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Proteome Analysis of Aniline-Induced Proteins in *Acinetobacter lwoffii* K24

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Abstract. *Acinetobacter lwoffii* K24 is a soil bacterium that can use aniline as a sole carbon and nitrogen source (by β-ketoadipate pathway genes (*cat* genes)) and has two copies of *catABC* gene separately located on the chromosome. In order to identify aniline-induced proteins, two-dimensional electrophoresis (2-DE) was applied to soluble protein fractions of *A. lwoffii* K24 cultured in aniline and succinate media. In the range of pH3–10, more than 370 spots were detected on the silver stained gels. Interestingly, more than 20 spots were selectively induced on aniline-cultured bacteria. Twenty-three protein spots of *A. lwoffii* K24 were analyzed by N-terminal microsequencing and internal microsequencing with in-gel digestion. Of 20 aniline induced protein spots, we identified six β-ketoadipate pathway genes, one subunit of amino group transfer (putative subunit of aniline oxygenase), malate dehydrogenase, putative ABC transporter, putative hydrolase, HHDD isomerase, and five unknown proteins. Especially in case of two catechol 1,2-dioxygenases (CDI_1 and CDI_2), more than three isotypes were detected on the 2D gel. This study showed that the proteome analysis of *A. lwoffii* K24 may be helpful for identification of genes induced by aniline and understanding of their function in the cell.

Aniline is a major industrial chemical and intermediate that is used in the manufacture of pesticides, dyes, plastics, and pharmaceuticals [10]. It has been reported to be degraded to catechol, which in turn is subject to *ortho*-(β-ketoadipate) pathway or *meta* pathway [13]. However, Aoki has reported that only 8% of aniline-assimilating bacteria metabolized aniline through the *meta* pathway and the rest used *ortho*-(β-ketoadipate) pathway [13]. The β-ketoadipate pathway genes (*cat* genes) are widely distributed genes for degradation of various aromatic compounds in soil bacteria. However, the complete β-ketoadipate pathway genes were reported only in *Acinetobacter calcoaceticus* and *Pseudomonas putida*, and functions of transport systems of β-ketoadipate pathway regulon were little known [4]. Even though the *cat* genes are highly conserved, their organization, regulation, and enzyme distribution (isozymes, points of branch convergence) are diverse in different bacterial groups [9].

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Acinetobacter lwoffii K24 is a non-motile soil bacterium that can aerobically grow on minimal media containing aniline as a sole nitrogen and carbon source [7]. *A. lwoffii* K24 was found to have two gene clusters of β-ketoadipate pathway and induce two different *catABC* genes under aniline media (Fig. 1) [8]. However, the regulation of two *cat* genes in *A. lwoffii* K24 was not clearly understood.

Recently, proteome analysis has become a powerful tool for investigating global changes in gene expression of prokaryotic organism. This approach provides a snapshot of the protein products at a given time under defined physiological conditions and gives invaluable biological information. Some bacterial groups for which proteomes have been determined are *Haemophilus influenzae* [11], *Bacillus subtillus* [15], *Mycobacterium tuberculosis* [6], and *Helicobacter pylori* [5].

