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Proteomic analysis of brown planthopper: application to the study of carbamate toxicity

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Abstract

Toxicity to *o-sec*-butylphenyl methylcarbamate compound (BPMC) was analyzed in the rice brown planthopper, *Nilaparvata lugens*, using a differential proteomics approach of identifying proteins on two dimensional-polyacrylamide gel electrophoresis (2D-PAGE). Proteome analysis from BPMC-treated brown planthopper resulted in the modulation of 22 proteins at the expression level as compared to control samples on coomassie brilliant blue (CBB) stained gels. Out of total 22 proteins, 10 proteins showed elevated expression, eight proteins showed decreased expression and four proteins showed specific expression after insecticide treatment. The N-terminal sequences of seven out of 22 proteins were determined by a gas-phase protein sequencer. The internal amino acid sequences of the 15 proteins were determined by the sequence analyses of peptides obtained by Cleveland peptide mapping method and were compared with those of the known proteins available in public databases and the EST database of the brown planthopper in our laboratory to understand the nature of the proteins. Sequence analyses revealed that the expression of putative serine/threonine protein kinase, paramyosin, HSP 90, β-tubulin, calreticulin, ATP synthase, actin and tropomyosin was elevated, and that of β-mitochondrial processing peptidase, dihydrolipoamide dehydrogenase, enolase and acyl-coA dehydrogenase was reduced due to the exposure of BPMC. The differential expression of these proteins reflects the overall change in cellular structure and metabolism after insecticide treatment.

Keywords: Insecticide; Planthopper; 2D-PAGE; Proteomics

1. Introduction

The brown planthopper *Nilaparvata lugens* is a serious pest of rice crop in the temperate and tropical regions of Asia and Australia (Heinrichs, 1994). Various insecticides (organophosphate, carbamate, pyrethroid, and neonicotinoide compounds) are being used to control the brown planthopper population in the rice fields (Dyck and Thomas, 1979; Hemingway et al., 1999). These groups of insecticides display neurotoxicity in insects and mammals, and are biodegradable (Sogorb and Vilanova, 2002).

Although discovery of genes has been greatly accelerated by recent progress in structural and functional genomics, the functions of proteins that depend considerably on post-translational modification and protein–protein interactions cannot be inferred through genome analysis. Proteomics has emerged as an enormously powerful method for gaining insight to different physiological changes at cellular level (Komatsu et al., 1999; Antelman et al., 2000; Konishi et al., 2001; Chen et al., 2002; Natera et al., 2000; Ishimura et al., 2002), but relatively no attempt have been made to apply this technique to study insect toxicity.

In the present study, an attempt has been made to comprehend the molecular basis of the toxicity in the brown planthopper to *o-sec*-butylphenyl methylcarbamate compound (BPMC), one of the most popular carbamate compound used commercially to control planthoppers, using a differential proteomics approach to identify proteins through two dimensional-polyacrylamide gel electrophoresis (2D-PAGE).

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2. Materials and methods

2.1. Planthopper strain

Planthopper strain of *N. lugens*, originally collected from Izumi city, Kagoshima prefecture, in 1982 was raised by weekly spraying of *o*-isopropoxyphenyl methylcarbamate (PHC, 250 ppm) and *S*-1,2-bis (ethoxycarbonyl) ethyl *O*,*O*-dimethyl phosphorodithioate (malathion, 500 ppm), kindly provided by Y. Otsu of Bayer Agrochemicals Ltd. (Japan).

2.2. Lethal dose response

To estimate the lethal dose response of the BPMC, dry film method was used. BPMC was coated on the inside of the glass tubes (13 cm × 1.5 cm), by applying 300 μl of acetone containing different concentrations (1–100 ppm) of BPMC and rolling the tubes until the acetone had evaporated. A total of 10 female brown planthoppers were placed in each tube for 3 h, and were subsequently transferred to the rice seedlings growing at 25 °C. Mortality was scored for each concentration of BPMC in triplicates 24 h after the treatment. Control mortality in the absence of insecticide was taken into account in deriving dose response. Raw bioassay data were analyzed by probit analysis using computer program POLO (Robertson et al., 1980) for calculating the LC_{10} and LC_{50}.

