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Production of Stress-shock Proteins in *Pseudomonas* sp. DJ-12 Treated with 4-Hydroxybenzoate

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***Pseudomonas* sp. DJ-12 can grow on 4-hydroxybenzoate (4HBA) at concentration of 5 mM or lower by degrading 4HBA for carbon and energy sources. The organisms were found to produce DnaK stress-shock protein when treated with several aromatic hydrocarbons including 4HBA. Those cells treated with 5 mM 4HBA exhibited increased tolerance to 10 mM concentration. In this study, the production of other stress-shock proteins besides DnaK was examined in *Pseudomonas* sp. DJ-12 exposed to various concentrations of 4HBA, comparing the production of the proteins with their survival and degradation of 4HBA. The organisms could degrade 4HBA at 0.5 to 5 mM concentrations after 60 to 90 minutes of incubation. The survival rate of the organism decreased when treated with 4HBA at 10 mM or higher concentrations. The stress-shock proteins of DnaK, GroEL, and GroES were produced in the cells which were treated with 4HBA at 0.5 mM or higher concentrations for 10 minutes. Fifteen additional stress-shock proteins were produced in the cells which were treated with 5 mM 4HBA for 40 minutes. The DnaK and GroEL proteins in the cells gradually decreased upto 6 hours after the stress was removed from the culture.**

Key words: Stress-shock proteins, DnaK, GroEL, 4HBA stress, *Pseudomonas* sp. DJ-12

Microorganisms can defend for themselves from environmental stresses by producing a variety of specific stress proteins when exposed to extreme environments. In general, such stress-shock proteins including DnaK, DnaJ, and GroEL, are known to be involveed in the maturation of newly synthesized proteins and in the refolding or degradation of denatured proteins (17, 21). Accordingly it is believed that the proteins play important roles during environmental stresses by rescuing or scavenging the proteins denatured by the stresses as the chaperone. The cellular responses to the environmental stresses have been widely studied in different bacteria against heat shock (9, 11), oxidative stress (1, 5), starvation (13, 27), and SOS stress (25).

Recently, the defense mechanisms of microorganisms against the stresses of pollutant chemicals and aromatic hydrocarbons have become major concerns in environmental molecular biology. Early work by VanBogelen *et al.* (29) showed that exposure of *E. coli* to chemical pollutants, such as ethanol, $CdCl_2$, H_2O_2, and 6-amino-7-chloro-5,8-dioxoquinoline (ACDQ) lead to induction of approximately thirty-five different stress-shock proteins including DnaK, GroEL, and Lon. Blom *et al.* (6) also reported that some stress-shock proteins induced by chemical pollutants overlap with proteins produced by heat shock and carbon starvation, but at least 50% of them are unique to the given chemicals. Aromatic hydrocarbons, such as benzene, toluene, ethylbenzene, and xylene (BTEX), were also reported to be the inducers of stress-shock proteins in *Pseudomonas putida* (2, 30). Lambert *et al.* (20) reported that exposure of *E. coli* to 20 mM benzoate led to induction of stress-shock proteins including DnaK, HtpG, and UspA. Vercellone-Smith and Herson (31) showed that the newly synthesized proteins by *Pseudomonas putida* in response to toluene overlapped with those induced by carbon starvation, but four other toluene-shock proteins are unique to the toluene-exposed cells.

Pseudomonas sp. DJ-12 isolated from Daejon industrial complex is an aerobic degrader of 4-chlorobiphenyl (4CB) and biphenyl (18). The organism is capable of transforming 4CB to 4-chlorobenzoate (4CBA) by *meta*-cleavage of the aromatic ring and 4CBA to 4-hydroxybenzoate (4HBA) by dechlorination process (8, 14). The strain DJ-12 could also degrade 4HBA extensively at the concentration of 5 mM or lower.