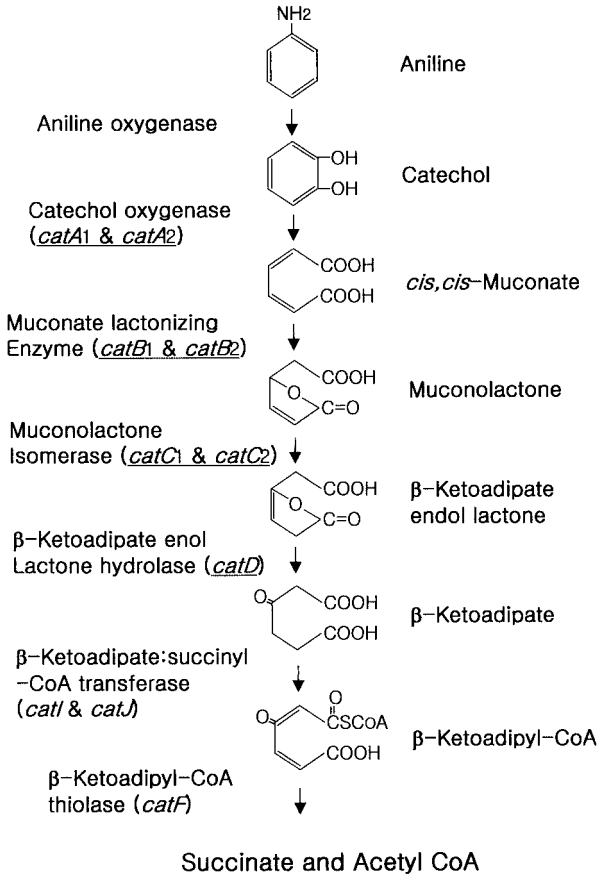

Fig. 1. Aniline degradation pathway and enzymes of *A. lwoffii* K24. The nucleotide sequence of underlined genes have been deposited in the GenBank database under the accession number U77658 and U77659.

In this study, we have performed comparative proteome analysis of protein profile expressed under aniline and succinate in *A. lwoffii* K24. These results will be the useful data for the identification of complete *cat* genes and other aniline-induced genes and the understanding of their functions in *A. lwoffii* K24.

Materials and Methods

Growth conditions and sample preparation. *A. lwoffii* K24, precultured in the potassium phosphate buffer (pH 6.25) containing 3.4 mM $MgSO_4$, 0.3 mM $FeSO_4$, 0.2 mM $CaCO_3$, 10 mM NH_4Cl, and 10 mM sodium succinate, was cultured in the aniline or the succinate medium for comparative proteome analysis [7]. The cells were cultured at 30°C with aeration until more than 1.0 optical density at 600 nm. The exponentially growing cells were harvested and washed with 20 mM Tis-HCl (pH 8.0). Harvested cells were disrupted by a French pressure cell-(SLM AMINCO, Urbana, IL, USA) at 20,000 lb/in^2. The supernatant (soluble fraction) was fractionated by centrifugation at 15000×g for 45 min and used for 2D gel electrophoresis. Protein concentrations of the soluble fraction were determined using Bradford protein assay (Bio-Rad, Hercules, CA, USA).

2-D PAGE electrophoresis (2-DE). Dried samples (about 200 μg) were resolved in 50 μl Buffer I (SDS 0.3%, DTT 0.2 M, Tris-HCl 50 mM) and were heated at 95°C for 5 min. After incubation with 5 μl 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 Buffer III (Urea 9.9 M, NP-40 4%, carrier ampolyte [pH 3–10], DTT 100 mM) for isoelectric focusing (IEF). IEF and SDS-PAGE were performed as described by Choi using PROTEAN II xi electrophoresis kit (BIO-RAD, Hercules, CA, UAS) [2]. Silver staining was carried out by the method of Moriesy [12]. Protein spots separated on the 2-D gel were transferred onto a PVDF membrane by a semi-dry blotting apparatus (BIO-RAD Trans-Blot SD) at 2 mA/cm^2 for 50 min. The PVDF membrane was stained with a Coomassie Brilliant Blue R250 and washed with 50% methanol. Stained 2-D gels were scanned by an image scanner of Amersham pharmacia biotech (Uppsala, Sweden).

N-terminal sequencing and analysis. Coomassie stained protein spots were excised from the PVDF membrane and installed into the blot cartridge of a model 491A protein sequencer (Perkin-Elmer, Foster City, CA, USA) for sequencing analysis. The obtained N-terminal sequence was used for protein identification by BLAST search of NCBI.

In-gel digestion and peptide mapping. Gel pieces of protein spots 3 and 9 were dehydrated 3 times by 50% acetonitrile and 0.2 M ammonium bicarbonate. Trypsin digestion was performed in 0.2 M ammonium bicarbonate containing 0.02% Tween-20 with 1 μg trypsin at 30°C for 24 h. The digested peptides were extracted with 60% acetonitrile containing 0.1% TFA and applied to an OD-300, Aquapore C-18 columm (2.1 × 300 mm) of PE Brownlee (Foster City, CA, USA) in Model 172 HPLC of Perkin Elmer and separated with an increasing acetonitrile gradient of 16–72% at the flow rate of 210 μl/min for 60 min. Each peak was manually collected and used for N-terminal sequencing.