2.3. Insect treatment with BPMC for protein extraction

Female planthoppers were treated with the LC_{10} and LC_{50} doses of the BPMC for 3 h in the BPMC coated tubes and were transferred to the rice seedlings. Alive insects were separated from dead insects at the time of sampling. Samples of control and BPMC-treated insects were collected at 6, 12 and 24 h after treatment and were frozen for protein extraction.

2.4. Protein extraction and 2D-PAGE

For 2D-PAGE, whole insects were homogenized in lysis buffer (1:6 w/v) containing 9.5 M urea, 2% NP-40 (Nacalai Tesque, Kyoto, Japan), 2% ampholine (pH 3.5–10 and pH 5–8; equal volumes), 5% β-mercaptoethanol and 0.05% PVP-40 (Sigma), using a chilled glass mortar and pestle to extract soluble proteins. The homogenate was twice subjected to centrifugation at $20,000 \times g$ for 5 min, and the resulting supernatant was used as the crude protein extract. The supernatant (75 μl) was subjected to 2D-PAGE. Isoelectric focusing (IEF) pH 3.5–7.0 was carried out in a glass tube with a length of 13 cm and a diameter of 3 mm by applying current at 200 V for 30 min followed by 400 V for 18 h and 600 V for 1 h in first direction using Bio-Rad (Richmond, CA, USA) Protean II electrophoresis equipment. Immobilized pH gradient (IPG) was carried out in pre-prepared tubes (Bio-Rad), pH 6.0–10.0 by applying current at 400 V for 1 h followed by 1000 V for 16 h and 2000 V for 1 h. SDS-PAGE in the second dimension was performed on a 15% separation gel. The separation gels were stained with coomassie brilliant blue (CBB) to visualize the protein spots. The isoelectric point and molecular mass of each protein were calibrated using 2D-PAGE standards (Bio-Rad). The molecular weight and p*I* values were evaluated automatically with Image Master 2D Elite software (Amersham Biosciences). The 2D-PAGE were repeated at least three times to confirm reproducibility, an important factor in this study.

2.5. N-terminal amino acid sequence analysis

After 2D-PAGE, proteins were electroblotted onto PVDF membrane (Fluorotrans; Pall BioSupport Division, Port Washington, NY, USA) using a semi-dry transfer blotter (Nippon Eido, Tokyo), and visualized by CBB staining. The proteins were excised from the PVDF membrane and analyzed by Edman degradation method using a gas-phase protein sequencer (Model 492; PE Applied Biosystems, Foster City, CA, USA).

2.6. Internal amino acid sequence analysis

The CBB stained protein spots of interest were excised from the separation gel using a scalpel and the proteins were electro-eluted from the gel pieces using an electrophoretic concentrator (ISCO, Lincoln, CA, USA) at 200 V for 2 h. After electro-elution, each protein solution was dialyzed for 48 h against milliQ. The proteins were dissolved in 30 μl of an SDS sample buffer containing 0.06M Tris–HCl, 2% SDS, 5% β-mercaptoethanol and 10% glycerol pH 6.8. Twenty microliter of the sample was applied to a sample well in an SDS-PAGE gel, overlaid by 20 μl of V8 protease (0.1 μg/μl). Electrophoresis was performed until the sample and protease were stacked in the stacking gel, and interrupted for 30 min for digestion of the protein (Cleveland et al., 1977). The electrophoresis was then continued, and the separated digests were electroblotted onto a PVDF membrane and subjected to amino acid sequencing as above. The sequences were compared with protein sequences in a Swiss-Prot database using the FASTA sequence alignment program.

