major enzyme preparation eluted with buffer A containing 0.3 M NaCl harbors both G6PDH-1 and G6PDH-2, whereas the minor preparation eluted with 0.1 M NaCl contained only G6PDH-2 activity at lower level. The major enzyme preparation contained both G6PDH-1 and G6PDH-2 was subjected to 2',5'-ADP Sepharose™ 4B affinity chromatography for further purification. In the ADP Sepharose affinity chromatography step, three activity peaks (each eluent obtained from buffer A containing 0.05, 0.1, and 0.2 M NaCl) were obtained (Fig. 3). The electrophoretic resolution of proteins at each purification step by SDS-PAGE showed that each eluent obtained from the ADP Sepharose chromatography revealed a single protein band (Fig. 4: lanes 3, 4, and 6). An eluent obtained with the buffer containing 0.05 M NaCl contained G6PDH-1 (lane 3), whereas the eluents obtained with 0.1 and 0.2 M NaCl buffers contained G6PDH-2 (lane 4 and 6). The electrophoretic mobilities of G6PDHs in the preparation obtained through the ADP Sepharose chromatography were quite different from those in the cell-free extracts or in the preparation of DEAE-cellulose chromatography (Fig. 5B: lanes 1, 2, 3, and 4), that is, the purified G6PDHs migrated very slowly. These results indicate that G6PDHs would aggregate during the ADP Sepharose chromatography. Additionally, the modification of G6PDHs during the sepharose chromatography was suggested, since G6PDH resolved on gel was visible by protein staining (lane 4 on A panel), but was invisible by activity staining (lane 4 on B panel), despite the fact that G6PDH activity was certainly measurable in the same enzyme preparation. The aggregation of G6PDH was evidenced with molecular weight estimated by native PAGE as described later. The purified G6PDH-1 and G6PDH-2, using NADP as a cofactor, gave 2,890 and 1,033 U/mg protein of specific activity with 25% and 38% recovery, respectively. The purified G6PDH-1 and G6PDH-2, using NAD as a cofactor, gave 3,078 and 1,077 U/mg protein of specific activity with 31% and 45% recovery, respectively (Table 1).

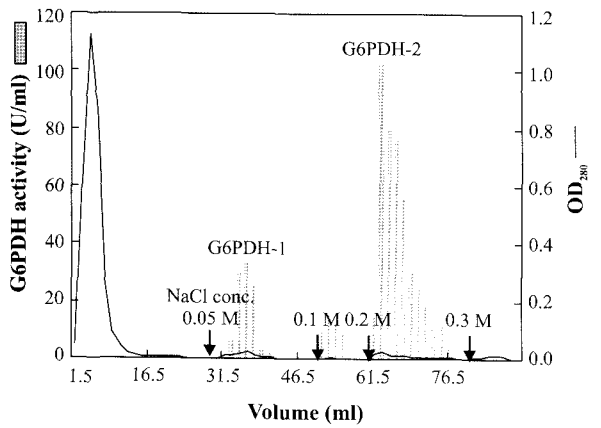


Fig. 3. Elution profile of protein by 2',5'-ADP Sepharose™ 4B chromatography. Proteins were eluted with a step-wise NaCl gradient (0.05~0.30 M) in 50 mM potassium phosphate, pH 7.0 at flow rate of 0.5 ml/min. Column size: 1×8 cm.

