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Contributed Paper

Proteomic Study of Recombinant *Escherichia coli* Expressing *Beauveria bassiana* Chitinase Gene

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ABSTRACT

This research aims to investigate the effect of *Beauveria bassiana* chitinase gene on the protein profile of recombinant *Escherichia coli* by using a proteomic approach. Total protein expressed in non-recombinant *E. coli* [pSE420] and recombinant *E. coli* [pDSM1] was analyzed by two-dimensional polyacrylamide gel electrophoresis. The expression profiles of recombinants were then compared with those of non-recombinants. Three protein spots were differentially expressed (2 up-regulate and 1 down-regulate). In addition, five proteins spots were also complementarily observed. Surprisingly, none of these protein spots were clearly unique to the theoretical pI and MW of *B. bassiana* chitinase. This evidence probably resulted from proteolytic cleavage during the post-translational processing in *E. coli* host cells. These protein spots were then identified using the TagIdent program. The results suggested that most of these proteins are probably involved in nucleotide biosynthesis.

Keywords: chitinase, *Beauveria bassiana*, recombinant DNA, proteomics, two-dimensional gel electrophoresis.

1. INTRODUCTION

Chitinase is a degrading enzyme of chitin substrate, the major component of insect, crustacean and fungi structure [1]. This enzyme is involved in the metamorphosis and hyphal growth of fungi, therefore, insects and fungi are major sources of chitinase. Chitinase also plays a major role in plant defense mechanisms [2]. Hence, it has been studied as the target for pest control agents worldwide [3, 4].

Proteomics is a technique used to investigate whole proteins expressed by an organism, tissue or a cell at a specific time point under defined environmental conditions. This technique is based on the combinations of two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS). Nowadays, proteomics has been used for many research purposes e.g. disease diagnosis, drug target and biomarkers. The biomarkers of Alzheimer and pancreatic cancer were elucidated by a proteomic approach [5, 6]. These studies provided the information for the early therapeutic treatment. In plant research, proteomic has been used as a tool to examine the disease tolerant plants [7, 8].

Proteomic analysis of some recombinant *E. coli* had been reported earlier [9, 10]. The expression level of some protein was different

from the native *E. coli* which could be a result of foreign gene. To better understand this evidence in genetically modified *E. coli*, this study is conducted. The objective of this study is to compare the protein profiles of recombinants *E. coli* expressing *B. bassiana* chitinase gene with those of non-recombinants by using 2-DE. In addition, the protein changes in *E. coli* caused by recombinant protein production are described.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Urea, 3-[(3 cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), dithiothreitol (DTT), immobilized pH gradient (IPG) strip (ImmobilineDryStrip, pH 3-10 nonlinear, 7 cm), IPG-buffer (pH 3-10 NL) were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Unless stated otherwise, all reagents and chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2 Bacterial Strain and Culture Condition

E. coli TOP10 carrying recombinant plasmid pDSM1 was used as a bacterial host strain. Plasmid pDSM1 consisted of a *Beauveria bassiana* chitinase gene fused with pSE420 expression vector under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG) inducible T7 promoter. The recombinant and non-recombinant *E. coli* were separately cultured at 37°C in Luria-Bertani (LB) medium supplemented with ampicillin at a final concentration of 50 μg/ml. When the OD_{600} of cultures reached around 0.5, IPTG was added to make a concentration to 0.8 mM. The cultures were grown for 2 h at the same conditions. Cells were then harvested by centrifugation at 12,000 rpm for 10 min at 4°C. The cell pellets were washed twice in washing buffer (10 mM Tris base, 5 mM $(CH_3COO)2Mg \cdot 4H_2O$) and then stored at -20°C for further analysis.

