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Molecules and Cells

Expression of a Glutathione Reductase from *Brassica rapa* subsp. *pekinensis* Enhanced Cellular Redox Homeostasis by Modulating Antioxidant Proteins in *Escherichia coli*

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Glutathione reductase (GR) is an enzyme that recycles a key cellular antioxidant molecule glutathione (GSH) from its oxidized form (GSSG) thus maintaining cellular redox homeostasis. A recombinant plasmid to overexpress a GR of *Brassica rapa* subsp. *pekinensis* (*Br*GR) in *E. coli* BL21 (DE3) was constructed using an expression vector pKM260. Expression of the introduced gene was confirmed by semi-quantitative RT-PCR, immunoblotting and enzyme assays. Purification of the *Br*GR protein was performed by IMAC method and indicated that the *Br*GR was a dimmer. The *Br*GR required NADPH as a cofactor and specific activity was approximately 458 U. The *Br*GR-expressing *E. coli* cells showed increased GR activity and tolerance to H_2O_2, menadione, and heavy metal ($CdCl_2$, $ZnCl_2$ and $AlCl_2$)-mediated growth inhibition. The ectopic expression of *Br*GR provoked the co-regulation of a variety of antioxidant enzymes including catalase, superoxide dismutase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase. Consequently, the transformed cells showed decreased hydroperoxide levels when exposed to stressful conditions. A proteomic analysis demonstrated the higher level of induction of proteins involved in glycolysis, detoxification/oxidative stress response, protein folding, transport/binding proteins, cell envelope/porins, and protein translation and modification when exposed to H_2O_2 stress. Taken together, these results indicate that the plant GR protein is functional in a cooperative way in the *E. coli* system to protect cells against oxidative stress.

INTRODUCTION

All aerobic organisms are exposed to reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O2^{\cdot-}$), alkyl hydroperoxide (ROOH; cumene hydroperoxide and *t*-butyl hydroperoxide), and hydroxyl radical ($HO^{\cdot}$), during the course of normal metabolism or following exposure to unfavorable conditions involving radical-generating agents (Wheeler and Grant, 2004). Oxidative stress occurs when cells are exposed to elevated levels of ROS. These ROS can lead to DNA damage and mutation, lipid peroxidation, the disassembly of iron-sulfur clusters, disulfide bond formation, and other types of protein oxidation (Carmel-Harel and Storz, 2000). To protect against cellular damage or adapt to environmental conditions, cells have evolved effective defense mechanisms involving antioxidant enzymes and free radical scavengers. A number of recent reports have highlighted the key role played by sulphydryl groups (-SH) in the response to oxidative stress and, in particular, the role of the glutathione/glutaredoxin and thioredoxin systems in maintaining the redox homeostasis of cells (Wheeler and Grant, 2004).

Tripeptide glutathione (GSH; γ-L-glutamyl-L-cysteinylglycine), a non-proteinous thiol compound, is found in almost all aerobic organisms, including bacteria, fungi, plants, and animals. GSH is an important molecule that protects cells exposed to environmental stresses. The redox-active sulphydryl group of GSH can protect cells from ROS by directly scavenging free radicals, and also in conjunction with glutaredoxin and acting as a cofactor for antioxidant enzymes such as glutathione peroxidase. In this manner, oxidized glutathione forms glutathione disulfide (GSSG), which is recycled back to GSH by the glutathione reductase enzyme (Wheeler and Grant, 2004). In addition to its role as an antioxidant, GSH has been implicated in a wide range of metabolic processes. The formation of GSH-conjugates and their subsequent export into the vacuole is an important method for the detoxification of xenobiotics and heavy metals such as cadmium. S-gluthathiolation, the reversible binding of GSH to sulphydryl groups, can protect proteins from irreversible oxidative damage. GSH has also been implicated in the synthesis of DNA precursors, protein folding, and amino acid transport (Sugiyama et al., 2000; Wheeler and Grant, 2004).

The intracellular level of glutathione is high in some cells, and enzymes involved in its antioxidant function, such as glutathione peroxidase and glutathione reductase, have been identified (Spickett et al., 2000). Glutathione reductase (GR) belongs to a family of flavin-containing pyridine-nucleotide-disulfide oxidoreductases. The major function of these homologous dimeric pro-

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teins is to catalyze the conversion of GSSG to GSH using NADPH as a source of reducing power. NADPH is regenerated by glucose-6-phosphate dehydrogenase. Accordingly, GR plays a key role in maintaining the redox balance of the cells by keeping high ratios of GSH/GSSG. Its physiological importance is indicated by studies demonstrating that cells with attenuated GR activities were more susceptible to oxidative stress (O'Donovan et al., 1999). To date, the genomic DNA or cDNAs encoding GR have been determined in various organisms, including *E. coli* (Greer and Perham, 1986), *Pseudomonas aeruginosa* (Perry et al., 1991), cyanobacterium *Anabaena* (Jiang et al., 1995), yeast (Collinson and Dawes, 1995), *Arabidopsis thaliana* (Kubo, 1993), pea (*Pisum sativum* L.) (Stevens et al., 2000), tobacco (Creissen and Mullineaux, 1995), and human (Bucheler et al., 1992). Transgenic lines that overexpress the GR of *Drosophila* (Mockett et al., 1999) and a plant (*Brassica juncea*) (Pilon-Smits, 2000) were shown to be more resistant to stress conditions when compared to the control lines or wild-type. The GR gene from *Brassica rapa* (previously referred to *B. campestris*) was previously cloned and characterized (Lee et al., 1998; 2002). The mRNA level of *Br*GR was found to increase upon exposure to oxidative stress such as ozone or paraquat. However, there is little molecular and structural information available for the GR protein of *B. rapa*, even though it would be necessary to obtain valid information on how *Br*GR is regulated in responses to environmental challenges. In this study, we overexpressed and purified the *B. rapa* GR using the *E. coli* BL21 system for basic biochemical studies. In addition, we examined whether the ectopically expressed *B. rapa* GR would function and protect *E. coli* cells against various oxidative stresses induced by exogenous stimuli.