Results and Discussion

Analysis of the 2-DE protein profiles of *A. lwoffii* K24. For comparative analysis, the soluble protein fraction of aniline- and succinate-induced *A. lwoffii* K24 was used for 2-DE (Fig. 2). Succinate is a common metabolite produced by the last β-ketoadipate enzyme, β-ketoadipyl-CoA thiolase (*catF*), and can be used as carbon source in the cell. We expected to identify aniline-induced protein spots by comparing the two 2D gels. About 370 protein spots were detected on the silver stained gels within the size range of 10-120 kDa and the pH range of 3–10. Because these protein spots were mainly located on the pH 3–7, the proteins most expressed in *A. lwoffii* K24 were suggested to be acidic proteins. Comparative analysis showed that more than 20 protein spots were significantly expressed only on aniline-induced protein fraction. The results of 2D patterns were reproducible with independent preparation of protein sample.

Identification of protein spots and analysis. To identify aniline-induced protein spots, protein spots developed on the 2D gel were electroblotted on PVDF membrane and 20 protein spots were selected for N-terminal

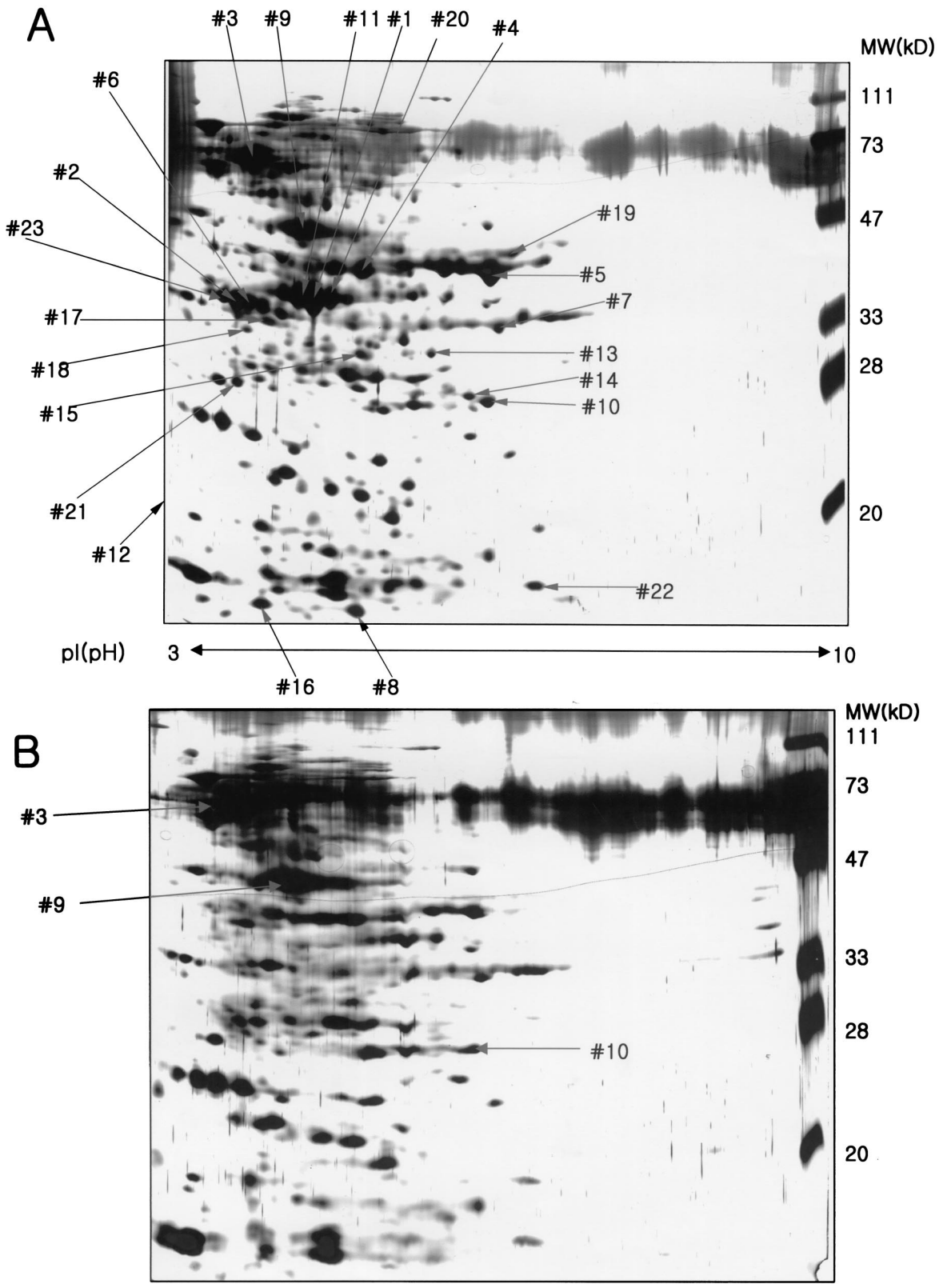

Fig. 2. 2-DE of soluble protein extracts of aniline (**A**) and succinate (**B**) induced *A. lwoffii* K24. Proteins were visualized by silver staining. Total soluble proteins were extracted from *A. lwoffii* K24 cultured in aniline and succinate media. The equivalent amounts (200 μg) of crude proteins were separated on 2DE. The protein spots indicated by arrows were analyzed by N-terminal and internal sequencing and listed in Table 1.

Table 1. Protein spot identification from the proteome of *Acinetobacter lwoffii* 24

Spot No.	Mw (kDa)	N-terminal & internal sequence	Homology	Identification
Induced by aniline				
1	36	SIKVF	100% with $catA_1$ of *A. lwoffii* K24 (U77658)	Catechol 1,2-dioxygenase I_1 ($catA_1$)
2	33	MNKQA	100% with $catA_1$ of *A. lwoffii* K24 (U77659)	Catechol 1,2-dioxygenase I_2 ($catA_2$)
6	33	MNKQA	100% with $catA_2$ of *A. lwoffii* K24 (U77659)	Catechol 1,2-dioxygenase I_2 ($catA_2$)