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The DnaK stress-shock protein was reported to be produced in the cells which were treated with several aromatic pollutants including 4HBA (24). In particular, those 4HBA-treated cells exhibited increased tolerance to 10 mM concentration of 4HBA. In this study, therefore, the production of the stress-shock proteins in *Pseudomonas* sp. DJ-12 exposed to various concentrations of 4HBA was examined by two-dimensional PAGE and Western blot methods.

Materials and Methods

Bacterial strain

The bacterial strain used in this study was *Pseudomonas* sp. DJ-12. The strain DJ-12 was isolated from Daejon industrial complex as reported by Kim *et al.* (18), and it can transform 4-chlorobiphenyl (4CB) to produce 4-chlorobenzoate (4CBA) by *meta*-cleavage of the aromatic ring under aerobic condition (14). The organism is also able to degrade 4-hydroxybenzoate (4HBA).

Degradation of 4HBA by strain DJ 12

Degradation of 4HBA by *Pseudomonas* sp. DJ-12 was examined by the resting cell assay as described by Arensdorf and Focht (3). The organisms were grown to log phase in Luria-Bertani (LB) broth at 30°C. The cells were harvested by centrifugation at 2,000 × g for 10 minutes and washed three times with 10 mM phosphate buffer (pH 7.0). The cells were inoculated to about 10^8 cells/ml in 100 ml of 10 mM phosphate buffer (pH 7.0) containing 4HBA (Sigma Co., USA) between concentrations 0.5 to 7 mM. During incubation at 30°C for 4 hours, the remaining amount of 4HBA in the buffer was examined at 246 nm with a UV spectrophotometer.

Stress treatment with 4HBA and viability test

The strain DJ-12 grown in LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) were harvested by centrifugation at 2,000 × g for 10 minutes. These cells were washed three times with 10 mM phosphate buffer (pH 7.0) and then inoculated to about 10^8 cells/ml in 30 ml MM2 medium in 100 ml Erlenmeyer flasks containing 4HBA at concentrations between 0.5 to 15 mM. The organisms were exposed to 4HBA in the flasks on a shaking incubator at 30°C. After exposure for appropriate period of time, the viable cells were enumerated by plating them on LB agar during experiment.

Western blot analysis

After the organisms were treated with 4HBA, the cells collected by centrifugation at 2,000 × g were suspended in 10 mM phosphate buffer (pH 7.0) and then disrupted with a sonicator (Fisher M-300). Prior to SDS-PAGE analysis, the proteins were quantified with a protein assay kit (Sigma Co., USA) according to the manufacturer's instruction. The SDS-PAGE of the proteins was performed according to the method described by Bollag *et al.* (7) using 10% and 4% acrylamide for separation and stacking gels, respectively. The 4HBA-treated cells were analyzed for the stress-shock proteins by Western blot as described by Sambrook *et al.* (26) by using anti-DnaK and anti-GroEL monoclonal antibodies (StressGen Biotechnologies Corp., Canada). The proteins on the gels separated by SDS-PAGE were transferred to Hybond™-PVDF membrane (Amersham International plc., England) with a Semiphor semi-dry transfer unit (Hoefer Scientific Instruments, USA). The blots were blocked with 0.1% bovine serum albumin for 1 hour at room temperature. Subsequently the blots were washed with phosphate buffered saline (PBS), and incubated with primary antibody diluted 5,000 times in PBS-0.08% Tween 20 for 1.5 hours. Subsequently, the blots were washed with PBS-0.08% Tween 20. The secondary antibody (anti-mouse IgG HRP conjugate, Promega, USA) diluted 5,000 times in PBS - 0.08% Tween 20 was applied for 1.5 hours, and the blots were washed with PBS-0.08% Tween 20. The immunocomplex was detected with an ECL Western analysis system (Amersham, England) according to the manufacturer's instruction. The density of the blot was quantified with a GelDoc 1000 densitometer (Bio-Rad Co., USA).