MATERIALS AND METHODS

Strains and growth conditions

E. coli BL21 (DE3) cells were stored in 10% glycerol stocks at -70°C, and were maintained on Luria-Bertani (LB) agar plates. Cells harboring recombinant plasmids were grown and maintained on LB media containing 100 μg/ml ampicillin (Amp).

Cloning of GR from *B. rapa* subsp. *pekinensis*

Total RNA was extracted from *B. rapa* subsp. *pekinensis* (Lee et al., 2002), and cDNA was synthesized via MMLV reverse transcriptase (Sigma). The cDNA was amplified by PCR via the specific primer *Br*GR-F-*Nco*I (GATTCTGCACAACCATGGCG-AGGAAGATGC) and *Br*GR-R-*BamH*I (GAGTGTGAATTGGA-TCCAAAGATAATGC) with restriction enzyme sites for *Nco*I and *Bam*HI (underlined), at the 5′ and 3′ ends, respectively. PCR reaction conditions were as follows: initial denaturation cycle at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 90 s, and a final extension of 7 min at 72°C. The PCR product was inserted into the TOPO-TA cloning vector (Invitrogen). The ligated plasmid was first transformed into DH5α competent cells. After confirmation of the sequence and in-frame insertion, the construct containing the *Br*GR gene was digested with *Nco*I and *Bam*HI, and the resultant DNA fragment was cloned into the expression vector, pKM260 (EUROSCARF), to generate pKM-*Br*GR. The pKM-*Br*GR construct was transformed to *E. coli* BL21 (DE3) competent cells for the subsequent expression and purification of the His-tagged fusion protein (named *Br*GR).

Semi-quantitative RT-PCR analysis

The total RNA from cells cultured by mid-log phase in LB broth with vigorous shaking was extracted via the Total RNA Purification System (Invitrogen). A two-step semi-quantitative RT-PCR method was used to measure *Br*GR gene expression in *E. coli* BL21. The RNA pellets were dissolved in diethyl pyrocarbonate (DEPC)-treated water after DNase I treatment. In the first step, cDNA was synthesized by the Superscript III first-strand synthesis system (Invitrogen) according to method described. In the second step, the yield of epimastigote cDNA was measured according to the PCR signal generated from the standard house-keeping gene, G3PD (glyceraldehyde-3-phosphate dehydrogenase). PCR reaction conditions are as follows: initial denaturation cycle at 94°C for 2 min, followed by 24 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s, and a final extension of 7 min at 72°C. The volume of each cDNA pool was adjusted to give the same exponential phase PCR signal strength for G3PD. The primer sets are as follows: *Br*GR-F (GTCTCAGCGAAGAAGAGGCT) and *Br*GR-R (CAAACTGT-GCTTTGGTTGCT) in *Br*GR; G3PD-F (ATGACGACCATT-CATGCCTA) and G3PD-R (ATCACCAGACCAATGGCTTT) in G3PD. PCR amplicons was separated onto a 1.5% agarose gel, stained with ethium bromide, visualized and photographed.

Expression and purification of *Br*GR

For expression analysis, *E. coli* BL21 (DE3) harboring the pKM-*Br*GR plasmid was grown in LB-medium supplemented with 100 μg/ml ampicillin with vigorous shaking (160 rpm) at 37°C. When the culture reached mid-log phase (A_{660} = 0.6), isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.2 mM, and the culture was grown for another 4 h at 37°C. Induced culture was harvested by centrifugation at 5000 rpm for 10 min at 4°C, and then washed twice with cold-PBS buffer. The resulting cell pellet was resuspended in cold lysis buffer (50 mM phosphate buffer, pH 7.5, 0.3 M NaCl, 10 mM imidazole, and 1 mM PMSF) and sonicated for disruption of cells. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C to remove cell debris, and was then analyzed for protein expression by SDS-PAGE on 10% gels as described by Laemmli (1970). The supernatant containing soluble protein was used for purification and determination of native structure and enzyme activity using immobilized metal affinity chromatography (IMAC). The clear supernatant was loaded by gravity onto a column containing a pre-equilibrated Ni-NTA affinity resin (Quiagen). The column was washed twice with cold wash buffer (50 mM phosphate buffer, pH 7.5, 0.3 M NaCl, and 50 mM imidazole). The bounded protein *Br*GR was eluted with cold buffer (50 mM phosphate buffer, pH 7.5, 0.3 M NaCl, and 0.25 M imidazole). The enzyme was concentrated to the desired volume by 50 kDa Centricon (Millipore) at 5,000 rpm at 4°C. Purity was checked by SDS-PAGE or native PAGE.

Glutathione reductase activity

The enzymatic activity of recombinant *Br*GR was assayed spectrophotometrically. The assay was performed at room temperature with a reaction mixture (total 1 ml) containing 50 mM phosphate buffer (pH 7.6) with 1.7 mM EDTA, 1.0 mM GSSG, 0.1 mM NADPH, or NADH and crude extract. The absorbance was monitored at 340 nm. The activity was calculated using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ of NADPH or NADH. One unit is the amount of enzyme that oxidizes 1 nmol of NADPH per min at 25°C (Yu and Zhou, 2007).

Western blot analysis

Protein extract was separated by 10% or 12% SDS-PAGE, and was electrophoretically transferred to a PVDF membrane. The membranes were blocked with 5% non-fat skim milk in PBS

(Amicon) containing 0.05% Tween 20 (PBST) for 1 h at room temperature, and incubated overnight at 4°C with anti-His tag (Millipore), anti-DNP (Sigma), anti-DnaJ, anti-DnaK, anti-GroES (Stressgen), and anti-GR (Sigma). Blots were washed four times for 40 min with PBST and incubated with horseradish peroxidase coupled to anti-rabbit (Promega) or anti-mouse (Millipore) secondary antibodies for 1 h at room temperature. After washing, the signal was visualized using the ECL Western blotting detection reagent (GE Healthcare).