2.7. MALDI-TOF MS

Protein spots of interest were excised from a gel; the gel slices were washed with 25% (v/v) methanol and 7% acetic acid for 12 h at room temperature and then

destained with 50 mM NH_4HCO_3 in 50% (v/v) methanol for 1 h at 40 °C. Proteins were reduced with 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 60 °C followed by incubation with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min at room temperature. The gel slices were then crushed and dried followed by tryptic digestion (1 pmol) in 100 mM NH_4HCO_3 at 37 °C overnight. The digested peptides were extracted from gel slices with 0.1% trifluoroacetic acid (TFA) in 50% (v/v) acetonitrile/water. The peptide solution, thus, obtained was dried up and reconstituted in 30 μl of 0.1% TFA in 5% acetonitrile/water. Peptides from each trypsin-digested sample were desalted using a ZipTip (C18 resin; P10, Millipore Corporation, Bedford, MA). For each sample, 1 μl of the desalted peptide mixture was mixed (1:1) with the matrix α-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) and spotted onto a 100 position stainless steel matrix-assisted laser desorption ionization (MALDI) plate. MALDI-MS was performed using a Voyager Elite XL time-of-flight mass spectrometer (Applied Biosystem, Framingham, MA, USA). Calibrations were carried out using a standard peptide mixture. The mass spectra were subjected to a sequence database search with Mascot software (Matrix Science, London, UK) and evaluated based on the obtained candidate sequence with MS-Match, a software aid for protein identification by MS (http://www.protein.osaka-u.ac.jp/organic).

2.8. Planthopper EST analyses

Peptide sequences obtained from above proteomic analyses were subjected to a search for corresponding gene in an expressed sequence tags (EST, single-pass cDNA sequences) database of *N. lugens*, in our laboratory. The database is now being prepared from various tissues of the planthopper by mRNA extraction, cDNA synthesis, cloning into plasmids, and sequencing the insert DNA. Each of about 20,000 EST nucleotide sequences was translated into six open reading frames and the translated amino acids sequences were searched for the peptide sequences from the proteomic analyses. Text data-based search was performed using Perl (Practical Extraction and Reporting Language) v.5.8.1 under Macintosh OSX system. The matched EST sequences were then subjected to a search for corresponding protein in the public database (NCBI) to confirm the nature of assigned protein.

3. Results and discussion

3.1. Evaluation of BPMC toxicity to planthoppers

The *N. lugens* strain raised by spraying PHC and malathion was subjected to BPMC treatment, because of its widespread use in controlling planthoppers in rice fields. The LC_{10} and LC_{50} dose for BPMC were calculated by recording the probit mortality after 24 h as described in Materials and methods using the computer program POLO (Robertson et al., 1980). LC_{10} and LC_{50} dose were found to be 2.66 and 9.66 ppm, respectively. Fig. 1 shows the dose–response curve of female planthoppers in response to BPMC.

3.2. Differential expression of proteins

In the present study, acute toxicity by BPMC administration was studied by determining the changes in the protein profiles of the planthopper with multiple doses of BPMC and time points of analysis. No significant changes were observed in the LC_{10}-treated planthopper protein pattern on the 2D-PAGE as visualized by CBB staining, when compared with the control (data not shown). We observed distinct differential pattern after treatment with LC_{50} dose for 22 different proteins after 24 h (Fig. 2), while after 6 and 12 h responses were not yet pronounced (data not shown). The differentially expressed proteins after 24 h of treatment were scored from CBB stained IEF and IPG gels. Out of these proteins, 10 proteins showed elevated expression, eight proteins showed decreased expression and four proteins showed specific expression only in BPMC-treated samples. The expression level was determined using Image Master 2D Elite software (Amersham Biosciences) by calculating the density of the protein spots in control gels, which were set as 1.0 and simultaneously in the LC_{50} treated gels. The 2D maps of control and BPMC-treated planthoppers showed a good reproducibility, and a representative experiment is shown in Fig. 2. The