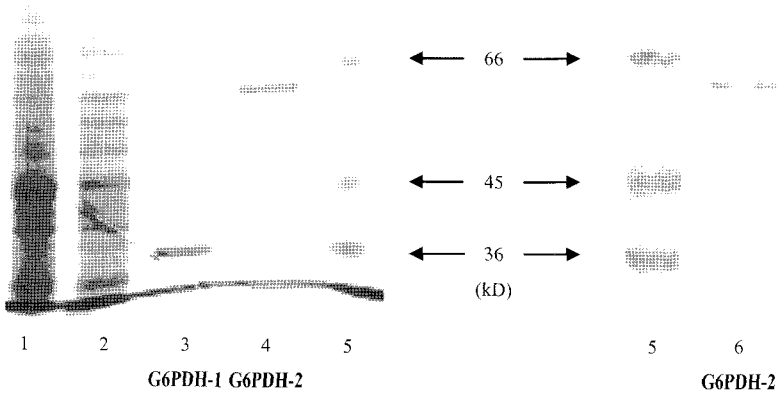


Fig. 4. Resolution of proteins during purification steps by SDS-PAGE. Lane 1, Cell-free extract (100 μg of protein loaded); 2, Enzyme preparation eluted during DEAE-cellulose chromatography with 50 mM potassium phosphate, pH 7.0 containing 0.3 M NaCl (100 μg of protein loaded); 3, Enzyme preparation eluted during 2',5'-ADP Sepharose™ 4B chromatography with 50 mM potassium phosphate, pH 7.0 containing 0.05 M NaCl (5 μg of protein loaded); 4, Enzyme preparation eluted during ADP Sepharose chromatography with 50 mM potassium phosphate, pH 7.0 containing 0.2 M NaCl (5 μg of protein loaded); 5, Size markers; 6, G6PDH-2 eluted with 50 mM potassium phosphate, pH 7.0 containing 0.1 M NaCl during ADP Sepharose chromatography (1 μg of protein loaded).

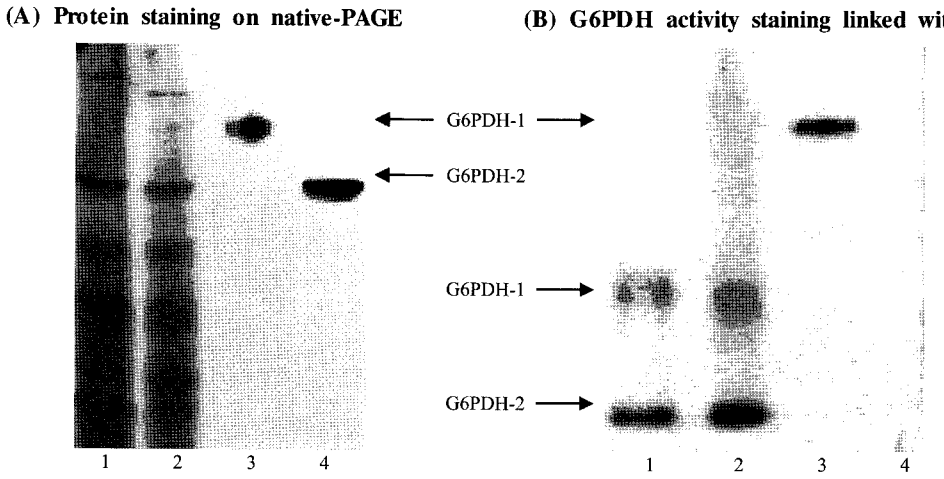


Fig. 5. Electrophoretic resolution of G6PDHs during purification steps. Lane 1, Cell-free extract (100 μg of protein loaded); 2, Enzyme preparation eluted with 50 mM potassium phosphate, pH 7.0 containing 0.3 M NaCl during DEAE-cellulose chromatography (100 μg of protein loaded); 3, Enzyme preparation eluted with 50 mM potassium phosphate, pH 7.0 containing 0.05 M NaCl during 2',5'-ADP Sepharose 4B chromatography (5 μg of protein loaded); 4, Enzyme preparation eluted with 50 mM potassium phosphate, pH 7.0 containing 0.2 M NaCl during ADP Sepharose chromatography (5 μg of protein loaded).