Response of *E. coli* to environmental stressors

Overnight cultures of *E. coli* BL21 (DE3) cells transformed with either pKM260 (empty vector) or pKM-*Br*GR plasmid were grown in fresh LB containing 100 μg/ml of ampicillin at 37°C with shaking. When the A_{600} reached a value of 0.6, 0.2 mM IPTG was added. The cells were grown for 4 h at 37°C, streaked onto LB agar plates containing 0.5 mM H_2O_2, 0.18 mM menadione (MD), 1.4 mM $CdCl_2$, and 2.0 mM $ZnCl_2$, and incubated at 37°C. For shaken-flask growth assays, overnight cultures were inoculated in fresh LB-Amp medium to a starting A_{600} of approximately 0.2 for each strain. Exogenous stressors (1.5 mM H_2O_2, 0.3 mM MD, 1.6 mM $CdCl_2$, and 1.7 mM $ZnCl_2$,) and 0.2 mM IPTG were also added simultaneously. Growth was monitored using a UV/visible spectrophotometer at 660 nm at different time intervals.

Assay of cellular hydroperoxide

When the culture reached mid-log phase (A_{660} = 0.6), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM, and the culture was grown for another 1 h at 37°C. Cells were exposed to 5.0 mM H_2O_2, 0.3 mM menadione (MD), 1.6 mM $CdCl_2$, or 2.0 mM $ZnCl_2$ for 0.5 h with shaking, washed twice with cold-PBS buffer, and then disrupted by sonication. The intracellular hydroperoxide level was determined by ferrous ion oxidation in the presence of a ferric ion indicator, xylenol orange (ferrous oxidation-xylenol orange assay) (Jiang et al., 1992).

Antioxidant enzyme activity

Enzyme activities of superoxide dismutase, catalase, glutathione peroxidase and glucose-6-phosphate dehydrogenase was measured by CALBIOCHEM®s Assay Kit (Calbiochem). One unit of each enzyme activity was defined as units per mg protein.

Two-dimensional gel electrophoresis and MALDI-TOF MS analysis

Mid-log phase *E. coli* cells were exposed to 5 mM H_2O_2 for 30 min, washed twice with cold PBS buffer, and suspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM PMSF and protease inhibitor cocktails). Cell lysis was carried out by sonication. Cleared supernatants by centrifugation were precipitated with TCA/acetone at -20°C overnight after DNase/RNase/Mg mix treatment at 15 min on ice. Protein pellets were washed 3 times with cold-ethanol, vaccum-dried, resuspended in sample buffer (9.5 M urea, 4% CHAPS, 0.1 M DTT, 40 mM Tris and 0.2% Bio-Lyte, 3-10) for 1 h at room temperature with shaking and then centrifuged at 15,000 rpm for 30 min. The supernatants were transferred to new eppendorf tube. The protein concentration was measured by modified method with Protein Assay Reagent (Bio-Rad). The first dimensional isoelectric focusing (IEF) was carried out on commercial 17 cm, pH 4-7 immobilized pH gradient (IPG) strips at 20°C with a maximum current limitation of 50 μA/strip in a PROTEAN IEF Cell (Bio-Rad). After active rehydration at 50 V for 12 h, 1.0 mg of the protein was loaded onto the bottom of an IPG strip. IEF was performed according to the following steps: 250 V for 0.5 h, 250-10,000 V for 5 h, and 90,000 V-h at 10,000 V. After focusing, the strips were equilibrated twice for each 15 min. The first equilibration solution contained 30% (w/v) glycerol, 6 M urea, 2% (w/v) sodium dodecyl sulphate (SDS), 50 mM Tris-HCl, pH 8.8, 65 mM dithiothreitol (DTT), and a trace of bromophenol blue. The second equilibration was performed with the same equilibration solution, except that DTT was replaced by 260 mM iodoacetamide. The gels were carried out second-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separation for the second dimension was performed on 1.0 mm thick 12% polyacrylamide gels at 10°C, at a constant current of 25 mA per gel in PROTEAN® II xi Cell (Bio-Rad). After protein fixation for 12 h in 40% methanol, the gels were stained with Coomassie Brilliant Blue R-250 (Sigma) for 4 h, and then destained. The interest spots were cut and performed the following mass spectrometry.

Protein spots were excised and destained with 30% methanol. Two-hundred mM ammonium bicarbonate was added to the gel piece and mixed for 30 min. The liquid was discarded and washing was repeated. The gel pieces were shrunk by dehydration in 100% acetonitrile, which was removed, and then the pieces were dried in Speed-Vac. Enzymatic digestion was performed by adding 20 μl of 0.0125 μg/μl sequence grade modified trypsin (Promega) in 50 mM ammonium bicarbonate and 5 mM calcium chloride, and incubated for 16 h at 37°C. Extracted digestion mixtures were dried using a Speed-Vac, and then suspended in 0.1% trifluoroacetic acid (TFA). Two μl of the extracted sample was dispensed onto the MALDI sample plate with 2 μl of matrix solution (40 mg/ml α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA and 50% acetonitrile) and dried under ambient conditions. Mass spectra were obtained using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems) with delayed extraction and reflectron. Spectra were calibrated upon acquisition using a ProteoMass Peptide MALDI-MS Calibration Kit, MS-CAL2 (Sigma) with angiotensin II (1046.5423), $P_{14}R$ (1533.8582), and ACTH fragment 18-39 (2465.1989) as external calibration. Database searches were carried out with ProFound (http://prowl.rockefeller.edu/ profound_bin/WebProFound.exe).