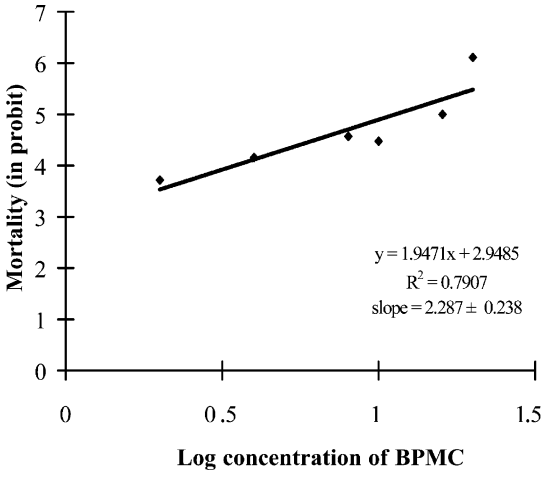

Fig. 1. Dose–response curve for the planthopper strain to BPMC. Ten female planthoppers were exposed to BPMC in triplicates for 3 h prior transferring to rice seedlings and mortality was scored after 24 h. Lethal dose value for the BPMC was determined using the computer program POLO (Robertson et al., 1980).

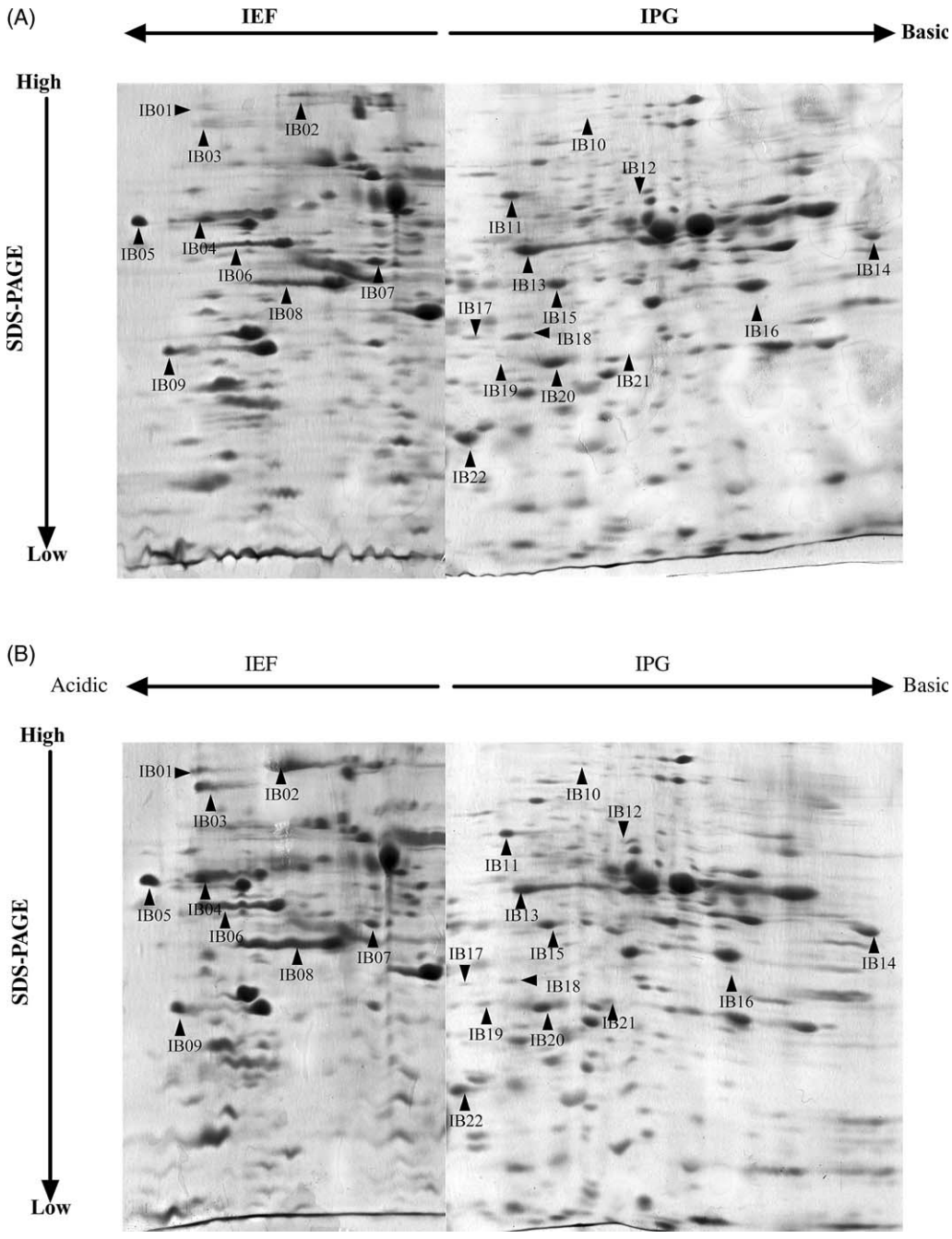

Fig. 2. A 2D-PAGE gel separation of proteins identified with CBB staining from (A) control and (B) BPMC-treated planthoppers after 24 h of treatment. The proteins were separated by isoelectric point (p*I*) in first dimension and by molecular weight (MW) in the second dimension. Two gels were run in the pH range of 3.5–7.0 and 6.0–10.0 as described in Materials and methods and the resulting pattern was overlapped.

spot density analysis of the marked proteins was performed for three gel images and the results are expressed as ±SD (Fig. 3). Quantification of spots revealed that protein expression for