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Cloning and Expression of *pcbC* and *pcbD* Genes Responsible for 2,3-Dihydroxybiphenyl Degradation from *Pseudomonas* sp. P20

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Pseudomonas sp. P20 was shown to be capable of degrading biphenyl and 4-chlorobiphenyl (4CB) to produce the corresponding benzoic acids which were not further degraded. But the potential of the strain for biodegradation of 4CB was shown to be excellent. The *pcbA, B, C* and *D* genes responsible for the aromatic ring-cleavage of biphenyl and 4CB degradation were cloned from the chromosomal DNA of the strain. In this study, the *pcbC* and *D* genes specifying degradation of 2,3-dihydroxybiphenyl (2,3-DHBP) produced from biphenyl by the *pcbAB*-encoded enzymes were cloned by using pBluescript SK(+) as a vector. From the pCK102 (9.3 kb) containing *pcbC* and *D* genes, pCK1022 inserted with a *Eco*RI-*Hin*dIII DNA fragment (4.1 kb) carrying *pcbC* and *D* and a pCK1092 inserted with *Eco*RI-*Xba*I fragment (1.95 kb) carrying *pcbC* were constructed. The expression of *pcbC* and *D* in *E. coli* CK102 and *pcbC* in *E. coli* CK1092 was examined by gas chromatography and UV-vis spectrophotometry. 2.3-dihydroxybiphenyl was readily degraded to produce meta-cleavage product (MCP) by *E. coli* CK102 after incubation for 10 min, and then only benzoic acid(BA) was detected in the 24-h old culture. The MCP was detected in *E. coli* CK1022 containing *pcbC* and *D* genes (by the resting cells assay) for up to 3 h after incubation and then diminished completely in 8 h, whereas the MCP accumulated in the *E. coli* CK1092 culture even after 6 h of incubation. The 2,3-DHBP dioxygenases (product of *pcbC* gene) produced by *E. coli* CK1, CK102, CK1023, and CK1092 strains were measured by native PAGE analysis to be about 250 kDa in molecular weight, which were about same as those of *Pseudomonas* sp. DJ-12, *P. pseudoalcaligenes* KF707, and *P. putida* OU83.

Aromatic hydrocarbons such as biphenyl, whether chlorinated or not, are a major group of chemical pollutants when found as contaminants in the environment. This is because of their recalcitrance and toxicity to wildlife and the humans (20). The benzene ring structures of biphenyl and chlorinated biphenyls are known as one of the main reasons why the chemicals are persistent. But many kinds of microorganisms have been found to degrade such recalcitrant chemicals, even though the extent of their degradation is lower and slower (11).

There have been two catabolic pathways reported in the upper steps of biodegradation by microorganisms: *meta*- and *ortho*-pathways (2, 3, 5, 10, 13). Four enzymes have been reported to be involved in the steps of aromatic ring-cleavage for degradation of biphenyl. The chemical is successively transformed to 2,3-dihydroxybiphenyl (2,3-DHBP) by biphenyl dioxygenase encoding by *bphA* and then dihydrodiol dehydrogenase encoding by *bphB*. The 2,3-dihydroxybiphenyl is continuously converted to *meta*-cleavage product (MCP) by 2,3-DHBP dioxygenase and then to benzoic acid(BA) by MCP hydrolase. The 2,3-DHBP dioxygenase and MCP hydrolase are reported to be encoded by *bphC* and *bphD* (*cbpC* and *D* in *Pseudomonas putida* OU83), respectively (1, 2, 6, 13, 21).