Statistical analysis

All biochemical experiments were carried out in at least three independent repetitions. Results were expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Characteristics of the *Brassica rapa* subsp. *perkinensis* GR sequence

A GR cDNA from *B. rapa* subsp. *perkinensis* was cloned previously (Lee et al., 1998). The complete *Br*GR ORF encodes a 502-amino-acid protein, with a predicted molecular weight of approximately 54 kDa. The deduced amino acid sequences were 93.2%, 80.1%, 71.5%, 53.7%, 34.7%, 35.6%, 40.8%, and 35.4% identical to those of *Arabidopsis thaliana* (Accession number: P48641), *Pisum sativum* (garden pea; Q43261), *Oryza sativa* subsp. *japonica* (rice; P48642), *Nicotiana tabacum* (tobacco; P80461), *Saccharomyces cerevisiae* (NP_015234), *Homo sapiens* (human; AAP88307), *Nostoc* sp. PCC7120 (CAA61856), and *E. coli* (BAE77794), respectively. It exhibited a high degree of conservation within the region responsible for the pyridine nucleotide-disulphide oxidoreductase active site and for the binding of FAD, GSSG, or NAD(P)H (Lee et al., 1998; Yoon et al., 2005). The observation that most motifs and

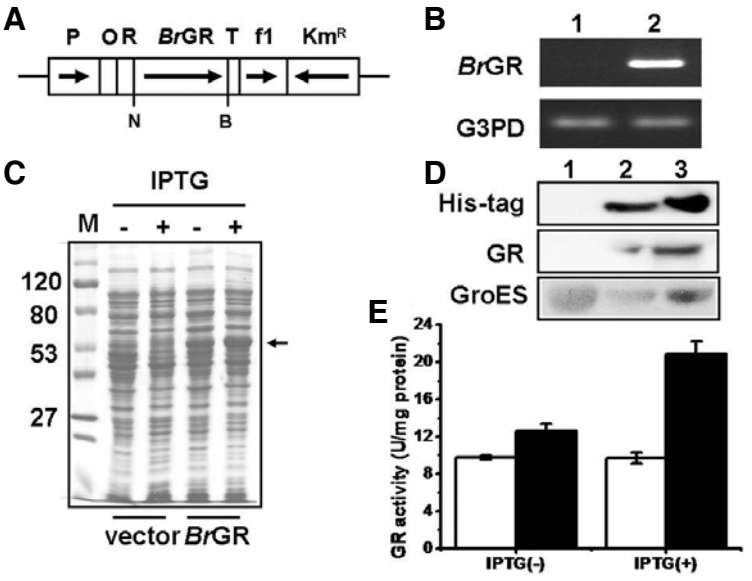

Fig. 1. Scheme of the pKM-*Br*GR construct. (A) Restriction sites used to fuse GR genes to the T7 promoter were displayed under the gene as N (*Nco*I) and B (*Bam*HI). Arrows indicate the direction of transcription (5′-3′). P, T7 promoter; O, *lac* operator; R, ribosomal binding site; T, T7 terminator; f1, origin of viral replication; Km^R, kanamycin-resistant gene. (B) mRNA expression of the transformed *E. coli* was detected by semi-quantitative RT-PCR analysis using housekeeping G3PD. PCR products were electrophoresed on a 1.5% agarose gel, and were visualized by ethidium bromide staining. Lane 1, wt cells with vector alone; lane 2, *Br*GR-transformed cells. (C) SDS-PAGE analysis of proteins expressed in *E. coli* containing the pKM-*Br*GR plasmid in the absence (-) and the presence (+) of 0.2 mM IPTG. Crude extracts (30 μg) were loaded, run on 12% SDS-PAGE, and visualized by Coomassie staining. M, broadway protein marker; vector, cells with vector alone; *Br*GR, pKM-*Br*GR-transformed cells. (D) Western blot analysis of *Br*GR recombinant protein samples. The Western blot was carried out using anti-His tag (His-tag) or anti-human GR (GR) antibody. Anti-GroEL antibody from *E. coli* was used for a housekeeping control. Lane 1, whole cell lysate with vector alone; lane 2, whole cell lysate with pKM-*Br*GR; lane 3, purified recombinant protein with pKM-*Br*GR, as described in "Materials and Methods". (E) Enzyme activity of GR expressed in *E. coli* containing the pKM-*Br*GR plasmid in the absence (-) and the presence (+) of 0.2 mM IPTG. The enzyme assay was performed as described in Materials and Methods. Enzyme activity was represented as U/mg protein. White bar, whole cell lysate with vector alone; black bar, whole cell lysate with pKM-*Br*GR.

domains in *Brassica rapa* subsp. *perkinensis* GR (*Br*GR) are conserved compared to those of other species suggests that this gene plays an important role in organisms upon exposure to environmental stress.

Overexpression and purification of the recombinant protein

A cDNA containing the open reading frame (ORF; 1509 bp) of the GR gene from *B. rapa* subsp. *perkinensis* was subcloned into an expression vector pKM260 at the *Nco*I-*Bam*HI sites thus it can be regulated by a T7 promoter (Fig. 1A). The construct was sequenced to confirm the absence of the deletion, insertion, and substitution of any nucleotide that could alter the amino acid sequence during the translation of the cloned genes. This plasmid was used to transform *E. coli* BL21 (DE3) and the expression of the *Br*GR gene was confirmed by semi-quantitative RT-PCR, SDS-PAGE, and immunoblotting analysis. Semi-quantitative RT-PCR analysis showed that one DNA fragment of 951 bp corresponding to the region between *Br*GR ORF was amplified in the *Br*GR-expressing strain (recombinant strain). No amplification signal was detected in a control strain with vector alone (Fig. 1B). SDS-PAGE gel showed that the *Br*GR protein was normally expressed (Fig. 1C). In addition, to exclude the detection of the endogenous GR of *E. coli*, we examined *E. coli* GR by Western blotting and found that the endogenous protein was non-reactive to the antibodies against His-tag or *Br*GR (Fig. 1D). *Br*GR expression was also supported by enzymatic kinetics before and after IPTG treatments. The GR activity of the *Br*GR transformed strain was 2.5-fold higher than that of the strain with vector alone upon IPTG treatment (Fig. 1E). It may be noted that both the vector alone and the pKM-*Br*GR transformed *E. coli* strains had endogenous GR genes in their chromosomes. Therefore, we can presume that *Br*GR contributed approximately 50% of the total GR activity, while half of the total GR activity may be due to the endogenous enzymes.

