J. of Basic Sciences, Cheju Nat'l Univ.
15(1), 45~54, 2002

기초과학연구 제주대학교
15(1), 45~54, 2002

A Proteomic Study on Catabolism of *p*-Hydroxybenzoate by *Acinetobacter lwoffii* K24 Capable of Aniline Degradation

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Acinetobacter lwoffii K24 capable of aniline degradation has been also found to utilize *p*-hydroxybenzoate as a sole carbon source. In this study, 2-DE using Q-sepharose column separation was attempted for fast screening of protocatechuate 3,4-dioxygenase for catabolism of *p*-hydroxybenzoate in *A. lwoffii* K24. Two protocatechuate 3,4-dioxygenase subunits, *pcaG* and *pcaH* were detected and identified with N-terminal and internal sequencing, suggesting proteomics using a column separation may be helpful for the identification of specific protein spots and maximizing the detectable protein spots on the 2-DE gel. The PCR process using degenerate primers for protocatechuate 3,4-dioxygenase and sequence analyses of the PCR products revealed the existence of *pcaH* and *pcaG* in *A. lwoffii* K24. These two subunits were found to be closely located and share extensive homology with *pcaHG* of *Pseudomonas marginata* or *Pseudomonas cepacia*, providing the evidence that *A. lwoffi* K24 has the protocatechuate branches as well as catechol branches of β -ketoadipate pathway.

INTRODUCTION

Acinetobacter lwoffii K24 capable of degrading aniline has been reported to produce two novel type I catechol 1,2-dioxygenases, CD I1 and CD I2 in response to aniline. These enzymes cleaves catechol into *cis,cis*-muconate, which is sequentially further metabolized *via* β -ketoadipate pathway (1-3). Notably, it has been also found in this study that protocatechuate can be catabolized by *A. lwoffii* K24 using β -ketoadipate pathway when grown in *p*-hydroxybenzoate.

Protocatechuate was converted into β -ketoadipyl coenzyme A by protocatechuate 3,4-dioxygenase (3,4-PCD). Our recent study reported that aniline-induced proteins in *A. lwoffii* K24 have been efficiently detected and characterized by proteomics using 2-DE (4). Despite useful analysis of proteins by 2-DE, it often can be limited by both the

number and type of proteins that can be resolved. In a crude cell extract, the most abundant proteins can dominate the gels, making the detection of low copy proteins difficult. In our efforts to overcome this problem, 2-DE analysis using a column separation has been firstly attempted to identify 3,4-PCD of *A. lwoffii* K24 in this study. Two subunits (*pcaHG*) of 3,4-PCD in *A. lwoffii* K24 have been also characterized.

MATERIALS AND METHODS

Bacterial strain and cultivation condition

A. lwoffii K24 was pre-cultured in the potassium phosphate buffer (pH 6.25) conta -ining 3.4 mM $MgSO_4$, 0.3 mM $FeSO_4$, 0.2 mM $CaCO_3$, 10 mM NH_4Cl and 10 mM sodium succinate as previously described and cultured in the *p*-hydroxybenzoate (10 mM) and the succinate medium for proteome analysis (4).

Purification steps and sample preparation

Harvested cells were suspended in 20mM Tris-HCl buffer (pH 8.0) and disrupted by a French pressure cell (SLM AMINCO, Urbana, IL, USA) at 20,000 lb/in2. The supernatant (crude cell extract) collected by centrifugation at 15,000 x g for 45min was used for 30 to 55% ammonium sulfate precipitation and dialyzed against Tris-HCl buffer (50mM, pH 7.0). The enzyme solution was applied to a Q-sephrose (HiLoad 16/10) column and was eluted with a 100 to 500 mM NaCl gradient at a flow rate 2ml/min for 40 min. The active fractions were pooled, dialyzed against MillQ water and used for 2D-PAGE. 3,4-PCD activity was measured spectrophotometrically by the procedure of Bull & Ballou (5) using Tris-HCl buffer (50mM, pH 7.0). One unit of enzyme activity is defined as the amount of enzyme that produces 1 mmol of β -carboxymuconate per min. The protein content were determined by the method of Bradford (6).