Two-dimensional PAGE of the proteins

Two-dimensional PAGE of the proteins which were purified from the 4HBA-treated cells was performed according to the methods described by O'Farrell (23) and Bollag *et al.* (7) by using the Hoefer 250 SE system (Hoefer Scientific Instruments, USA). The protein samples were mixed with equal volume of sample buffer (9.5 M urea, 2% Triton X-100, 5% 2-mercaptoethanol, 1.6% Phamalyte pH 4.0~6.5, 0.4% Phamalyte pH 3.0~10.0) and sample overlay buffer (9M urea, 0.8% Phamalyte pH 4.0~6.5, 0.2% Phamalyte pH 3.0~10.0, Bromophenol blue). To make 12 ml of isoelectric focusing gel, 6 g of urea was added to 100 ml side arm flask, and then 1.33 ml of 30% acrylamide and 288 ul of Phamalyte pH 4.0~6.5, 5.4 ml of H_2O were added. The flask was swirled until the urea completely dissolved, and then 25 μl of 10% amonium persulfate and 20 μl TEMED were added. The gel was placed into Hoefer 250 SE system. The lower reservoir was filled with 0.01 M H_3PO_4, and the upper reservoir was filled with 0.02 M NaOH.

The gel was then prerun at 200 v for 10 minutes, 300 v for 15 minutes, and 400 v for 15 minutes, consecutively. After the prerun, the lower and upper buffers were refilled. After the samples were loaded, the gel was run at 500 v for 15 min and then at 750 v for 3.5 hours. After isoelectric focusing, the gel was treated with equilibration buffer (5% 2-mercaptoethanol, 62.5 mM Tris-HCl pH 6.8, 2.3% SDS, 10% glycerol) for 2 hours. The second dimension was performed according to the SDS-PAGE method described above.

Results and Discussion

Degradation of 4HBA by strain DJ-12 and their survival

The degradation of 4HBA by *Pseudomonas* sp. DJ-12 is shown in Table 1. After incubation for 60 to 90 minutes the 0.5 to 5 mM concentration of 4HBA in MM2 began to degrade under aerobic condition. However, 4HBA at 6 mM or higher concentrations did not degrade even after 180 minutes of incubation. The survival of *Pseudomonas* sp. DJ-12 was examined during 6 hours incubation in MM2 broth containing 0 to 15 mM 4HBA, and the results are shown in Fig. 1. The survival rate of the organism was not effected by 4HBA at concentrations of 5 mM or lower. When the organisms were treated with 7 mM 4HBA, the number of the cells slightly decreased after 6 hours of incubation. However, the survival rates of cells treated with 4HBA at 10 mM or higher concentrations were drastically decreased by more than 4 order magnitude. The survival rates of the organisms did not decrease at 5 mM or lower 4HBA which was the concentration to be degraded by the organisms as seen in Table 1.

Several studies demonstrated that degradation and survival rates of organisms diminish at high concentration of aromatic hydrocarbons (4, 15). The toxic effects of the compounds were recognized to be due to the permeability change in membrane which was attributed to change in fatty acids by the phenolic compounds (15, 22), and to energy depletion in the cells caused by change of glycolytic metabolite levels in case of benzoates (32). *Pseudomonas* sp. DJ-12 was proved to grow on 4HBA only at 5 mM or lower concentrations by degrading 4HBA for utilization as carbon and energy sources. Therefore, it can be explained that the survival rates of the organism decreased at 7 mM or higher concentrations of 4HBA and 4HBA at those concentrations was not degraded.

Table 1. Degradation of 4-hydroxybenzoate by *Pseudomonas* sp. DJ-12

Incubation time (min)	Degradation at different concentrations						
	0.5	1	2	3	5	6	7
30	−	−	−	−	−	−	−
50	+	+	+	−	−	−	−
90	++	+	+	+	+	−	−
120	+++	++	++	++	+	−	−
150	+++	+++	+++	+++	++	−	−
180	+++	+++	+++	+++	++	−	−

+++, 80% degradation of the initial amount of 4HBA; ++, 60% degradation; +, 40% degradation; −, no degradation.