Table 1. Purification of two isoforms of G6PDH from *Deinococcus radiophilus*

Purification step		Total activity (Unit)[a]		Total protein (mg)	Specific activity (U/mg)		Yield (%)		Purification fold	
		NADP	NAD		NADP	NAD	NADP	NAD	NADP	NAD
Cell-free extract		1364.6	1109.4	57.6	23.7	20.7	100	100	1	1
DEAE-Cellulose		1295.7	1064.5	6.0	216.0	177.5	95.0	89.4	9.1	8.6
2',5'-ADP Sepharose 4B	G6PDH-1	231.2	246.3	0.12	2889.9	3078.1	25.4	31.0	122.0	148.9
	G6PDH-2	206.5	215.3	0.5	1032.5	1076.5	37.8	45.2	43.6	52.1

[a]One unit of G6PDH is defined as an amount of enzyme required to yield 1 μmol of NAD(P)H from NAD(P) per min at room temperature.

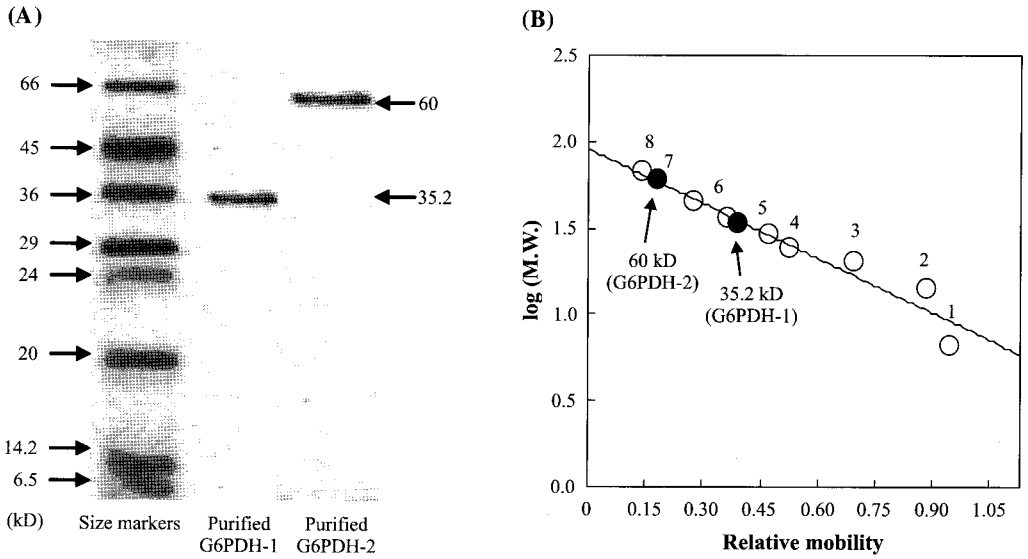


Fig. 6. Molecular weight determination of purified G6PDHs on SDS-PAGE. (A) Resolution of G6PDHs and protein markers on 0.1% SDS-15% PAGE. (B) Determination of molecular weight of G6PDHs on SDS-PAGE. Size markers: 1, aprotinin (6.5 kD); 2, bovine -lactalbumin (14.2 kD); 3, soybean trypsin inhibitor (20 kD); 4, bovine trypsinogen (24 kD); 5, bovine erythrocytes carbonic anhydrase (29 kD); 6, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kD); 7, chicken egg albumin (45 kD); 8, bovine serum albumin (66 kD).