spots IB01, IB02, IB03, IB04, IB05, IB06, IB08, IB09, IB14 and IB19 increased ~3.6-, 6.4-, 4.1-, 2.5-, 1.3-, 6.3-, 2.6-, 1.7-, 1.8- and 2.2-fold, respectively (Fig. 3A), while the protein expression for spots IB07, IB11, IB13, IB15, IB17, IB18, IB20, and IB22 decreased ~0.4-, 0.25-, 0.28-, 0.35-, 0.88-, 0.7-, 0.35- and 0.5-fold, respectively (Fig. 3B). The protein spots IB10, IB12, IB16 and IB21 were observed only after BPMC treatment.

3.3. *Characterization of proteins by Edman sequencing, Cleveland peptide mapping and MS*

To obtain the sequence tags by Edman sequencing following separation by 2D-PAGE, gels were electro-

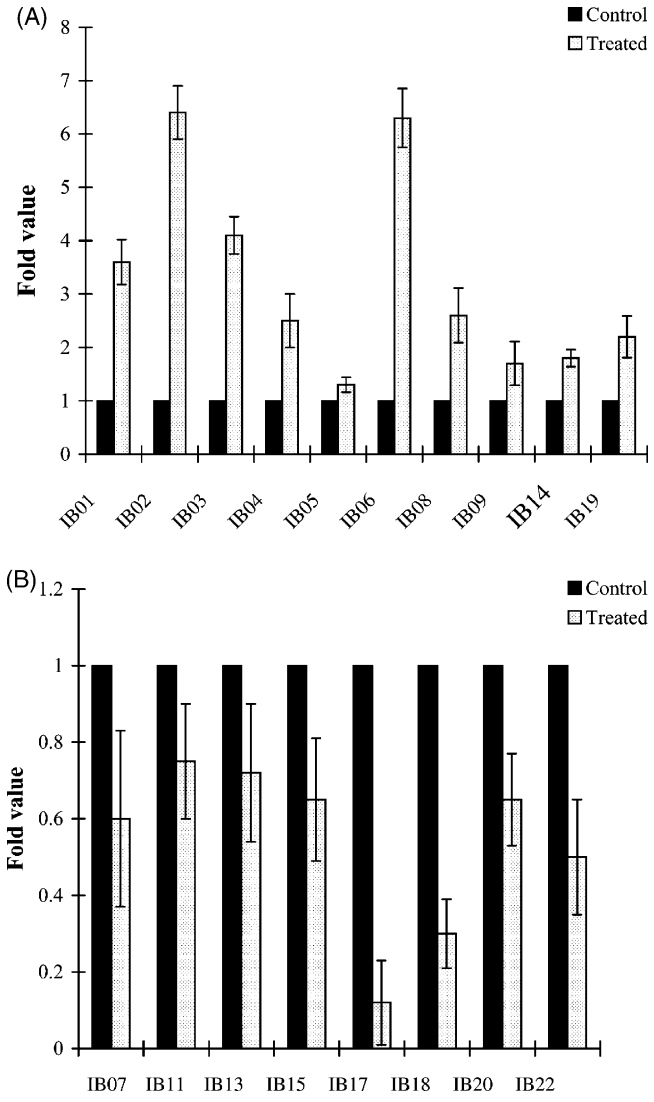

Fig. 3. Quantification of the protein expression (A) proteins showing increased expression (B) proteins showing decreased expression. Protein spots were quantified by scanning the intensities on the treated gels and compared to the control value, which was set as 1.0 using a 2D Elite software. Values are shown as mean ± SD.