Pseudomonas sp. DJ-12 and P20 were the natural isolates as previously reported (11, 22). Both strains were capable of degrading biphenyl and 4-chlorobiphenyl (4 CB) through the *meta*-cleavage pathway and catabolizing the four enzymes encoded by *pcbA, B, C* and *D* genes. The gene clusters of *pcbA, B, C* and *D* in both strains were cloned in this laboratory and some characteristics of the genes have been studied (7, 12, 14, 15, 18). *Pseudomonas* sp. DJ-12 could continuously degrade 4-chlorobenzoic acid (4CBA) produced as the end product of the *pcbABCD*-encoded enzymes, whereas *Pseudomonas* sp. P20 could not degrade the end product

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accumulating in the culture and rendering toxicity to the organism. Nevertheless *Pseudomonas* sp. P20 did show much stronger activity for degradation of 4CB than *Pseudomonas* sp. DJ-12. The genetic characteristics of the *pcbA, B, C* and *D* genes in *Pseudomonas* sp. P20 have not been much studied.

In this study, therefore, the *pcbC* and *D* genes responsible for *meta*-cleavage of 4CB and biphenyl were cloned from *Pseudomonas* sp. P20, and the expression of the genes in *E. coli* was examined by UV-vis spectrophotometry and gas chromatography.

MATERIALS AND METHODS

Bacterial Strains, Chemicals, and Culture Condition

Pseudomonas sp. P20 is a bacterial isolate capable of degrading biphenyl and 4-chlorobiphenyl (4CB) as reported previously (22). The clones of pCK1 and pCK101 containing *pcbABCD* and *pcbCD* genes, respectively, were originally cloned from the chromosomal DNA of the strain (14). The *pcbCD* genes were further subcloned in pCK102 and pCK1022, and *pcbC* was in pCK1092. The sources and relevent characteristics of bacterial strains and plasmids used in this study are listed in Table 1. Recombinant plasmids of pCU1 (7), pAW313 (9), and pKTF20 (4) were used to compare the molecular weight of 2,3-DHBP dioxygenase in native PAGE. The chemicals used in this study were as follows: 2,3-dihydroxybiphenyl (Wako Chemical Ind. Ltd., Osaka, Japan) and benzoic acid (Sigma Chemical Co.). The recombinant strains were cultivated in a Luria-Bertani (LB) broth supplemented with ampicillin (100 μg/ml) and tetracycline (15 μg/ml).

DNA Manipulation

The recombinant plasmids were isolated by an alkaline lysis procedure described by Sambrook *et al.* (16). The enzymes of *Eco*RI, *Xho*I, *Sal*I, *Hin*dIII, *Sac*I, *Xba*I, and T4 DNA ligase were purchased from KOSCO Co. (Seoul) and used as instructed by the supplier. Electrophoresis was carried out in 0.8% agarose gel and the gel was reacted with 0.5 μg/ml ethidium bromide for 40 min and then photographed.

Cloning of 2,3-DHBP-degrading Genes

The plasmid DNA of pCK101 was completely digested with *Eco*RI and mixed with pBluescript SK(+) vector digested with the same enzyme at a ratio of 1 : 3 as seen in Fig. 1. The ligations were carried out by adding T4 ligase and incubating the mixture at 16℃ for 16 hrs. The recombinant plasmids were then transformed into *E. coli* XL1-Blue according to the procedures of Sambrook *et al.* (16). The subclones of pCK102, pCK1022, and pCK1092 were constructed in the same way with various endonucleases. The transformants were selected on the LB agar medium containing ampicillin (100 μg/ml) and tetracycline (15 μg/ml). The medium was added with 40 nl of X-gal (20 mg/ml) and 4 μl of isopropylthio-β-D-galactoside(IPTG; 200 mg/ml). The expression of *pcbC* and *D* genes in the recombinant strains was identified by spraying 0.1% of 2,3-dihydroxybiphenyl (2,3-DHBP) solution on the colonies. The colonies possessing *pcbC* gene turned yellow, resulting from conversion of 2,3-DHBP to the yellow-colored *meta*-cleavage product (MCP).