8	16	MLFHVRM	100% with $catC_2$ of *A. lwoffii* K24 (U77659)	Muconolactone isomerase$_2$ ($catC_2$)
11	36	SIKVF	100% with $catA_1$ of *A. lwoffii* K24 (U77658)	Catechol 1,2-dioxygenase I_1 ($catA_1$)
12	20	SIF(A/G)(D/Y)IV(N/K)K(L/Q)FGKA(K/L)PDQPA		Unknown
13	30	PYAAVXGTELHYRID(?)GN(?)RHXXA	66% (15 a.a) with putative hydrolase of *Streptomyces coelicolor* A3(2) (CAB95984)	Putative hydrolase
14	26	MINKIFESLQSAVADVHDGATVMIG	56% with β-ketoadipate:succinyl-CoA transferase α subunit (*pcaI*) of *P. putida* (M88763)	β-ketoadipate:succinyl-CoA transferase α subunit (*catI*)
15	30	N-terminal blocked		Unknown
16	16	MLFHVEMT	100% with $catC_1$ of *A. lwoffii* K24 (U77658)	Muconolactone isomerase$_1$ ($catC_1$)
17	32	MKLLRYGPKGQEKPXLLDAQXK	68% with putative 2-hydroxyhepta-2,4-diene-1,7-dioate isomerase of *Streptomyces Coelicolor* A3(2) (AL 136058)	2-hydroxyhepta-2,4-diene-1,7-dioate (HHDD) isomerase
18	31	MKKYAVIWETNFPEDNPLLARM	50% with amino group transfer (*tdnT*) of *Pseudomonas putida* (BAA12806)	Putative aminotransferase (involved in the conversion of aniline to catechol)
19	43	XTVDVTVAHYXDATAXYF		Unknown
20	36	SIKVF	100% with $catA_1$ of *A. lwoffii* K24 (U77658)	Catechol 1,2-dioxygenase I_1 ($catA_1$)
21	28	MRVLTRDEMAXRVAQXIPEGAY	73% with β-ketoadipate:succinyl-CoA transferase β subunit-(*pcaJ*) of *Acinetobacter calcoaceticus* (B44570)	β-ketoadipate:succinyl-CoA transferase β subunit-(*catJ*)
23	33	MNKQA	100% with $catA_2$ of *A. lwoffii* K24 (U77659)	Catechol 1,2-dioxygenase I_2 ($catA_2$)
Increased by aniline				
4	40	DEVV(?)KIGHVAPLTGG(?)IAHLG	55% with branched-chain amino acid ABC transporter (AAF09862)	Putative ABC transporter
5	40	AKPAKRVAVTGAAGQIAYSL	100% with malate dehydrogenase of *Burkholderia pseudomallei* (P80536)	Malate dehydrogenase
7	32	ADAKIPVVAAENFYGDVVQQL		Unknown
22	18	N-terminal blocked		Unknown
Commonly induced by aniline & succinate				
3	60	N-terminal blocked, XIEVGKENTTII (In) XXDNAGDGTTXATVLAQRIV(In)	83% with 60 kDa chaperonin (Protein CPN60/GroEL) of *Pseudomonas putida*-(P48216)	Putative chaperonin
9	45	N-terminal blocked, XXXXXFLMPVVE(?) (In)		Unknown
10	25	DEKMMSAAXSKTNF		Unknown