2.3 Preparation of Protein Extracts

Cells were resuspended with 200 ml lysis solution (8M urea, 4% w/v CHAPS, 2% v/v IPG buffer 3-10 NL, 40 mM DTT) containing protease inhibitor. The cell suspensions were placed in ice for 30 min and subsequently subjected to three freeze-thaw cycles. After the final thaw cycle, samples were centrifuged for 10 min at 12,000 rpm and 4°C. The supernatant was then cleaned using the 2D Clean up kit (GE Healthcare Bio-Sciences). The total protein content in the supernatant was then determined using the 2D Protein quantification kit (GE Healthcare Bio-Sciences).

2.4 Two-dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

For, isoelectric focusing (IEF), 15 μg of each protein extract was added to the rehydration buffer (8M Urea, 2% w/v CHAPS, 0.002% w/v bromophenol blue) and 7 cm, non-linear pH 3-10, Immobilized pH gradient (IPG, GE Healthcare) were rehydrated at 20°C for 12 h. Focusing was then performed at 20°C on a IPG phor II (GE Healthcare Bio-Sciences) using a voltage slope from 300 to 5000 V during the first 3 h, then stabilized at 8000 V for 36 min. Before the second dimension separation, the gel strips were equilibrated for 15 min in SDS equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M Urea, 30% v/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue) supplemented with 50 mg DTT. Strips were then re-equilibrated in SDS equilibration buffer, freshly added by 125 mg IAA for 15 min, and then washed by SDS-PAGE running buffer for 5 sec. Strips were placed on the pre-cast polyacrylamide gel (12.5%), and electrophoresis was performed on MiniVE (GE Healthcare Bio-Sciences) for 1.5 h at 200 V or until the

dye front migrating to the bottom of the gel.

2.5 Gel Staining and Analysis

The gels were visualized using a silver staining kit (GE Healthcare Bio-Sciences). Silver stained gels were then acquired by scanning with UTA-1100 (Amersham Biosciences) at 300 dpi resolution. The acquired images of TIFF format were analyzed using an ImageMaster™ 2D Platinum software version 5.0 (Amersham Biosciences). Manual editing and removal of artifacts were done after automatic spot detection at default parameters. Gels were analyzed in triplicate.

2.6 Protein Identification

The protein spots of interest were identified by comparing their pI and MW values with the *E. coli* HAMAP (High-quuality Automated and Manual Annotation of microbial Prepteomes) database using Tag Ident (http://www.expasy.ch/tools/tagident.html) program by choosing MW range ±5% and pI range 0.1 (spot no.1,2,3,7,8) and 0.25 (spot no.4,5,6).

3. RESULTS AND DISCUSSIONS

3.1 Proteomic Analysis

Two-dimensional gel electrophoresis analysis was performed on recombinant and non-recombinant *E. coli* in order to evaluate the effect of *B. bassiana* chitinase gene expression on physiological changes at a total protein level. In this study, IEF was performed with nonlinear pH 3-10 IPG strips to analyze a broad range of *E. coli* proteome and to cover the acidic pI of *B. bassiana* chitinase. As three replicates of gels were carried, ImageMaster™ 2D Platinum software was used to construct the two reference gels representing expressed proteins from recombinants and non-recombinants, respectively. The results showed that the protein patterns of recombinants and non-recombinants were similar with approximately 500 protein spots (Fig.1a, 1b). The number of protein spots was different from another recombinant *E. coli* 2D pattern [11]. This could explain that the type of plasmid DNA may have an effect on protein expression in *E. coli*.

The reference gels were then compared. Two protein spots were up-regulated (Fig.1, spot no.1 and 2) and 1 down-regulated (Fig.1, spot no.3). Interestingly, spot no.2 exhibited a significant change in abundance (>2-fold difference). Five protein spots were newly present in recombinant *E. coli* (Fig.1 spot no.4-8). The pI and MW values of these protein spots were compared with calculated *B. bassiana* chitinase. Although the overexpression of *B. bassiana* chitinase was confirmed in previous study [12], none of these protein spots were clearly unique to theoretical pI and MW of *B. bassiana* chitinase. This may be resulted from the proteolytic cleavage during the post-translational processing in *E. coli*. The proteolytic cleavage of expressed chitinase in other recombinant *E. coli* had been reported elsewhere [13, 14].