Production of stress-shock proteins by 4HBA stress

The production of DnaK and GroEL stress-shock proteins was detected by Western blot with anti-DnaK and anti-GroEL monoclonal antibodies as about 70 kDa and 68 kDa in molecular weight, respectively (Fig. 2 and 3). The DnaK proteins were increasingly induced in the cells treated at 0.5 mM or higher concentrations of 4HBA for 10 minutes (Fig. 2). However, the production of DnaK was observed only at 3 mM or higher concentrations when the cells were treated for 40 minutes. The GroEL proteins of the cells were analyzed by Western blot with anti-GroEL antibody as shown in Fig. 3. The GroEL was induced by treatment with 4HBA for 10 minutes at 0.5 mM or higher concentrations. but the cells treated for 40 minutes showed the protein only at 3 mM or higher concentration. The production of DnaK and GroEL proteins in *Pseudomonas* sp. DJ-12 treated with the 4HBA at different concentrations for 60 minutes is

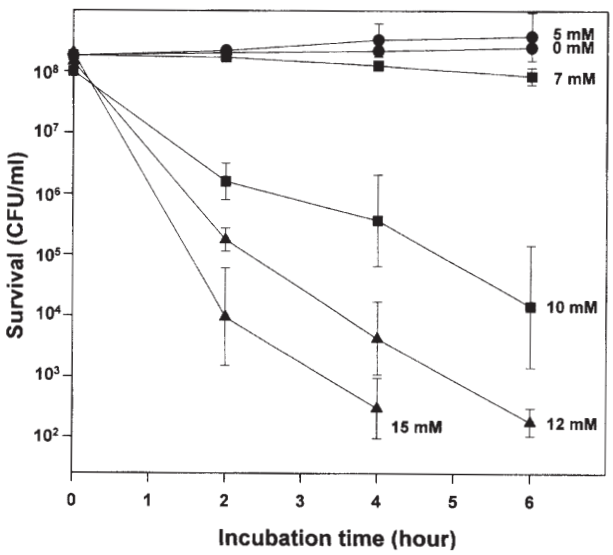

Fig. 1. Survival of *Pseudomonas* sp. DJ-12 in MM2 broth containing various concentrations of 4-hydroxybenzoate.

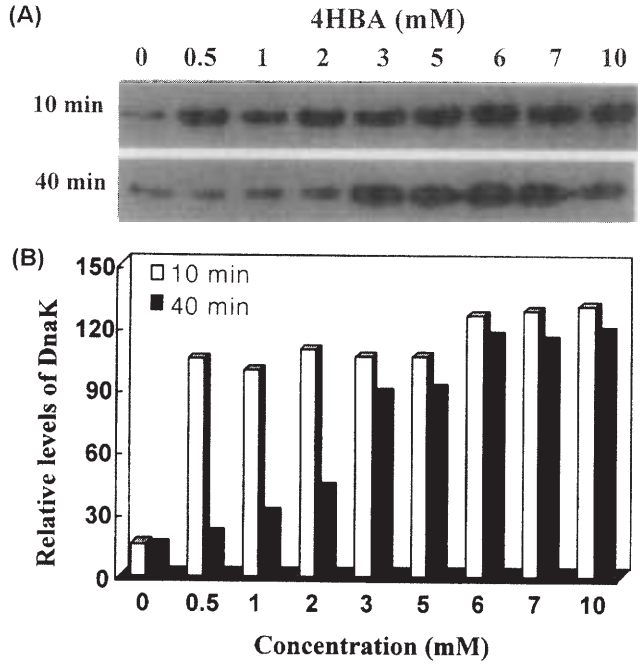

Fig. 2. Western blot (A) of the stress-shock proteins with anti-DnaK monoclonal antibody and their densitometric analysis (B). The cells were treated with 4-hydroxybenzoate at different concentrations for 10 or 40 minutes.