sequencing. The sequencing results showed that two protein spots (15 and 22) were N-terminal blocked and N-terminal Mets of the other eight protein spots were removed, which suggesting about 50% of proteins were processed by post-translational modification (PTM) in *A. lwoffii* K24 (Table 1).

Six *cat* gene products ($catA_1$, $catA_2$, $catC_1$, $catC_2$, *catI*, and *catJ*) were identified, but four *cat* gene products ($catB_1$, $catB_2$, *catD*, and *catF*) were not detected on the 2D gel of soluble protein fraction. *CatI* and *catJ* are the two subunits of β-ketoadipate succinyl-CoA transferase and are closely linked as a unit in all reported bacteria. Because *catI* and *catJ* of *A. lwoffii* K24 have not been cloned yet, N-terminal sequences of two proteins will be useful for cloning and location of the genes on the chromosome. Interestingly, protein spot 18 has 50% homology with one subunit of amino group transfer (*tdnT*) of *P. putida* (Table 2). A novel plasmid (pTDN1) of *P. putida* UCC22 is known to encode six genes (*tdnQTA1A2BR*), which are involved in the conversion of aniline to catechol [3]. These seven proteins related to aniline degradation were not detected on the 2D-gel of

Table 2. N-terminal sequence analysis of putative aniline degradation related protein spots

Spot 14	^{1}MINKIFESLQSAVADVHDGATVMIG^{25} ^{1}MINKTYESIASAVEGITDGSTIMVG^{25} (M88763) β-ketoadipate:succinyl-CoA transferase α subunit (*pcal*) of *P. putida*
Spot 18	^{1}MKKYAVIWETNFPEDNPLLARM^{22} ^{4}MKKYAVIWCSDASGDLELQEKM^{25} (BAA12806) Amino group transfer (conversion of aniline to catechol) of *P. putida*
Spot 21	^{1}MRVLTRDEMAXRVAQXIPEGAY^{22} ^{6}LTRDOIAORVAQDIPEGSY^{24} (B44570) β-ketoadipate:succinyl-CoA transferase β subunit (*pcaJ*) of *A. calcoaceticus*

succinate-induced protein fraction, which imply expression of these genes is tightly regulated in the cell. Unexpectedly $catA_1$ and $catA_2$ have more than three isotypes of which N-terminal sequences are same but pIs are different. Further study will be needed for why *catA*s have isotypes in *A. lwoffii* K24 and what make the different pIs of the isotypes.