2-D PAGE

For isoelectric focusing(IEF) dried samples (about 250 mg) were resolved in 50ml Buffer I (SDS 0.3%, DTT 0.2M, Tris-HCl 50mM) and were heated at 95℃ for 5 min. After incubation with 5 ml Buffer II ($MgCl_2$ 50 mM, DNase I 10 unit, RNase 3.75 unit, Tris-HCl 50 mM) on ice for 10 min, samples were resolved in 350ml IPG buffer (Urea 8 M, CHAPS 2% (W/V), 0.5% IPG buffer). The protein sample solution was applied on immobilized pH3-10 nonlinear gradient strips using IPGphor (Pharmacia). Focusing was performed with 3 steps (500V for 1hr, 1000V for 1hr and 8000V for 8hr). The second dimension was run on a 12% polyacrylamide SDS gel using PROTEAN II xi electrophoresis kit (BIO-RAD, Hercules, CA, UAS). Silver staining was carried out by the method of Heukeshoven and Dernick (7) using Silver Staining Kit of Pharmacia Biotech.

In-gel digestion

The stained protein spots were excised from the gel and digested with trypsin. After washing with 10 mM ammonium bicar -bonate and 50% acetonitrile, gel pieces were swollen in digestion buffer containing

50 mM ammonium bicarbonate, 5 mM $CaCl_2$ and 12.5 ng/L trypsin, and incubated at 37°C for 12 to 16 hr. The peptides were recovered by two step extractions with 50 mM ammonium bicarbonate and 100% acetonitrile. The resulting peptide extracts were pooled and lyophilized in a vacuum centrifuge and stored at -20°C.

Internal sequencing by ESI-Q TOF mass spectrometry and analysis

All MS/MS experiments for internal sequencing were performed on a Q-TOF2 mass spectrometer (Micromass, U.K) equipped with a nano-ESI source and coated glass capillary (Protana, Denmark). After desalting by Zip-Tips (Millipore), approximately 5L of the sample was placed into a glass capillary nanospray needle. The applied voltage to the union to produce an electrospray was 1500 eV, and cone voltage was 30 eV. Collision energy was increased to 25-40 eV from 10 eV for CID experiments to obtain fragment ions. Argon was introduced as a collision gas at a pressure of 10 psi. MS/MS spectra were analyzed using the MS-Tag program (htt://prospector.ucsf.edu/) against the NCBInr database.

N-terminal protein sequencing

Protein spots on the 2-D gel were transferred onto a PVDF membrane and stained with a Coomassie Brilliant Blue R250 as described before (4). Protein sequencing was performed using the Edman degradation method in Applied Biosystems (ABI) model 491A protein sequencer. The obtained N-terminal sequences were analyzed for protein identification by BLAST search of NCBI.

PCR amplification of the protocatechuate 3,4-dioxygenase

Degenerate primers for *pcaH* and *pcaG* were synthesized using N-terminal & internal amino acid sequences (Table 3). Two oligonucleotides (forward and reverse primers) were designed from PcaH and PcaG, respectively. PCR reactions were performed for 25 cycles of 1min at 95℃, 1min at 50℃ and 1 min at 72℃. PCR products were purified on the 1% agarose gel for sequencing.

DNA sequencing and analysis

The DNA sequences of about 1.1-kb PCR product containing *pcaHG* genes were determined by *Taq* cycle sequencing using the DyeDeoxy Terminator method (Applied Biosystems Inc.). Sequencing reactions were prepared according to the supplier's instructions and analyzed by electrophoresis using the Perkin Elmer Model 377 DNA sequencer. DNA analysis and dendrogram analysis were done using Mac DNASIS of Hitachi Software and CLUSTAL W (1.82).

RESULTS AND DISCUSSION

Enzyme activity assay of Acinetobacter lwoffii K24

A. lwoffii K24 was found to use the *p*-hydroxybenzoate as a sole carbon source and grow up to O.D. 1.0 for 48 hr in our culture condition (Data not shown). The activity of protocatechuate 3,4-dioxygenase (3,4-PCD) was detected in a crude extract