After we confirmed that the introduced *Br*GR was transcribed and translated in *E. coli*, we performed the purification of the recombinant protein. The *Br*GR gene was found to be optimally expressed after the induction of 0.2 mM IPTG. When IPTG was treated with concentrations of 0.3 mM or greater, we found that more than 70% of the protein was localized in the insoluble inclusion bodies (data not shown). pKM-*Br*GR with the histidine tag at the N-terminal was purified by a protocol involving IMAC as described in Materials and Methods. The purification resulted in near homogenicity as determined from the Coomassie blue-stained gel (Fig. 2A). The subunit molecular weight (MW) and isoelectric point (pI) of the protein were analyzed by using ProFound software with MALDI-TOF data. The score, coverage, MW, and pI of the protein (accession number: AAC49980) were 2.26, 36%, 54.04 kDa, and 6.0, respectively (Fig. 2B). As the expectation of many researchers, the recombinant *Br*GR protein was found to be active as a homodimer. The purified enzyme migrated as a single band of ~110 kDa on 8% native gel electrophoresis (Fig. 2C). The protein on SDS-PAGE using a non-reducing sample buffer migrated as one band of about 110 kDa (Fig. 2D, lane 1). The purified protein on SDS-PAGE with a reducing buffer containing DTT migrated as a fragment around 55 kDa. The purified polypeptides indicated in Figs. 2C and 2D were identified as correct *Br*GR by MALDI-TOF MS analysis (data not shown). These results indicate that *Br*GR is a dimer in the cell. To date, most GR proteins of prokaryotes and eukaryotes exist as dimers in tissues and cells (Seo, 2006) as seen in the case of *Br*GR.

Next, we characterized the specific activity of the *Br*GR protein (approximately 455.7 U/ mg protein) using its NADPH oxidation rate (Fig. 2E). The activity was found to be two-fold higher than that of GR from human erythrocytes and the cyanobacterium *Nostoc* sp. PCC7120 (Jiang et al., 1995), and three-fold and 3.6- fold higher than that of GR from yeast (Yu and Zhou, 2007) and intertidal copepod *Tigriopus japonicus* (Seo, 2006), respectively.

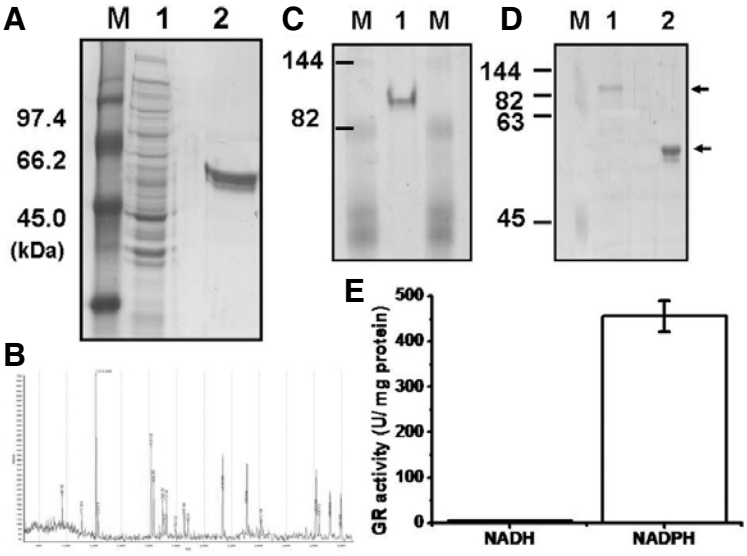

Fig. 2. Purification of the overexpressed *Br*GR protein and specific enzyme activity. The expressed protein was purified by IMAC as described in "Materials and Methods". (A) SDS-PAGE analysis of *Br*GR protein expressed in *E. coli* containing the pKM-*Br*GR plasmid in the presence of 0.2 mM IPTG. Protein samples were loaded, run on 10% SDS-PAGE, and visualized by Coomassie staining. M, broad-range protein marker; lane 1, crude extract (10 μg) of pKM-*Br*GR cells; lane 2, His-tag purified protein (5 μg). (B) The purified protein was identified by MALDI-TOF analysis. (C) His-tag purified *Br*GR protein was separated by 8% non-denaturing gel electrophoresis and visualized by Coomassie staining. M, broad-range protein marker; lane 1, His-tag purified protein (5 μg). (D) His-tag purified *Br*GR protein (5 μg) was separated by 10% denaturing gel electrophoresis (SDS-PAGE) and visualized by Coomassie staining. The position standard size marker is shown on the left. M, broad-range protein marker; lane 1, protein with non-denaturing sample buffer; lane 2, protein with denaturing sample buffer. (E) Specific enzyme activity of *Br*GR protein. Enzyme activity was assayed with 0.2 mM NADH or NADPH as a substrate, and is represented as U/mg protein.

Stress tolerance of the *Br*GR-transformed cells upon exposure to oxidative stresses