Nine protein spots (4, 5, 7, 12, 13, 15, 17, 19, and 22) that are not directly related with aniline degradation genes, were also induced or increased by aniline. It may be possible for these proteins to be components of stimulon for global control in response to aniline. Protein spot 13 was identified as putative hydrolase and protein spot 17 was identified as putative hydrolase and 2-hydroxyhepta-2,4-diene-1,7-dioate (HHDD) isomerase. HHDD isomerase is an enzyme of catabolic pathway for homoprotocatechuic acid (HPC), which use the *meta* cleavage pathway [14]. Protein spots 7 and 19 may be novel proteins because they did not display any significant similarity to proteins currently available in the database. These proteins will be analyzed for identification and function by internal sequencing and gene cloning. Protein spots 5 and 4, increased on aniline-induced 2D gel, were identified as malate dehydrogenase and putative ABC transporter, respectively.

Protein spots 3 and 9 were commonly induced major proteins in aniline and succinate media. Because N-terminal amino acids of two proteins (3 and 9) were blocked, in-gel digestion and peptide mapping were performed for internal sequencing (Fig. 3). Internal sequence of protein spot 3 showed 83% homology to 60kDa chaperonin (GroEL) of *P. putida*. Protein spot 10, induced in aniline and succinate media, was not detected on the 2D gel of LB cultured *A. lwoffii* K24. This protein was not identified by N-terminal sequence.

From these study, we found that aniline induced not only aniline degradation genes but also other related genes including novel genes. For the searching of other aniline-induced proteins, 2D analysis of insoluble protein fraction will be performed. *A. lwoffii* K24 can use ben-

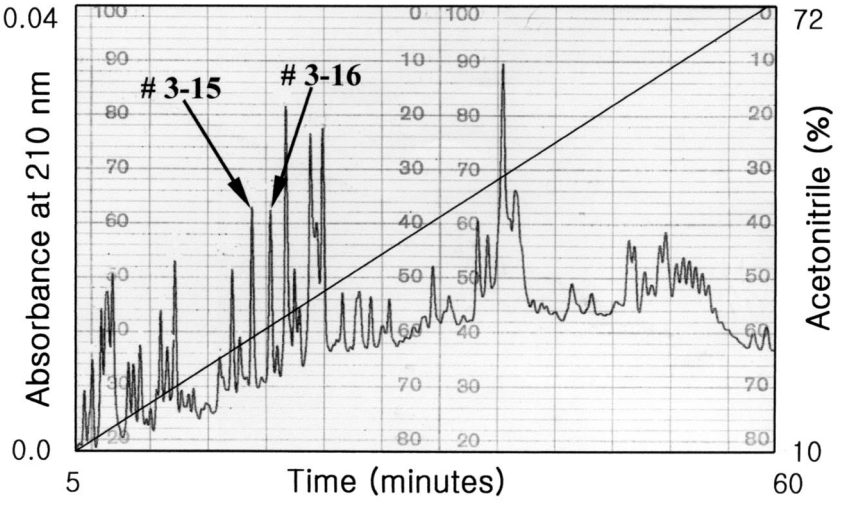

Fig. 3. RP-HPLC separation of trypsin digestion of spot 3 protein of *A. lwoffii* K24. Peaks of 3–15 and 3–16 were manually collected at 210 nm and used for N-terminal sequencing. N-terminal amino acid sequences of trypsin-digested peptides were analyzed by automatic sequencer (Perkin-Elmer 491A). 3–15: XIEVGKENTTII^{12}, 3–16: ^{1}XXD NAGDGTTXATV-LAQRIV^{20}.

zoate, *p*-hydroxybenzoate as a sole carbon source as well as aniline (unpublished data). Proteome analysis of *A. lwoffii* K24 cultured with various aromatic compounds will give us more clear information for biodegradation-related genes and their regulation.

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