Table 1. Bacterial strains and plasmids used in this study

Strain and plasmid	Relevant characteristics	Reference or source
Strains		
Pseudomonas sp. P20	$4CB^+$, BP^+, $4CBA^-$, $2,4DCB^+$	21
Pseudomonas sp. DJ-12	$4CB^+$, BP^+, $4CBA^+$, $2,4DCB^+$	11
E. coli XL1-Blue	Host strain	Stratagene Ltd.
Plasmids		
pCK1	*pcbABCD*, Ap^r, 14 kb *Eco*RI fragment of Pseudomonas sp. P20 cloned in pBluescript SK(+)	14
pCK101	*pcbCD*, subclone containing 7.9 kb *Eco*RI fragment of pCK1	14
pCK102	*pcbCD*, subclone containing 6.3 kb *Eco*RI fragment of pCK101	This study
pCK1022	*pcbCD*, subclone containing 4.2 kb *Eco*RI-*Hin*dIII fragment of pCK102	"
pCK1092	*pcbC*, subclone containing 1.95 kb *Eco*RI-*Xba*I fragment of pCK1022	"
pCU1	*pcbC*, 6.4 kb *Eco*RI fragment of *Pseudomonas* sp. DJ-12	7
pKTF20	*bphABC*, 7.2 kb *Xho*I fragment of *P. pseudoalcaligenes* KF707	4
pAW313	*cbpC*, 2.1 kb *Eco*RI fragment of *P. putida* OU83	9
pBluescript SK(+)	Cloning vector, AP^r	Stratagene Ltd.

Abbreviations: 4CB, 4-chlorobiphenyl; BP, biphenyl, 4-chlorobenzoic acid; 2,4DCB, 2,4-dichlorobiphenyl.

Analysis of Metabolites

Expression of *pcbC* and *D* was analyzed basically by resting cells assay using UV-vis spectrophotometry and gas chromatography. The cells containing *pcbC* and *D* or *pcbC* gene were incubated to be 10^9 cells/ml in the LB broth and reacted in the MM2 broth containing 2 mM 2,3-DHBP at 37℃. After removal of the cells from the broth after reaction for the appropriate time, the metabolites were scanned with a UV-vis spectrophotometer.

For gas chromatography of the metabolites, culture

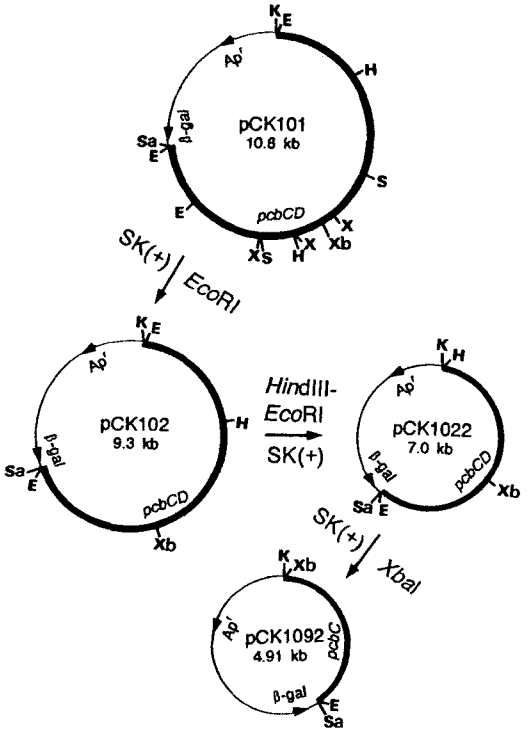

Fig. 1. Contruction scheme of recombinant plasmids containing *pcb* genes.
Abbreviation: Sa, *Sac*I; E, *Eco*RI; X, *Xho*I; S, *Sal*I; H, *Hin*dIII; Xb, *Xba*I, K, *Kpn*I; SK(+), pBluescript SK(+) vector