Characterization of two isoforms of G6PDH

(1) The molecular weights of G6PDH-1 and G6PDH-2 as estimated by gel filtration were 130 kD and 110 kD, respectively. SDS-PAGE revealed that the subunit molecular weights of G6PDH-1 and G6PDH-2 were ca. 35.2 kD and 60 kD, respectively (Fig. 6). These results suggest that G6PDH-1 and G6PDH-2 were tetrameric and dimeric proteins, respectively. It is generally known that the G6PDHs in most bacteria, fungi, parasites, and eucaryotic cells exist in a dimeric or tetrameric form. The 60 kD subunit of *D. radiophilus* G6PDH-2 would be similar to the majority of bacterial G6PDHs, including *Bacillus licheniformis* (Opheim and Bernlohr, 1973), *Thermotoga maritima* (Hansen *et al.*, 2002), and *Pseudomonas multivorans* (Vander Wyk and Lessie, 1974). Interestingly, the subunit of *D. radiophilus* G6PDH-1 (ca. 35.2 kD) is rather smaller, as was reported in *Mycobacterium smegmatis* (Purwantini and Daniels, 1996). However, the purified enzymes seemed to be aggregated upon 2',5'-ADP Sepharose chromatography, as the molecular weights of purified G6PDH- 1 and G6PDH-2 determined by native polyacrylamide gel electrophoresis were ca. 398 kD and 452 kD, respectively (data not shown). These results indicate that G6PDH-1 and G6PDH-2 formed a dodecamer and an octamer, respectively, during the affinity chromatography. Similar instances of aggregated forms of G6PDHs occurring during 2',5'-ADP Sepharose chromatography were reported with *Anabaena* sp. PCC 7120 (Gleason, 1996) and *Chlorella vulgaris* C-27 (Honjoh *et al.*, 2003). The aggregation of G6PDH may also be attributable to high substrate concentration (Cacciapuoti and Lessie, 1977), enzyme concentration (*in vitro*), or lower pH (*in vivo*) (Sundaram *et al.*, 1998). The isoelectric points of purified G6PDH-1 from *D. radiophilus* turned out to be 6.5, a value similar to that reported in *P. multivorans* (Vander Wyk and Lessie, 1974), whereas the pI of G6PDH-2 is ca. 5.7, similar to that of G6PDH purified from *T. maritima* (5.8, Hansen *et al.*, 2002) or *P. aeruginosa* (5.7, Ma *et al.*, 1998).

(2) Inhibition of G6PDHs by ATP and oleic acid

Adenine nucleotide-linked control of G6PDHs has been documented in many literatures (Lessie and Neidhardt, 1967; Lessie and Vander Wyk, 1972; Opheim and Bernlohr, 1973; Cacciapuoti and Lessie, 1977; Anderson and Anderson, 1995; Anderson *et al.*, 1997; Ibraheem *et al.*, 2005). Besides, the regulation of G6PDHs by long chain fatty acids and their acyl coenzyme A esters was reported with the NAD- and NADP-linked G6PDHs of *P. multivorans* (Cacciapuoti and Lessie, 1977), *Neisseria gonorrhoeae* (Cacciapuoti and Morse, 1980), and *A. vinelandii* (Anderson *et al.*, 1997). They also suggested the regulatory role of G6PDHs might be attributable to the availability of reducing power for fatty acid synthesis. Both ATP and oleic acid inhibited the activities of G6PDH-1 and G6PDH-2 in *D. radiophilus*. But a more drastic inhibition of both enzyme by oleic acid, as depicted in Table 2. Thus, considering the results of a variety of previous studies, we assumed that *D. radiophilus* G6PDHs may be involved in the regulation of ATP or fatty acid synthesis via the modulation of $NAD(P)H+H^+$ supply. The profound inhibitory effect of oleic acid on G6PDH-1 suggests a more regulatory role of G6PDH-1 in fatty acid synthesis.

Table 2. Effect of ATP or oleic acid on activities of G6PDHs

Reagents (mM)	Relative activity (%)*	
	G6PDH-1	G6PDH-2
ATP		
0.0	100.0	100.0
0.5	75.2	78.2
1.0	55.0	45.5
2.0	35.0	22.7
Oleic acid		
0.0	100.0	100.0
0.4	71.1	68.0
0.8	30.2	36.7
1.0	17.4	30.5

*Relative activity was calculated as 100× (enzyme activity in the presence of chemicals/enzyme activity in the absence of chemicals) in the standard assay mixture composed of 100 mM Tris-HCl (pH 8.0), 0.2 mM NADP, 10 mM $MgCl_2$, and 2 mM glucose-6-phosphate.