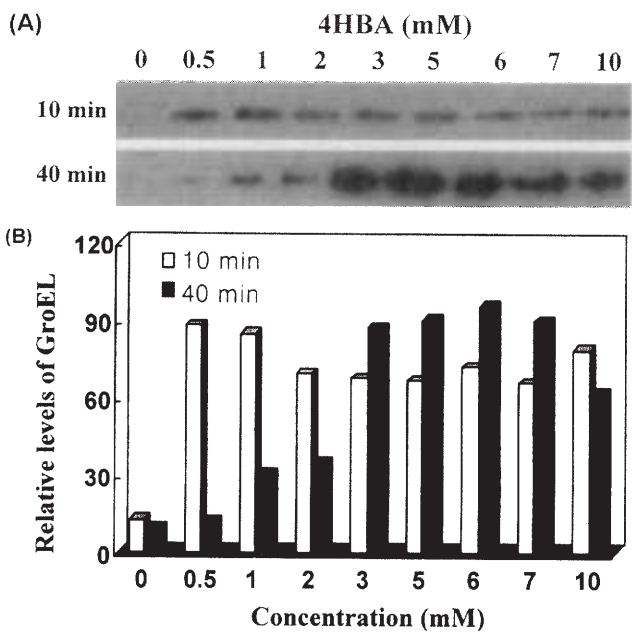

Fig. 3. Western blot (A) of the stress-shock proteins with anti-GroEL monoclonal antibody and their densitometric analysis (B). The cells were treated with 4-hydroxybenzoate at different concentrations for 10 or 40 minutes.

summarized in Table 2. The GroEL protein began to appear in the cells treated with 4HBA for only 5 minutes at 1 to 12 mM concentration, but the production of DnaK was induced 5 minutes later than GroEL. However, both DnaK and GroEL proteins were not detected in the cells which were treated between 0.5 to 2 mM 4HBA for 40 minutes or longer, 4 mM 4HBA for 50 minutes or longer, or 6 mM 4HBA for 60 minutes or longer.

The stress-shock proteins induced by several aromatic hydrocarbons were studied by many researchers (19, 20, 31). Thirty-three kinds of stress-shock proteins including DnaK, HtpG, and UspA were analyzed by two-dimensional PAGE in *E. coli* treated with benzoate at pH 6.5 (20). Jeon and Lee (19) reported that DnaK and GroEL were synthesized in *E. coli* treated with phenol at 1 and 2 mM con-

Table 2. Production of stress shock protein in *Pseudomonas* sp. DJ-12 by treatment with 4-Hydroxybenzoate at different concentrations

Treatment time (min)	Stress-shock protein	Production of stress-shock protein by 4-hydroxybenzoate (mM)						
		0.5	1	2	4	6	9	12
5	DnaK	–	–	–	–	–	–	–
	GroEL	–	+	+	+	+	+	+
10	DnaK	+	+	+	+	+	+	+
	GroEL	+	+	+	+	+	+	+
20	DnaK	+	+	+	+	+	+	+
	GroEL	+	+	+	+	+	+	+
30	DnaK	+	+	+	+	+	+	+
	GroEL	+	+	+	+	+	+	+
40	DnaK	–	–	–	+	+	+	+
	GroEL	–	–	–	+	+	+	+
50	DnaK	–	–	–	–	+	+	+
	GroEL	–	–	–	–	+	+	+
60	DnaK	–	–	–	–	–	+	+
	GroEL	–	–	–	–	–	+	+

+, heavy production; –, no production.