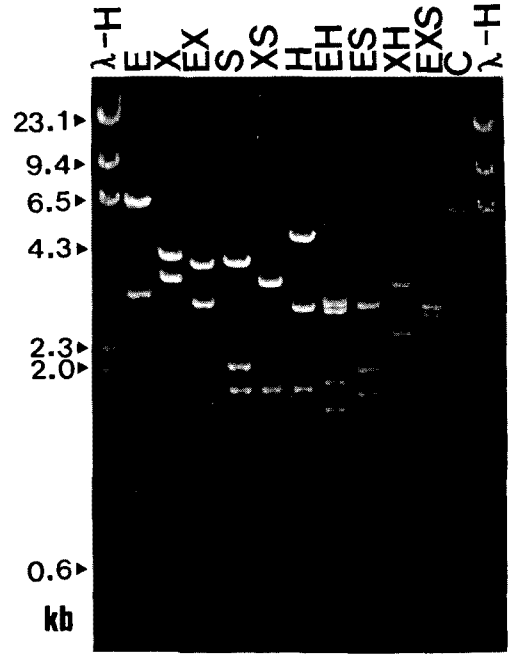

Fig. 2. Digestion profiles of recombinant plasmid pCK102 by various endonucleases.
Abbreviations: λ-H, λ-*Hin*dIII size marker; E, *Eco*RI; X, *Xho*I; S, *Sal*I; H, *Hin*dIII; C, *Cla*I.

solutions were acidified with H_2SO_4 and extracted with diethyl ether of the same volume. Analyses were carried out on a silicone OV17 (3% on chromsorb W, mesh 80~100). The nitrogen gas was injected at 45 ml/min and hydrogen gas was injected at 35 ml/min. The temperature program was 1.5 min at 150℃, followed by a ramp rate of 10℃/min to 250℃. Detection of the metabolites was performed with a flame ionization detector.

Polyacrylamide Gel Electrophoresis of Proteins

All procedures for preparation of crude cell extracts were performed at 4℃. *Pseudomoans* sp. P20 and *E. coli* strains harboring pCK1, pCK102, pCU1, pKTF20, and pAW313 were grown in LB broth for 16 hours and harvested by centrifugation at 25,000 ×g. The suspended cells in the 10 mM phosphate buffer (pH 7.0) were broken by sonication and centrifuged at 25,000 ×g for 30 min. The supernatants were loaded onto 10% non-denatured polyacrylamide gel. Gel electrophoresis was performed at 30 mA for 5 hour according to the methods of Silhavy *et al.* (19). The gel was sprayed with 0.1% of 2,3-DHBP to produce MCP by the 2,3-DHBP dioxygenase as yellow coloring on the band of the enzyme. And then the proteins were visualized by staining the gel with Coomassie brilliant blue R-250.

RESULTS AND DISCUSSION

Cloning of *pcbC* and *D* Genes

The hybrid plasmid of pCK102 (9.3 kb) was cloned from the recombinant plasmid of pCK101 by using pBluescript SK(+) as a vector, and then pCK1022 (7.0 kb) and pCK1092 (4.91 kb) were further subcloned as seen in Fig. 1. The pCK102 contained an *Eco*RI DNA fragment of 6.3 kb carrying *pcbCD* genes. The pCK1022 and pCK1092 were inserted with the *Eco*RI-*Hin*dIII fragment (4.1 kb) harboring *pcb*C and *D* and a *Eco*RI-*Xba*I fragment (1.95 kb) carrying *pcb*C, respectively. Digestion profiles of pCK1022 and pCK1092 by several endonucleases are shown in Fig. 2 and Fig. 3. The pCK1092 had two restriction sites for *Sac*I and one site each for *Hin*dIII, *Sal*I, and *Pst*I endonucleases.

The generalized physical maps of pCK1 and the subclones, and expression of *pcbA, B, C* and *D* genes are shown in Fig. 4. The *pcbA, B, C* and *D* genes were determined to be organized in the order of *ABCD*. Among *Pseudomonas* strains degrading biphenyl and 4-chlorobiphenyl, the *bphABC* genes were separated from *bphD* gene in *P. pseudoalcaligenes* KF707 (5) and *Pseudomonas* sp. LB400 (8). The *bphK, H, J* and *I* genes were placed between them in *Pseudomonas* sp. LB400, which were determined to be involved in degradation of the aliphatic compounds produced by *bphABCD*-

encoded enzymes. But pAW6194 of *P. putida* OU83 was reported to have gene structures of *cbpA, X, D, C* and *B*, the X of which was not identified for its function (9).