3.2 Protein Identification

The pI and MW of eight interested protein spots were compared with protein identified in *E. coli* 2D database using TagIdent as presented in Table 1. Most of the affected proteins in recombinant *E. coli* are involved in metabolic pathway. Such evidence was also observed in other recombinant *E. coli* [11, 15]. From table 1, two protein spots were up-regulated. These proteins probably are uridylate kinase and cation efflux protein cusF precursor. Uridylate kinase is an enzyme function in nucleotide biosynthesis. As the recombinants contain more gene than non-recombinants, the up-regulation of uridylate kinase may be essential for DNA replication. Cation efflux protein cusF precursor is part

Table1. List of proteins identified on 2D map, as shown in Figure1.

Spot no.	Entry name	Description	pI/MW (Experiment)	pI/MW (Theoretical)	Accession no.	Fold change[1]
1	PYRH_ECO57	Uridylate kinase	6.93/26.00	7.07/25.80	P0A7F1	1.64
2	CUSF_ECOLI	Cation efflux protein cusF precursor	6.20/9.60	6.19/9.91	P77214	20.9
3	NANK1_ECOL6	Putative-N-acetylmannosamine kinase I	5.88/30.80	5.89/29.70	Q8FD60	0.89
4	ILVB_ECOLI	Acetolactate synthase isozyme I large subunit	5.29/60.50	5.30/60.40	P08142	Δ
5	GLPK_ECO57	Glycerokinase	5.38/55.00	5.36/56.10	P0A6F4	Δ
6	K6PF1_ECO57	Phosphofructokinase	5.54/34.60	5.47/34.80	P0A797	Δ
7	DYR9_ECOLI	Dihydrofolate reductase type 9	7.75/20.20	7.81/19.90	Q59397	Δ
8	WZB_ECO57	LMW protein-tyrosine-phosphatase wzb	7.40/16.70	7.65/16.70	P0AAB3	Δ

[1]Fold change of expression level indicates the ratio of a spot density of the chitinase producing strain, *E. coli* [pDSM1] to the control strain, *E. coli* [pSE420]. New spots are indicated by symbol (D).