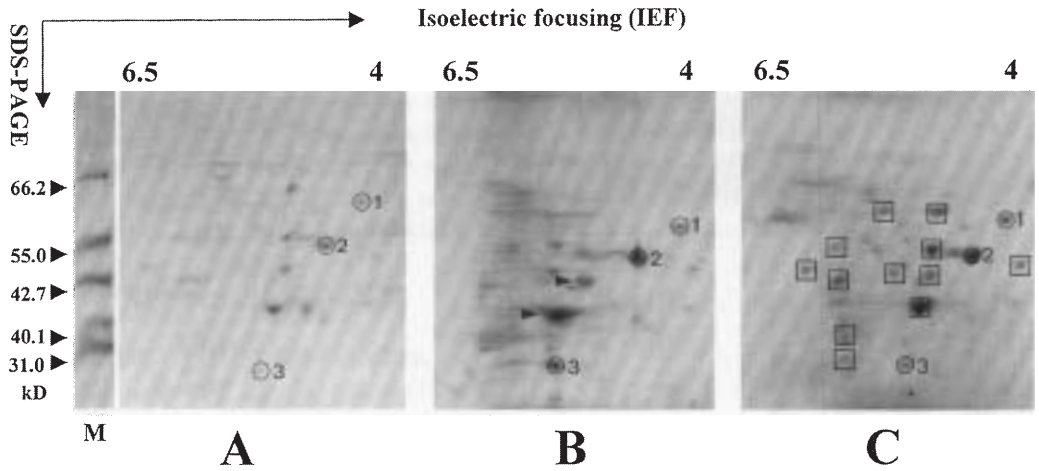

Fig. 4. Two-dimensional PAGE of the proteins produced in *Pseudomonas* sp. DJ-12. A, controll cells; B, cells treated with 5 mM 4HBA for 10 min; C, cells treated with 5 mM 4HBA for 40 min. The circled spots marked with 1, 2, and 3 indicate DnaK, GroEL, and GroES, respectively. The spots marked with arrow head and rectangle also indicate the proteins positively induced by 4HBA.

centrations, respectively. Vercellone-Smith and Herson (31) reported that *Pseudomonas putida* mt-2 cells exposed to toluene as sole carbon source at growth-supporting (4 mg/l), inhibitory (130 mg/l), and lethal (267 mg/l) levels produce twenty-six new proteins, twenty-two of which overlapp with those induced by carbon starvation. The cells provided with 4 mg/l toluene reflected a period of induction and adaptation prior to growth on toluene, and the cells exhibited production of stress-shock proteins at inhibitory level (130 mg/l) of toluene. They described that the level of toluene influenced the length of time that the stress-shock protein was synthesized. Kanemori *et al.* (16) reported that synthesis rates of DnaK and GroEL increased with a little lag, reached a maximum at about 30 to 60 minutes, and decreased gradually when azetidine was added in *E. coli* culture as a stress chemical. Therefore, the results that 4HBA at 0.5 to 5 mM concentration was not degraded by *Pseudomonas* sp. DJ-12 for the first 30 to 60 minutes incubation (Table 1) and that DnaK and GroEL proteins were produced during the same period in this study (Table 2) could be explained by adaptation mechanism required prior to growth on 4HBA as indicated by Vercellone-Smith and Herson (1997). The fact that the proteins were not detected in the cells treated with 0.5 to 6 mM 4HBA for 40 to 60 minutes (Table 2) could be due to that the cells were adapted to the stress and got ready for growth on 4HBA, which in turn, they did not need to produce the stress-shock proteins, as described by Kanemori *et al.* (16) and Vercellone-Smith and Herson (31).

Two-dimensional PAGE analysis of the proteins

The total proteins produced in *Pseudomonas* sp. DJ-12 which were treated with 5 mM 4HBA for 10 or 40 minutes were analyzed by two-dimensional PAGE as shown in Fig. 4. The cells treated with 4HBA for 10 min (Fig. 4B) and 40 min (Fig. 4C) exhibited fifteen stress-shock proteins which were newly produced. Three of those proteins were identified as DnaK, GroEL, and GroES proteins, respectively, as observed in Fig. 2 and 3 by the Western blot analysis. The pI values of the three proteins were 4.5, 4.7, and 4.8, respectively, which were identical to those of DnaK, GroEL, and GroES produced in *Lactococcus latics* by salt stress (17).