But the gene order of *pcbA, B, C* and *D* in *Pseudomonas* sp. P20 (15) was found to be similar to those in *Pseudomonas* sp. DJ-12 (7) and was capable of degrading BA and 4CBA while *Pseudomonas* sp. KKS102 (10, 23) was not capable of degrading BA and 4CBA.

Expression of *pcbC* and D in *E. coli*

Expression of *pcbC* and *D* genes of pCK102 in *E. coli* XL1-Blue was examined by gas chromatography with the metabolites extracted from the cultures containing 2 mM 2,3-dihydroxybiphenyl after incubation for 10 minutes and 24 hours. The 2,3-DHBP and meta-cleavage product (MCP) were detected in the 10 min old culture as seen in Fig. 5A. On the other hand, those substances were remarkably reduced in the 24 h culture although benzoic acid was increased (Fig. 5B). These results indicated that the *pcbC* and *D* genes in pCK102 cloned from *Pseudomonas* sp. P20 were well expressed in *E. coli* XL1-Blue.

The *pcbC* and *D* genes in *E. coli* CK1022 and the *pcbC* gene in *E. coli* CK1092 were tested for their expression by the resting cell assay method for degradation of 2,3-DHBP and MCP. In the case of *E. coli* CK1022 including *pcbC* and *D* genes (Fig. 6), the MCP produced from 2,3-DHBP by the dioxygenase encoding by the

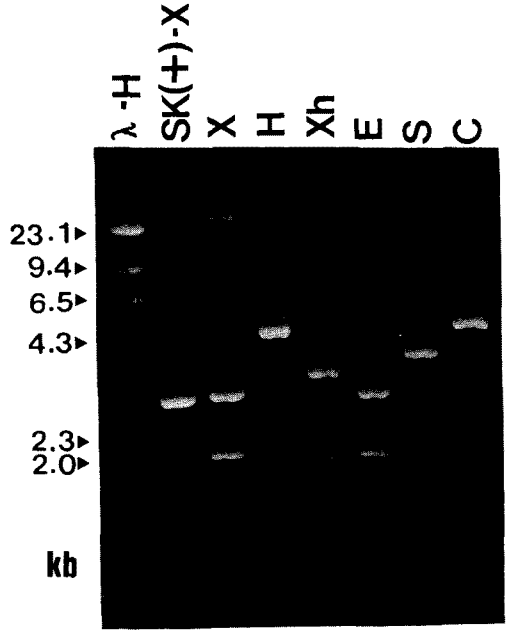

Fig. 3. Digestion profiles of recombinant plasmid pCK1092 by various endonucleases.
Abbreviations: λ-H, λ-*Hin*dIII size marker; X, *Xba*I; H, *Hin*dIII; Xh, *Xho*I; E, *Eco*RI; S, *Sal*I; C, *Cla*I.