Oxidative stress can alter the cellular environment, allowing exposed cellular thiols to become oxidized. Aerobic prokaryotic and eukaryotic organisms have developed a set of cell defense systems to minimize or prevent the detrimental effects of ROS produced by endogenous- or exogenous stimuli. Among antioxidant enzymes, GR participates in protection against oxidation by maintaining the adequate redox state in the intracellular environment and, thus, regulating various cellular activities (Li et al., 2005). *E. coli* has been used as a favorable model system for testing because its genetic response systems have been studied extensively. Antioxidant capacity of *Br*GR protein can be examined by the induction of cell protection via the introduction of a variety of stressors including hydrogen peroxide, menadione (MD), or heavy metals. MD is a quinone that is extensively used in studies of cellular oxidative stress. The main postulated mechanism of its toxicity is oxidative stress caused by the process of redox cycling, thus yielding peroxide radicals (Castro et al., 2007). Though zinc usually appears to be protective against oxidative stress and apoptosis, exposure of cells to this metal in excess is cytotoxic. The molecular mechanism underlying zinc toxicity is not fully understood, but in some cases, zinc has been reported to act as a pro-oxidant molecule by enhancing the production of ROS (Martelli and Moulis, 2004). Cadmium is an inducer of oxidative stress, particularly lipid peroxidation (Mendoza-Cozatl et al., 2005). First, we compared the stress tolerance of the *E. coli* transformed with either pKM-*Br*GR or vector alone in the presence of 1.5 mM H_2O_2, 0.3 mM MD, 1.6 mM $CdCl_2$, and 1.7 mM $ZnCl_2$. In drug-amended LB medium, *E. coli* BL21 exhibited biphasic growth kinetics, as measured by changes in A_{660}. First, the growth of the *Br*GR-expressing strain occurred at almost the same rate as that of the strain with the vector alone, but the final A_{660} in the *Br*GR strain was considerably higher (Figs. 3A, 3C, 4A, and 4C). In addition to the altered growth kinetics, an attenuation of stress sensitivity was seen in agar plate assay. The viability of the *Br*GR strain rapidly recovered in LB medium supplemented with 0.5 mM H_2O_2, 0.18 mM MD, 1.4 mM $CdCl_2$, and 2.0 mM $ZnCl_2$ as compared to that of the control strains (Figs. 3B, 3D, 4B, and 4D). These results suggest that the *Br*GR expression in BL21 cells undergoes cellular physiological changes that enhance their capacity to withstand stresses. Thus, *Br*GR expression seems to affect the redox status of the host cell, and the *Br*GR expressing cells should exhibit different responses of oxidative stress than control cells. To test this notion, we analyzed the cellular hydroperoxide levels and protein oxidation as described to "Materials and Methods". Oxidant concentrations were set to a mild stress condition; 40 to 60% of cell viability than normal growth conditions. The increase of the intracellular hydroperoxide level (Fig. 5A) and protein oxidation (Fig. 5B) by H_2O_2, MD, Cd, and Zn stress was significantly blocked in the *Br*GR-transformed cells as compared to the transformed cells with vector alone. In the case of protein oxidation, carbonylation of phosphoglycerate kinase, an important member of glycolytic pathway (Nellemann et al., 1989), was not observed in the *Br*GR-transformed cells (Fig. 5B, V1). The proteins that are irreversibly inactivated by carbonyl derivatives cannot be repaired and have to be recognized and degraded by cellular proteolytic processes. If early removal of protein oxidation fails, cells may allow for oxidized proteins to become more severely oxidized and/or cross-linked. These detrimental effects include mitochondrial damage, resulting in decreased ATP synthesis and enhanced production of ROS, and proteasome inhibition. The selective degradation of oxidized proteins is required to maintain cellular homeostasis. The progressive accumulation of oxidized and cross-linked proteinacious materials has been implicated in the aging process that is characterized by a gradual functional decline and an increase in the probability of disease and death (Castro et al., 2007). We hypothesized that if *Br*GR exerts antioxidant capacity against oxidative stress *in vivo*, as indicated by above results, this should be further reflected in its effect on cellular antioxidant metabolites. We measured enzyme activity of other antioxidant proteins during oxidant treatment. As seen in Fig. 6, the expression of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPX), and glucose-6-phosphate dehydrogenase (G6PDH) were significantly higher in the *Br*GR-transformed cells than in the control cells during MD, Cd, and Zn stress. However CAT, SOD,

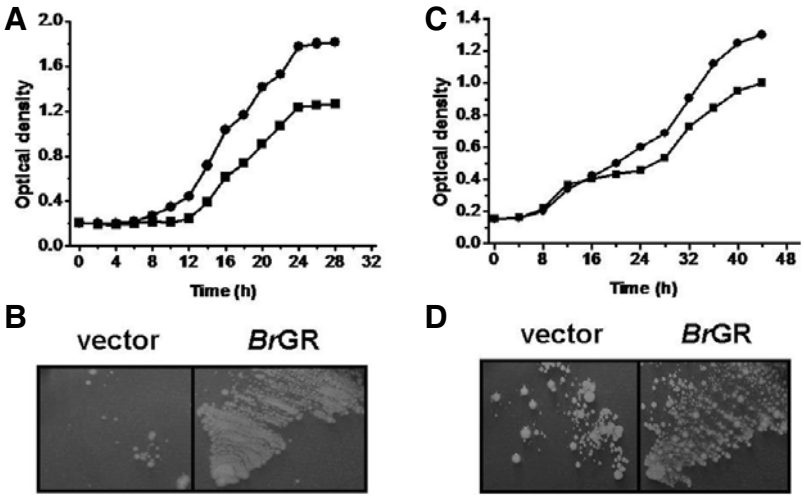

Fig. 3. Effects of the *Br*GR overexpression against hydrogen peroxide and menadione. The bacterial cell growth rate was monitored at 2 h intervals for the indicated time in LB medium containing 0.2 mM IPTG in the presence of 1.5 mM H_2O_2 (A) and 0.3 mM menadione (C). Wt (Wild-type) cells with vector alone (square); *Br*GR-expressing cells (circle). Cell survival was determined by a streaking assay on LB medium supplemented with 0.5 mM H_2O_2 (B) and 0.18 mM menadione (D) as described in "Materials and Methods". Wt cells with vector alone (left); *Br*GR-expressing cells (right).

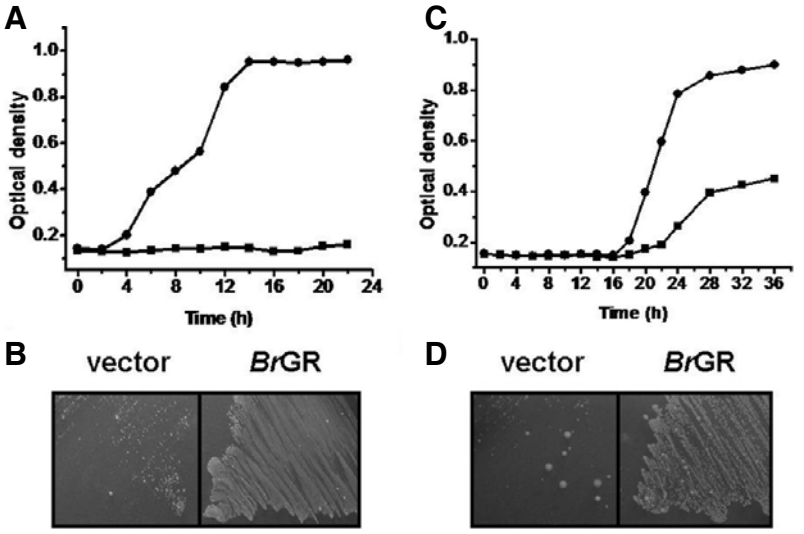

Fig. 4. Effects of *Br*GR overexpression during cadmium and zinc exposure. The bacterial cell growth rate was monitored at 2 h intervals for the indicated time in LB medium containing 0.2 mM IPTG in the presence of 1.6 mM $CdCl_2$ (A) and 1.7 mM $ZnCl_2$ (C). Wt cells with vector alone (square); *Br*GR-expressing cells (circle). Cell survival was determined by a streaking assay on LB medium supplemented with 1.4 mM $CdCl_2$ (B) and 2.0 mM $ZnCl_2$ (D). Wt cells with vector alone (left); *Br*GR-expressing cells (right).