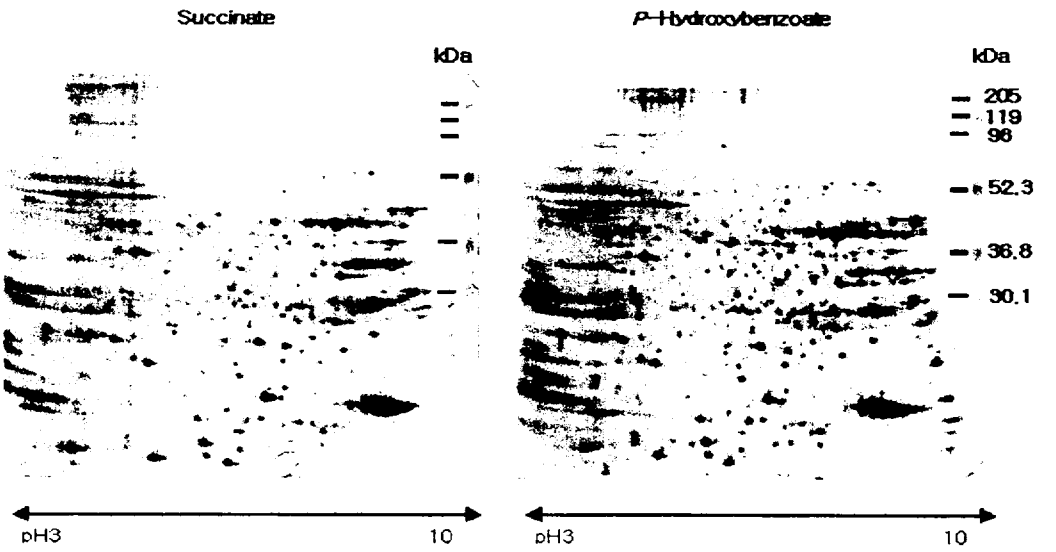

FIG.1. Two-dimensional gel of the total soluble protein extract of A. *lwoffii* K24 cultured in succinate medium (A) and *p*-hydroxybenzoate medium (B). The protein extracts were separated on pH3-10 nonlinear IPG strips, followed by 12% SDSpolyacryl amide gel.

of cells grown in *p*-hydroxybenzoate , but no catechol 1,2-dioxygenase (1,2-CD) activity was measured. Our previous study demonstrated that the crude extract from K24 cells grown on aniline showed only 1,2-CD activity (1). These findings suggested that the two branches (catechol and protocatechuate) of β -ketoadipate pathway in strain K24 might be stringently controlled by different inducers.

2-D PAGE analysis of crude proteins from Acinetobacter lwoffii K24

To screen and detect the proteins induced by *p*-hydroxybenzoate, 2-DE of crude cell extracts of *A. lwoffii* K24 grown on the medium containing *p*-hydroxybenzoate was compared with that of crude extracts of cells grown in succinate (Fig. 1).

Our previous study reported that the proteome analysis using 2-DE of crude extract was efficiently performed to screen and detect proteins induced by aniline (4). However, 2-DE analysis of crude extract induced by *p*-hydroxybenzoate was found ineffective for screening proteins for *p*-hydroxygenzoate catabolism because hundreds of protein spot displayed on the 2-D gel of crude extract made the detection of specific proteins induced by *p*-hydroxybenzoate difficult as shown in Fig. 1. Even though many protein spots were observed to be highly expressed by *p*-hydroxybenzoate, more elaborate procedure was needed to pick up the spots which were expected enzymes for catabolism of *p*-hydroxybenzoate. In general, when a total-cell lysate is used 2-DE analysis potential can be limited by both the number and type of proteins that can be resolved.

Therefore, cell crude extracts from *p*-hydroxybenzoate were applied to the Q-sepharose column after ammonium precipitation to reduce the complexity of the total protein mixture as well as to collect and concentrate the cell fraction exhibiting 3,4-PCD activity.

Screening of protocatechuate 3,4-dioxygenase by enrichment using Q-sepharose Chromatography

The active fractions that were found to have 3,4-PCD activity by spectrometric analysis were collected through Q-sepharose column. Table 1 shows that the fractions obtained from Q-sepharose column chromatography demonstrated 5-fold higher specific activity of 3,4-PCD than that of crude extract.

The protein expression profiles were shown by 2-DE of Q-sepharose fractions of *A. lwoffii* K24 cultured in succinate and *p*-hydroxybenzoate media (Fig. 2).