blotted onto a PVDF membrane and 22 selected protein spots were analyzed by a gas-phase protein sequencer. N-terminal sequence for seven out of 22 proteins were successfully determined in this manner and rest of the proteins for which N-terminal sequence cannot be sequenced were inferred as having blocking groups at the N-terminal (Table 1). Blockage is common for proteins, and is caused by acetylation or the addition of a pyrrolidone carboxylate group to the N-terminal amino acid residue (Wellner et al., 1990). The N-terminal sequence of IB17, IB19 and four proteins (IB10, IB12, IB16 and IB21) showing specific expression after BPMC treatment might not be determined due to very less amount of protein samples. The internal amino acid sequences of the 15 proteins were determined by Cleveland peptide mapping method (Cleveland et al., 1977) using *Staphylococcus aureus* V8 protease (Table 1). Two fragments were analyzed to determine the internal sequence for each spot, while the internal sequence of only one fragment was available for some spots. For spot IB11, we were unable to get any of the internal sequence. The amino acid sequences determined here were compared with those of known proteins of insects and other organisms in Swiss-Prot database using FASTA sequence alignment program. We assigned the homology on the basis of the experimental p*I* and molecular weight of our proteins with the known proteins with in the same range. The N-terminal sequence of IB07 (β-MPP), IB11 (no homology), IB13 (enolase), IB14 (hypothetical protein) and IB15 (acyl-coA dehydrogenase) showed very less or no similarity at the N-terminal regions of the assigned protein. For spot IB20, two fragments analyzed using Cleveland method had same sequence VAAAKAAHFS suggesting the repetition of this sequence in the protein.

Also, peptide sequences from each of the spots were searched in the EST database (~20,000 sequence data) of *N. lugens* in our laboratory using Perl program to translate nucleotides to amino acids and to search for the peptide sequences to confirm the assigned proteins. As shown in Table 1, we found no homology in the EST database for IB01, IB05, and IB15 while a 100% homology was found for other sequenced peptides in the translated EST data confirming the nature of assigned proteins. Sequenced peptide from spot IB11 and IB18 showed 100% homology in the EST database (accession no. AB158284 and AB158286, respectively). Earlier, the IB11 was assigned as unknown protein because of no homology at the N-terminal region of the proteins in the database but as the peptide sequence is 100% match with the translated EST (ca. 210 amino acids) and this sequence showed 64% homology with dihydrolipoamide dehydrogenase of *Bombyx mori* (accession no. AAM93255), we can surely assign it as dihydrolipoamide dehydrogenase. Also, the translated EST (ca. 266 amino acids) showing 100% match with IB18 was found to show 50 % homology with HSP 30 of *Neurospora crassa* (accession no XP_332056), suggesting the cross contamination of this protein from yeast-like symbiotes of the brown planthopper (Suh et al., 2001) as high homology was observed with *Neurospora* and no homology was observed in the database with HSP proteins from insects. Thus, sequence tags determined by Edman sequencing method for proteins showing differential expression pattern by BPMC administration (Table 1) resulted in three major groups of cytoskeleton proteins (IB02, IB04, IB08 and IB09), chaprone proteins (IB03 and IB05) and mitochondrial proteins (IB06, IB07, IB11 and IB15).