G6PDH and GPX were not notably different under hydrogen peroxide (H_2O_2) stress (Fig. 6A). Elevated induction of chaperone proteins DnaK, DnaJ, and GroES in the transformed cells were also identified by immunoblotting analysis (Fig. 6B). Even though differences in expression levels exist, striking contrast in scavenging ability was observed. *Br*GR-expressing *E. coli* cells appeared to lead to increase in activities of antioxidant and chaperone proteins thus decreasing the H_2O_2 concentration and protein damages.

Systematic and comparative analysis of the proteome under H_2O_2-induced oxidative stress

To gain further knowledge on the physiological effects of *Br*GR-overexpression, we compared the 2-D gel pattern of two different cell types in the presence of 5 mM H_2O_2 for 0.5 h at 37°C. Proteomics can be used to compare and analyze changes in protein expression profiles in cellular response resulted from a recombinant protein synthesis. The identification of selected protein spots were also determined, as described in "Materials and Methods", and is summarized in Table 1. H_2O_2 exposure in the *Br*GR-transformed cells resulted in up-regulation of nine proteins with antioxidant- (alkyl hydroperoxide reductase and CysK), potential antioxidant- (inorganic pyrophosphatase), energy metabolism- (fructose-1,6-biphosphate aldolase, maltoporin and glycerol kinase), and protein synthesis-roles (elongation factor G) (Fig. 7). Enteric bacteria respond to a variety of environmental challenges by coordinately controlling the expression of regulons and stimulons. *Escherichia coli* cells respond to the redox stress imposed by ROS-generating agents by increasing the expression of different polypeptides. These proteins include many components of multilevel antioxidant enzymes, with inducible functions for ROS-scavenging (CAT, SOD, GR and GPX), NADPH production (G6PDH), protein synthesis, and metabolic pathway (Chou et al., 1993). A major outer membrane porin protein OmpF permits passive diffusion of small and hydrophilic molecules into the periplasm. The reduced OmpF may evade the protection action under unfavorable conditions (Bernstein et al., 1999). An iron-regulated alkyl hydroperoxide reductase (AhpC) is the primary scavenger of endogenous hydrogen peroxide and reactive sulfur species (RSS) in *E. coli*. The AhpC is known to be a more efficient scavenger of trace of H_2O_2 than catalase, and confered aerotolerance and oxidative stress resistance (Seaver and Imlay, 2001). AhpC from *Anabaena* sp. PCC 7120 protects *E. coli*

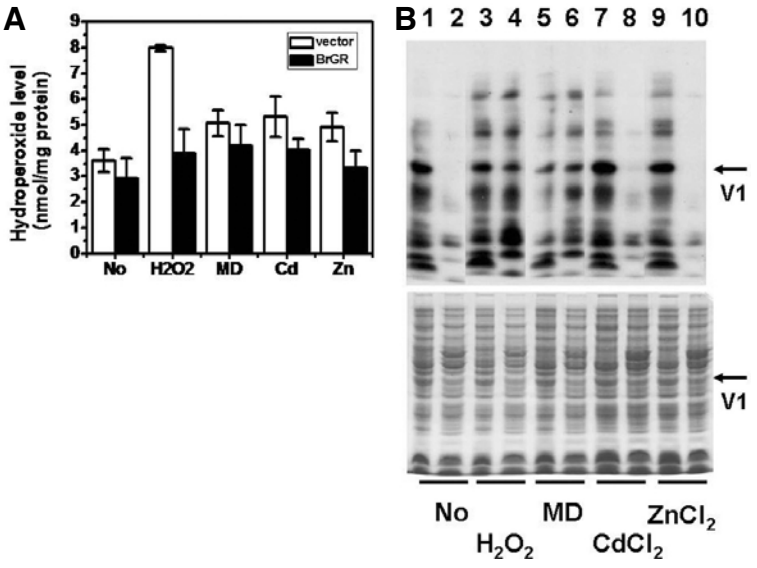

Fig. 5. Intracellular hydroperoxide levels and protein oxidation under multiple stressors. (A) Intracellular hydroperoxide levels were measured by using FOX reagent either under normal condition (No) or after exposure to 5.0 mM H_2O_2, 0.3 mM menadione (MD), 1.6 mM $CdCl_2$ (Cd), and 2.0 mM $ZnCl_2$ (Zn) for 0.5 h at 37°C. White bar, wt cells with vector alone; black bar, *Br*GR-expressing cells. (B) Protein oxidation was analyzed by Western blot using anti-DNP. Upper panel, Immuno blot analysis; Bottom panel, 12% SDS-PAGE.1,3,5,7,9: the transformed cells with empty vector; 2,4,6,8,10: the *Br*GR-transformed cells.