The 2-D gel was electorblotted on PVDF

Table 1. Partial purification of protocatechuate 3,4-dioxygenase for catabolism of *p*-hydroxybenzoate in *A. lwoffii* K24

Step	Total activity (Unit)	Specific activity (Unit/mg)	Total protein (mg)	Yield (%)	Purification (fold)
Crude extract	276.1	1.07	258	100	1
Ammonium precipitation (30-55%)	177.1	1.64	108	64.1	1.53
Q-sepharose	153.8	5.23	29.4	55.7	4.89

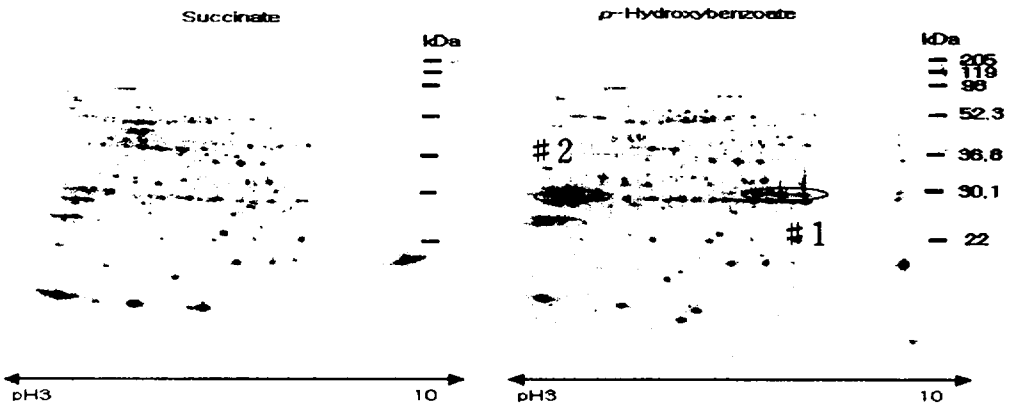

FIG. 2. Two-dimensional gel of Q-sepharose fractions of A. *lwoffii* K24 cultured in succinate medium (A) and *p*-hydroxybenzoate medium (B). Q-sepharose fractions of *p*-hydroxybenzoate-grown cells were selected by protocatechuate 3,4-dioxygenase activity assay and pooled. The Q-sepharose fractions of cells grown in succinate medium were used as control for comparative proteomic analysis. Putative two subunits of 3,4-PCD were designated by circles (#1, #2).

membrane, and differently expressed proteins marked in circle were excised from the membrane and applied to N-teminal amino acid sequencing. Sequence analysis of N-teminal amino acids revealed *pcaH* and *pcaG* of 3,4-PCD for the conversion of protocatechuate to bketoadipyl coenzyme A in *A. lwoffii* K24 (Table 2).

The three protein spots in each circle were found to have the identical N-terminal sequences, as similarly in proteomic analysis of aniline-induced proteins in *A. lwoffii* K24 (4). In gel digestion for the 2D gel of the identical protein spots was carried out for internal sequencing, followed by MS/MS analysis by ESI-Q TOF mass spectrometry (Table 3). Sequencing results confirmed that 3,4-PCD could be induced and expressed by *p*-hydroxy benzoate (Data not shown).

The PCR process using the primers, Oligo#181 and Oligo#183 resulted in the production of approximately 450-bp DNA fragment which was similar in size with *pcaG* (Fig. 3). When Oligo#178 and Oligo#183 were used, hydroxybenzoate.

Comparative analysis of 2-DE images between total protein extract and Q-sepharose fraction showed that several other proteins were enriched in Q-sepharose fraction (Fig. 2) and sample preparation using a column separation was useful for the detection and identification of low copy proteins. Therefore, further 2-DE analysis for the other Q-sepharose fractions has been being performed to screen the other enzymes involved in *p*-hydroxybenzoate catabolism.

Table 2. Identification of the protocatechuate 3,4-dioxygenase in *A. lwoffii* K24 by N-terminal and internal sequencing

Circle No.	Mr (kDa)	Amino acid sequences	Homologous proteins	Identity (%)	Accession No.
1	26	GTEDLGALDHDLTR (In1)	PcaH (β -subunit)	93	AAC99961
		GYDFDIVLR (In)	PcaH (β -subunit)	89	AAC99961
		MDESFLTKRDFASHPAYVY(N2)	PcaH (β -subunit)	63	AAC99961
2	22	PDEAPHLNV (In)	PcaG (α -subunit)	75	AE004453
		FEDEAEAN (In)	PcaG (α -subunit)	88	D35119
		TTLKQTPSQTVGPYFAYGLC	(N)PcaG (α -subunit)	100	AAA25925

[1]In indicates internal amino acid sequence; [2]N, N-terminal amino acid sequences