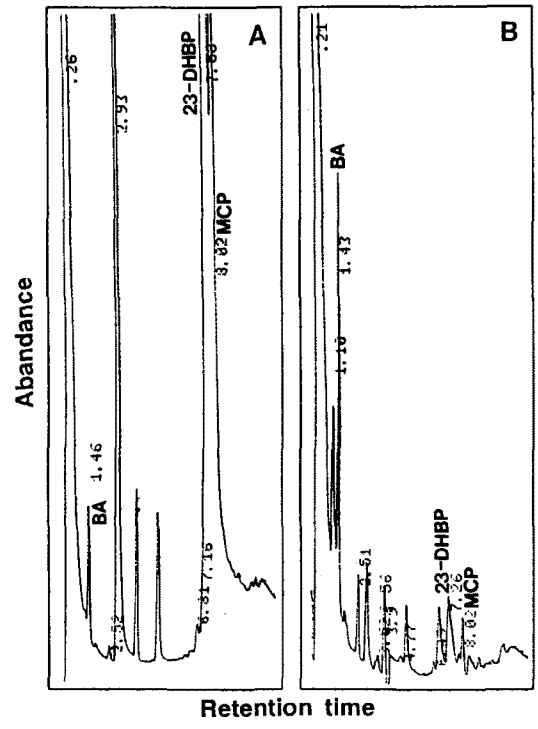

Fig. 5. GC spectra of the metabolites produced from 2,3-dihydroxybiphenyl by E. coli CK102.
Panels show the metabolite profile extracted from the cultures incubated for 10 min (A) and 24 h (B) culture. Abbreviation: 2,3-DHBP, 2,3-dihydroxybiphenyl; B, MCP, *meta*-cleavage compound (2-hydroxy-penta-2,4-dienoic acid); BA, benzoate

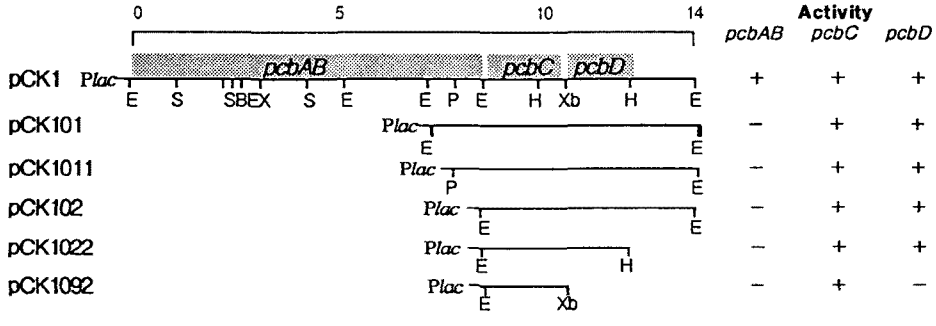

Fig. 4. Physical maps of the recombinant plasmid pCK1 and its subclones.
Abbreviations: E, *Eco*RI; S, *Sal*I; B, *Bst*EII; X, *Xho*I, H, *Hin*dIII; Xb, *Xba*I; pB-SK, pBluescript SK(+) vector; +, express; +, not express.

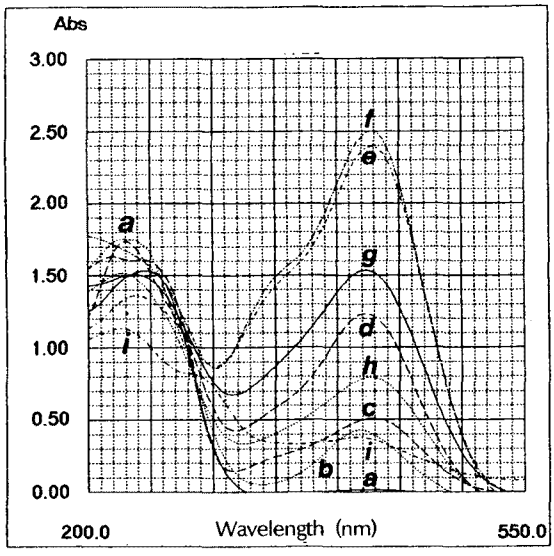

Fig. 6. Degradation of 2,3-dihydroxybiphenyl by *E. coli* CK-1022.

The resting cell assay was conducted in 10 mM phosphate buffer containing 2 mM 2,3-DHBP. The symbols indicates reaction times: a, 5 min; b, 10 min; c, 20 min; d, 30 min; e, 1.5 h; f, 3 h; g, 4.5 h; h, 6 h; i, 8 h.