The stress-shock proteins induced by aromatic hydrocarbons have been analyzed by two-dimensional PAGE in the microorganisms which were treated with 2-chlorophenol (10, 22) and with 2-nitrophenol (12). Faber *et al.* (10) detected fifteen and twenty-seven proteins in *E. coli* treated with 10 mg/l pentachlorophenol and 200 mg/l monochlorophenol for 60 minutes, respectevely. Gage and Neidhardt (12) reported that 53 stress-shock proteins were produced in *E. coli* exposed to 0.5 mM 2,4-dinitrophenol (DNP). These proteins included all of the heat-shock proteins except LysU, HtpE, and HtpN. Twenty-four of the fifty-three proteins were not induced by any other stresses and thus were unique to the DNP stress. Lupi *et al.* (22) classified the stress-shock proteins into four categories by the changes in the protein concentration in *Pseudomonas putida* KT2442 during treatment with 2-chlorophenol: those proteins which increased continuously during exposure, those which decreased in concentration, those which showed a concentration peak at some point following exposure, and those which were essentially unaffected. Among the fifteen stress-shock proteins produced in *Pseudomo-*

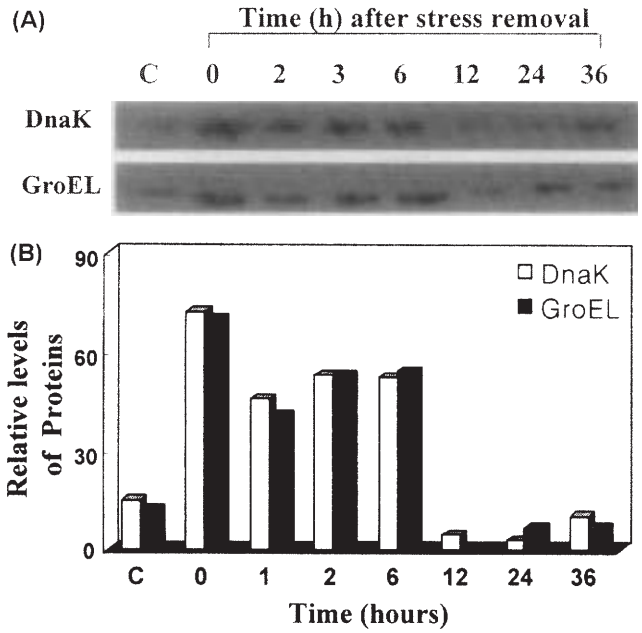

Fig. 5. Retaining period of the stress-shock proteins in *Pseudomonas* sp. DJ-12 cells induced by 5 mM 4HBA treatment for 10 min. A, Western blot of the proteins with anti-DnaK and anti-GroEL antibodies; B, densitometric analysis of the proteins.

nas sp. DJ-12 which was treated with 5 mM 4HBA in this study, thirteen belong to those which increase continuously and two (marked with arrow head) belong to those which show peak at some point during exposure.

Retaining period of the proteins in the cells

The *Pseudomonas* sp. DJ-12 cells treated with 5 mM 4HBA for 10 min were transferred to LB broth with no 4HBA and then placed at 30°C for over 36 hours to examine the retaining period of the stress-shock proteins in the cells induced by 4HBA. The presence of the stress-shock proteins were examined by Western blot and densitometric analysis as shown in Fig. 5. The DnaK and GroEL proteins gradually decreased upto 6 hours after the stress was removed from the culture, continued to decrease up until the lewel lower than the control after 12 hours. Streips and Polio (28) reported that the heat-shock proteins produced in Bacilli cells by exposure at 42°C were retained for 40 minutes after shifting to 37°C. This implies that the organisms adapted to the stress environment by producing some particular proteins could return to the normal physiological state when the stress is removed. However, the retaining period of the proteins in the cells may depend on the intensity of the stress and the exposure time to the stress.

Therefore, 4-hydroxybenzoate induced *Pseudomonas* sp. DJ-12 to produce at least fifteen stress-shock proteins as shown by two-dimensional PAGE technique. Among them, DnaK and GroEL were confirmed by Western blot analysis. The production of such stress-shock proteins need to be investigated further for their functions, besides survival tolerance, and for development of bacterial monitoring system for environmental pollutant chemicals.

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