Characterization of pcaH and pcaG

PCR products of genes specifying *pcaH* and *pcaG* of 3,4-PCD in *A. lwoffii* K24 were obtained using two sets of degenerate primers synthesized for the identification *pcaH*, *pcaG*, or *pcaHG*. Approximately 650-bp PCR product that was assumed pcaH was obtained through PCR process using the primers, Oligo#178 and Oligo#180 as expected approximately 1100-bp PCR product was obtained as expected. The DNA sequence of approximately 1100-bp PCR

Table 3. Degenerate PCR primers for amplification of *pcaH* and *pcaG*

Primer	Amino acid sequences	Deduced primer sequences
PcaH		
Oligo#178	MDESFLTK	3-ATG GAY GAR TCN TTY CTN CAN AA-5
Oligo#180	GYDFDIV (reverse)	3-ACN AGR TCR AAR TCR TAN CC-5
PcaG		
Oligo#181	MTTLKQTF	3-ATG ACN ACN CTN AAR CAR CAN CC-5
Oligo#183	YFEDEAEA (reverse)	3-GCY TCN GCY TCR TCY TCR AAR TA-5

product was determined, which contain *pcaH* and C-terminal truncated *pcaG*. These sequencing results provided the evidence that *pcaG* encoding α -subunit 3,4-PCD might be located proximally to *pcaH* encoding β -subunit (Fig. 3), suggesting the arrangement of 3,4-PCD genes in strain K24 might be similar with that found in many other bacteria (8, 9-13). The deduced dendrogram revealed that *pcaH* and *pcaG* from *A. lwoffii* K24 was closest to those found in *Pseudomonas marginata* (84% and 67%) and *Pseudomonas cepacia* (84 and 72%) (Fig. 4). Unexpectedly, each subunit of 3,4-DCP in *A. lwoffii* K24 shared low homology (approx 30%) with other known 3,4-DCP from the same *genus Acinetobacter calcoaceticus* (14) or *Pseudomonas putida*

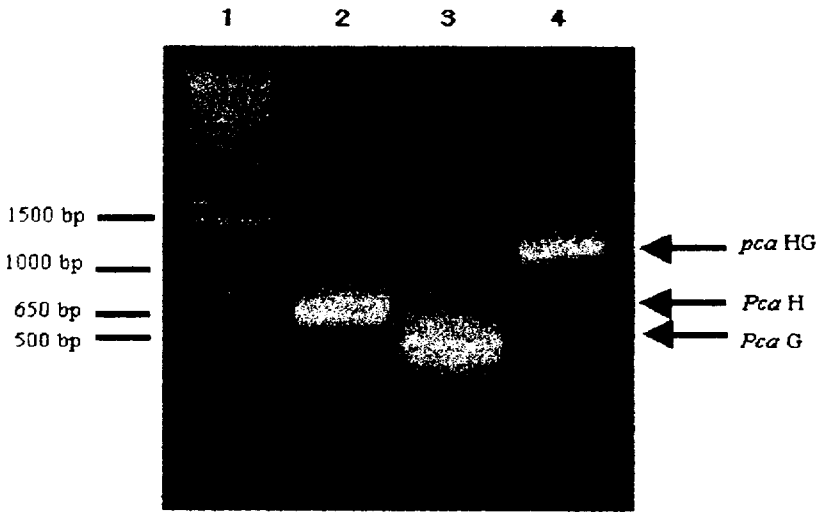

FIG. 3. PCR products of genes specifying *pcaH* and *pcaG*. Degenerate primers were used for PCR. Lanes: 1, molecular size marker; 2, *pcaH* PCR product obtained with primers Oligo#178 and Oligo#180; 3, *pcaG* PCR product obtained with primers Oligo#181 and Oligo#183; 4, *pcaHG* PCR product obtained with primers Oligo#178 and Oligo#183.

(15). Gene organization and convergence point of *pca* branches of β-ketoadipate were known to be very diverse in different bacteria (8, 9). We expect that *pcaHG* identified in this study will be used as a probe in the screening of other pca genes for the understanding of β-ketoadipate pathway in *A. lwoffii* K24. In conclusion, *A. lwoffii* K24 has been proved to have the protocatechuate branches as well as catechol branches of β-ketoadipate pathway and proteomic approach combined with column purification was very useful for screening the proteins for biodegradation in soil bacteria.

ACKNOWLEDGEMENT

This work was supported by Korea Research Foundation Grant (KRF-2001-050-D00033).