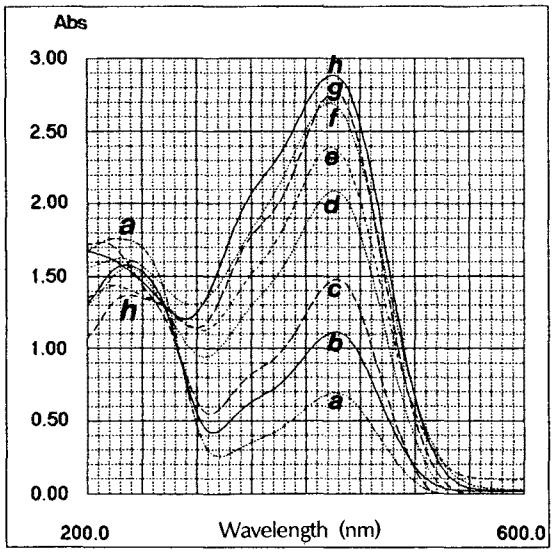

Fig. 7. Degradation of 2,3-dihydroxybiphenyl by *E. coli* CK-1092.

The resting cell assay was conducted in 10 mM phosphate buffer containing 2 mM 2,3-DHBP. The symbols indicates reaction times: a, 5 min; b, 10 min; c, 20 min; d, 30 min; e, 1.5 h; f, 3 h; g, 4.5 h; h, 6 h.

pcbC gene was detected at 434 nm for the first three hours of reaction, and then completely diminished by action of MCP hydrolase encoded by *pcbD* gene. This indicated clearly that the *pcbC* and *D* genes in *E. coli* CK1022 were excellently expressed and specified degradation of 2,3-DHBP. But the MCP produced from 2,3-DHBP by *E. coli* CK1092 possessing only *pcbC* gene was accumulated in the culture as shown in Fig. 7 and not decreased even after 6-hour reaction.

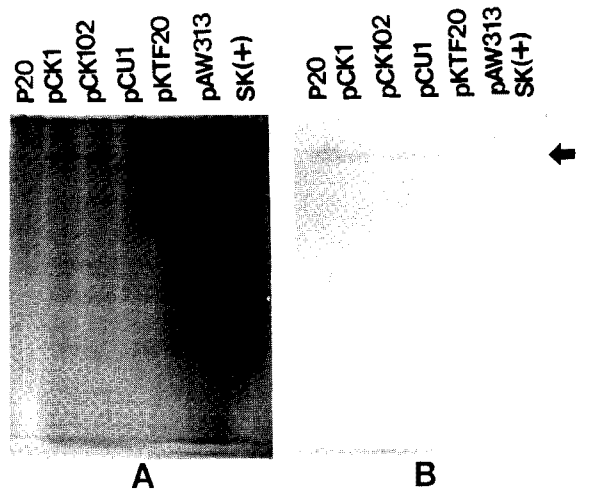

Fig. 8. Native-PAGE of 23-DHBP dioxygenase.

A, Gel electrophoresis of the proteins stained with Coomassie blue. B, The gel reacted with 0.1% solution of 2,3-DHBP in 50 mM Tris hydrochloride-10% acetone buffer, pH 7.5. The arrow indicates the position of the protein bands showing 2,3-DHBP dioxygenase activity.

2,3-DHBP Dioxygenase

The proteins produced in *E. coli* CK1, CK102, and the natural isolate of *Pseudomonas* sp. P20 were analyzed in SDS-PAGE. Their 2,3-DHBP dioxygenases were compared with those produced in *Pseudomonas* sp. DJ-12 (18), *P. pseudoalcaligenes* KF707 (4), *P. putida* OU83 (9) as seen in Fig. 8A. When the native PAGE was treated with 1% 2,3-DHBP solution, the yellow-colored bands (arrow) were detected to be the MCP which was converted from 2,3-DHBP by the dioxygenases produced by all the strains as shown in Fig. 8B.

The 2,3-DHBP dioxygenases produced by *Pseudomonas* sp. P20 and its cloned cells of *E. coli* CK102 were determined to be about 250 kDa in molecular weight. The molecular mass was about the same as those reported in *P. pseudoalcaligenes* KF707 (21), *P. putida* OU83 (9) and *Pseudomonas* sp. DJ-12 (18).

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