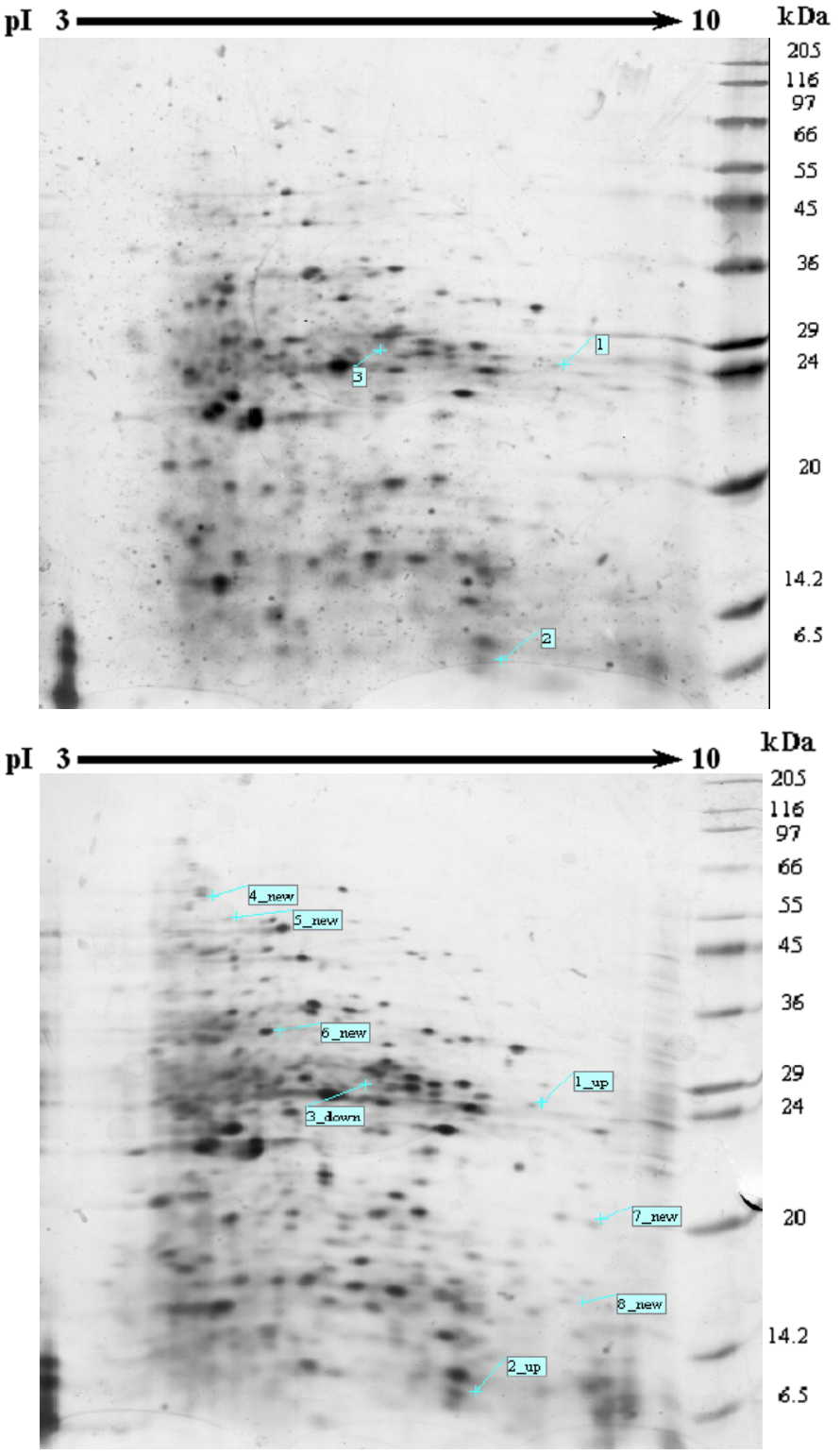

Figure 1. 2-DE profiles of total proteins expressed in non-recombinant *E. coli* [pSE420] (A) and recombinant *E. coli* [pDSM1] (B). Numbered notations refer to representative protein spots listed in Table 1.

of a cation efflux system that mediates resistance to copper and silver. The expression of gene encoding cusF was elevated in *E. coli* grown in high metal condition [16]. However, the reason why cation efflux protein is up-regulated in recombinant *E. coli* expressing *B. bassiana* chitinase gene is uncertain.

Putative-N-acetylmannosamine kinase I was the only one protein showing down-regulated. This enzyme catalyzes the phosphorylation of N-acetylmannosamine liberated from N-acetyl-neuraminic acid by nanA protein. The decrease of putative-N-acetylmannosamine kinase I in recombinant *E. coli* has not been reported elsewhere. In addition, five protein spots which are glycerokinase, phosphofrutokinase, acetolactate synthase isozyme I large subunit, dihydrofolate reductase type 9 and LMW protein-tyrosine-phosphatase wzb were found. These proteins function in metabolic pathway. Interestingly, acetolactate synthase isozyme I large subunit and dihydrofolate reductase type 9 are involved in amino acid (Val, Leu, Ile) and deoxyribonucleotide synthesis (*de novo* pathway), respectively. The presence of these five proteins in recombinant was unexpected. It might be a result of the inserted gene which possibly interrupts the metabolic pathway of host *E. coli*. However, these eight protein spots need to be further analyzed by mass spectrometry in order to confirm their types and functions.

4. CONCLUSIONS

The analysis of gene expression in recombinant *E. coli* using the proteomic approach suggests some changes in the metabolic pathway including amino acid and nucleotide synthesis. Although the expected protein spot of *B. bassiana* chitinase could not be detected, these results help us to better understand the physiological properties of genetically modified *E. coli*.

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