Table 3. Effect of chemicals on activities of G6PDHs

Chemicals (Conc.)	Relative activity (%)*	
	G6PDH-1	G6PDH-2
None	100.0	100.0
β-Naphthoauinone-4 sulfonic acid (1 mM)**	31.8	18.9
EDTA (1 mM)	70.4	103.3
EDTA (5 mM)	14.6	2.8
$AgNO_3$ (1 mM)	78.5	155.9
$CdCl_2$ (1 mM)	46.5	128.6
$CuSO_4$ (1 mM)	75.7	22.1
$HgSO_4$ (1 mM)	13.9	53.2
$MnCl_2$ (1 mM)	19.5	35.1
$ZnCl_2$ (1 mM)	6.2	16.8

*Relative activity was calculated as 100× (enzyme activity in the presence of chemicals/enzyme activity in the absence of chemicals) in the standard assay mixture composed of 100 mM Tris-HCl (pH 8.0), 0.2 mM NADP, 10 mM $MgCl_2$, and 2 mM glucose-6-phosphate.
**β-Naphthoauinone-4 sulfonic acid is a lysine modifier.

(3) Influence of EDTA, cationic metals, and group-specific reagents on enzyme activities

A remarkable inhibition of both G6PDH-1 and G6PDH-2 by β-naphthoquinone-4-sulfonic acid, a lysine modifier (68% and 81% inhibition, respectively) suggested lysine involvement at the active sites of *D. radiophilus* G6PDHs. The activity of both G6PDH-1 and G6PDH-2 were inhibited by 5 mM EDTA ca. 85% and 97%, respectively (Table 3), thereby indicating that Mg^{2+} is required for the activities of *D. radiophilus* G6PDH-1 and G6PDH-2. This fact is not surprising since the Mg^{2+} requirement for bacterial G6PDH activity is well documented (Opheim and Bernlohr, 1973; Vander Wyk and Lessie, 1974; Moritz *et al*, 2000; Espitose *et al.*, 2001). As depicted in Table 3, Ag^+ and Cd^{2+} ions exerted different effects on two isoforms; both ions caused a reduc-

tion in G6PDH-1 activity as compared with an increase in G6PDH-2 activity. *D. radiophilus* G6PDHs appear to be sensitive to a number of cations, including Cu^{2+}, Hg^{2+}, Mn^{2+}, and Zn^{2+}, but with different sensitivities. G6PDH-2 activity was inhibited remarkably by Cu^{2+}, as compared with G6PDH-1, whereas the other metal ions showed more profound inhibitory effects on G6PDH-1. Considering different sensitivity of the G6PDHs toward EDTA and a group of cations, the iso-G6PDHs of *D. radiophilus* would be distinguishable from each other in terms of their physiochemical properties, in addition to their molecular size and isoelectric points.

(4) pH and temperature effect on G6PDHs

Both G6PDH-1 and G6PDH-2 were active in a pH range of 7.0~11.0 with the highest level of activity observed at pH 8.0. At pH values below pH 6, no enzyme activity could be detected. Both enzymes were relatively stable in a pH range between 6 and 8. An optimum temperature for the activities of both G6PDHs was 30°C. The activities of both enzymes decreased at above 40°C, but at 70°C, the activity of G6PDH-1 was decreased more profoundly (87%) than was the activity of G6PDH-2 (59%). These data indicate that G6PDH-1 was more thermo-sensitive than G6PDH-2. The thermostability of G6PDHs was rather heat-unstable, as G6PDH-1 and G6PDH-2 retained 40% and 35% of their activities, respectively, after 30 min incubation at 70°C.

In this study, we did not address the kinetics of the G6PDHs purified via affinity chromatography for substrate and coenzyme specificity, since we considered the aggregated form of iso-G6PDHs resulting from purification to be experimental artifacts.

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