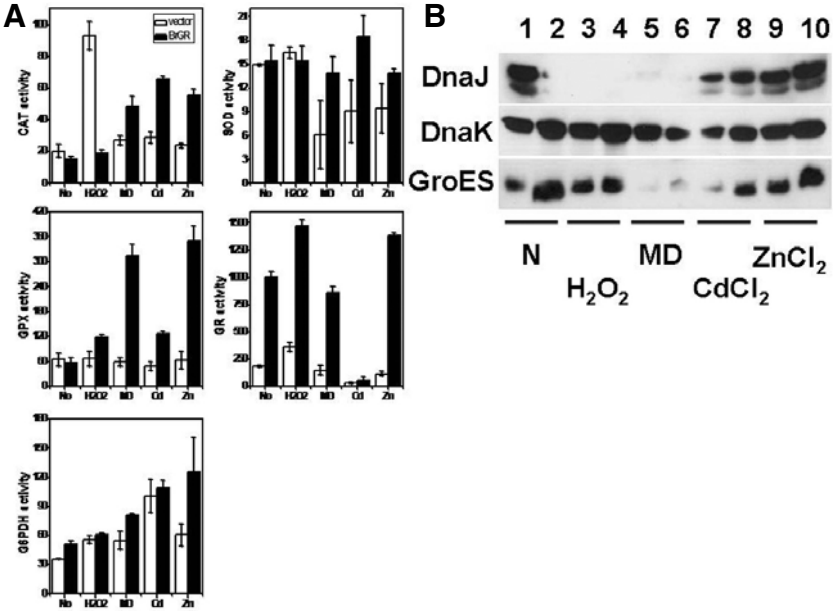

Fig. 6. Antioxidant enzyme activity and expression of chaperone proteins. The bacterial cells grown to mid-log phase were exposed to 5.0 mM H_2O_2, 0.3 mM menadione (MD), 1.6 mM $CdCl_2$ (Cd), and 2.0 mM $ZnCl_2$ (Zn) for 0.5 h at 37°C. Crude protein extracts were separated by sonication, and then used for enzyme activity measurement. (A) Each enzyme activity was represented as a U/mg protein. CAT, catalase; SOD, superoxide dismutase; GPX, glutathione peroxidase; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase. White bar, wt cells with vector alone; black bar, *Br*GR-expressing cells. (B) Western blot analysis of chaperone proteins under a variety of stress conditions. The analysis was performed under basis of SDS-PAGE (Fig. 5B). 1,3,5,7,9: the transformed cells with empty vector; 2,4,6,8, 10: the *Br*GR-transformed cells.

from multiple abiotic stresses such as heat (47°C), 6% NaCl, 4 mM $CdCl_2$, and 1 mM $CuCl_2$ exposure (Mishra et al., 2009). A cysteine synthase CysK protein involved in cysteine biosynthesis can augment cellular thiol pools and thereby involved in countering oxidative stress and heavy metals (Ackerley et al., 2006). Maltoporin (LamB), a high-affinity receptor for maltose and maltose oligosaccharides, serves as a channel for sugar migration across the outer membrane to utilize energy metabolism (Nishino et al., 2005) and expresses as a function of pH (Yohannes et al., 2004). Fructose-1,6-biphosphate aldolase (FBA) is a key metabolic enzyme catalyzing the cleavage of fructose-1,6-phosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, which is involved in both the glycolysis/gluconeogenesis and the pentose phosphate pathway in prokaryotes and eukaryotes. Overexpression of recombinant FBA from *Sesuvium portulacastrum* (*SpFBA*) increased stress tolerance to abiotic stimuli in transgenic *E. coli* (Fan et al., 2009). Inorganic pyrophosphatase (PPase) plays an important role in macromolecular biosynthesis and is essential for the viability of *E. coli* in order to counterbalance pyrophosphate synthesis (Chen et al., 1990). Glycerol kinase (GlpK) is an inducible enzyme whose activity is very low if glucose is present in the medium (Benov and Al-Ibraheem, 2002). Besides metabolism-related proteins, elongation factor G (EF-G) is a translation-related protein and is needed for protein synthesis (Huang et al., 2007). When anaerobically grown *E. coli* cells were exposed to hydrogen peroxide and menadione stress, elongation factor G is a major target of the oxidatively damaged proteins (Tamarit et al., 1998). In addition, AhpC, EF-G and GlpK have been known to be stress-responsive proteins in *E. coli* BL21 (DE3) (Han et al., 2008). *E. coli* mutants lacking some antioxidant defense proteins, such as those encoded by *sodB*, *cysK*, and *katE*, exhibit increased sensitivity to ROS stress (Ackerley et al., 2006). Collectively, *Br*GR-mediated stress toler-

Table 1. Proteins overexpressed in the transformed cells with *Br*GR under H_2O_2 stress

Spot no.	Est,d Z	Protein information	%	pI	MW
1	2.41	Chain A, Ompf porin mutant Y106f	62	4.6	37.05
2	2.38	alkyl hydroperoxide reductase subunit C	39	5.0	20.86
3	2.35	inorganic pyrophosphatase	49	5.0	19.83
4	2.36	alkyl hydroperoxide reductase subunit C	61	5.0	20.86
5	2.41	CysK protein	55	5.6	34.56
6	2.39	Chain A, Class Ii fructose-1,6-bisphosphate aldolase in complex with phosphoglycolohydroxamate	44	5.5	39.23
7	2.41	Chain G, Structure of the regulatory complex of *Escherichia coli* iiiglc with glycerol kinase	38	5.3	56.37
8	2.22	elongation factor G	28	5.2	77.73
9	2.42	Chain A, maltoporin maltose complex	63	4.7	47.48

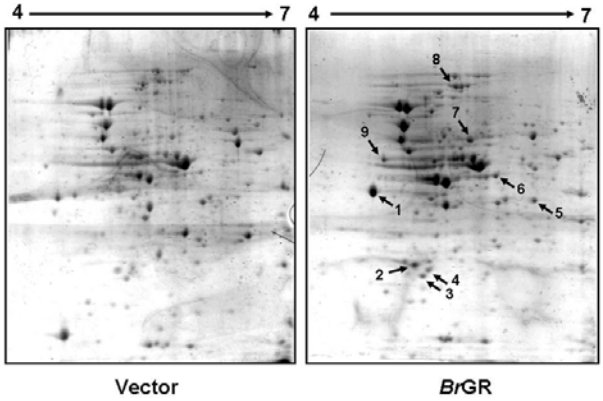

Fig. 7. Coomassie blue-stained two-dimensional electrophoresis gels of total cellular proteins from wild-type cells with empty vector alone (vector) and *Br*GR-transformed cells (*Br*GR) in the presence of 5.0 mM H_2O_2 for 0.5 h at 37°C. Distinct protein spots whose expression was up-regulated in the *Br*GR cells were excised and identified by MALDI-TOF MS analysis.

ance appears to be involved in cells' effective adaptation of stress response system by inducing antioxidant proteins and other related proteins.

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