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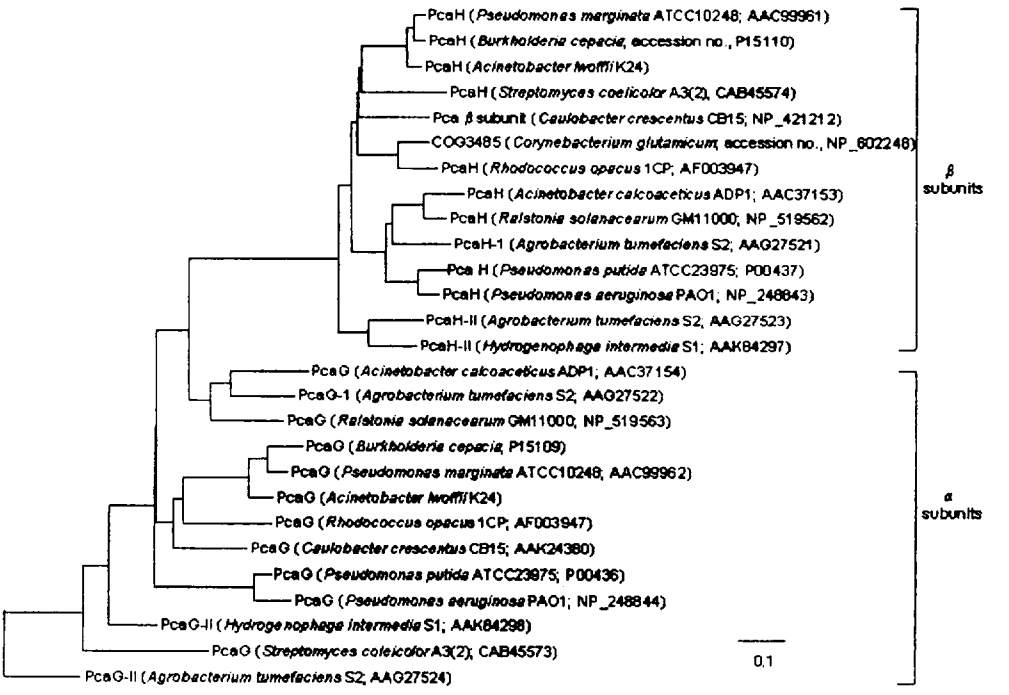

FIG. 4. Dendrogram showing the phylogenetic relationship for α and β subunits of protocatechuate 3,4-dioxygenase from *A. lwoffii* K24 and other bacteria. The dendrograms were constructed with the CLUSTAL method based on deduced amino acid sequences. The bar scale represents 10 nucleotide substitution per 100 nucleotides. The accession numbers for the published PcaG and PcaH sequences are within brackets.

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아닐린을 분해할 수 있는 *Acinetobacter lwoffii* K24에 의한 p-Hydroxybenzoate의 분해에 관련된 프로테옴 연구

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아닐린을 분해할 수 있는 *Acinetobacter lwoffii* K24는 단일 탄소원으로 *p*-hydroxybenzoate를 이용할 수 있는 것으로 밝혀졌다. 본 연구에서는 *A. lwoffii* K24에 있는 *p*-hydroxybenzaote의 분해에 관련된 protocatechuate 3,4-dioxygenase의 빠른 탐색을 위하여 Q-sepharose column을 이용한 2-DE가 처음 시도되었다. N-terminal 아미노산 분석과 내부염기서열 분석에 의하여 두 개의 protocatechuate 3,4-dioxygenase 소단위 *pcaG*와 *pcaH*가 탐지되고 동정되어, column분리를 이용한 프로테옴분석이 특정 단백질을 동정하고 2-DE 겔 상에서 탐지할 수 있는 단백질의 수를 최대한도로 확장하는 데 있어 상당한 도움이 될 것으로 제시되었다. Protocatechuate 3,4-dioxygenase에 대한 degenerate primer를 이용한 PCR과 얻어진 PCR 산물에 대한 염기서열 분석을 통하여 *A. lwoffii* K24에 *pcaH*와 *pcaG*가 존재하고 있음이 규명되었다. 이들 두 개 소단위는 서로 이웃해 위치해 있었으며 *Pseudomonas marginata* 또는 *Pseudomonas cepacia*의 *pcaHG*와 상당한 상동성을 갖고 있었다. 위의 결과들은 *A. lwoffii* K24가 β -ketopathway를 운용하는 데 있어 catechol 가지뿐만 아니라 protocatechuate 가지를 갖고 있다는 증거를 제시해 주었다.