Table 1
Identification of proteins showing differential expression in response to BPMC by gas-phase protein sequencer

Spot no.	Experimental p*I*/mol wt. (kDa)	N-terminal/ internal sequence	Database accession no./ homologous protein	% homology	EST accession no./homologous protein to translated EST	% homology of sequenced peptide with translated EST
IB01	4.12/74.5	N-blocked	–	–	–	–
		I-VIKPNNLLL	XP_342107/putative serine–threonine protein kinase	77	–	No homology
IB02	4.5/75.2	N-blocked	–	–	–	–
		I-SLHEEQEXVI	P13392/paramyosin	66[a]	AB158279/paramyosin	100[a]
IB03	4.1/70.5	N-blocked	–	–	–	–
		I-FRALLFVPRR	AAB05638/HSP 82	100	AB158280/HSP 90 analog	100
		I-SGKLLFIKI	AAB05638/HSP 82	88	–	No homology
IB04	4.21/50.1	N-MREIVHIQAG	NP_942113/β-tubulin	100	–	No homology
		I-TYXIDNEALY	NP_942113/β-tubulin	100[a]	AB159214/β-tubulin	100[a]
IB05	3.99/48.3	N-EVFFEENFAD	AAN73309/calreticulin	80	–	No homology
		I-YSEHPGKEFG	AAN73309/Calreticulin	100	–	No homology
IB06	4.5/45.4	N-blocked	–	–	–	–
		I-SGVISLKDKT	NP_726631/ATP synthase β-subunit	90	AB159215/ATP synthase β-subunit	100
		I-LGIYPAVDPL	NP_726631/ATP synthase β-subunit	100	AB158282/ATP synthase β-subunit	100
IB07	5.2/42.1	N-STAAAHAEVT	–	No homology	AB158281/β-mitochondrial peptidase	100
		I-MQEVETNLQE	AAC39915/β-mitochondrial peptidase	100	AB158281/β-mitochondrial peptidase	100
IB08	4.9/40.2	N-blocked	–	–	–	–
		I-ITALAPSTIK	AAK25829/actin	100	AB158283/actin	100
IB09	4.13/32.4	N-blocked	–	–	–	–
		I-VAALNRRIQL	P31816/tropomyosin	100	AB158285/tropomyosin	100
		I-VSEEKANQRE	P31816/tropomyosin	100	AB158285/tropomyosin	100
IB11	5.69/54.5	N-TSGEADLVV	–	No homology	AB158284/dihydrolipoamide dehydrogenase	100
IB13	5.79/45.3	N-IKQEKARQI	–	No homology	–	No homology
		I-ALELXDGEKN	NP_038537/enolase	77[a]	AB158432/enolase	100[a]
		I-FYKDNKYDLD	NP_038537/enolase	80	AB158432/enolase	100
IB14	8.68/44.5	N-GGPAAYSTYS	–	No homology	AB159216/unknown	100
		I-LLLKGYAGD	EAA06989/hypothetical protein	77	–	No homology
IB15	6.08/39.93	N-AHXYANGXAF	–	No homology	–	No homology
		I-ASGLGQTPVL	NP_648149/acyl-coA dehydrogenase	90	–	No homology
IB18	5.49/31.9	N-blocked	–	–	–	–
		I-IQISGTITQG	ZP_00052823/methionine synthase I	–	AB158286/fungal HSP 30	100
IB20	5.79/29.3	N-blocked	–	–	–	–
		I-VAAAKAAHFS	P42852/cuticle protein	70	AB158287/unknown	100
		I-VAAAKAAHFS	P42852/cuticle protein	70	AB158287/unknown	100
IB22	5.20/22.2	N-blocked	–	–	–	–
		I-LAGFGPXAAG	XP_344141/putative solute carrier protein	88[a]	AB159217/unknown	88[a]

N-, N-terminal amino acid sequence; I-, internal amino acid sequence; homology, amino acid sequence homology.
[a] Score for X was deleted for % homology.

3.3.1. Cytoskeleton proteins

All of the cytoskeleton proteins (IB02, IB04, IB08 and IB09) were upregulated by BPMC treatment. the Cytoskeleton, being involved in a number of vital processes such as cell division plane determination, cell elongation and shape determination, cytoplasmic streaming, locomotion anchorage and cellular polarity, is one of the important targets of neurotoxic chemicals (Damodaran et al., 2002). Earlier, an increased expression of α-tubulin was reported in response to sarin, an organophosphate compound, in rats (Damodaran et al., 2002). Increased expression of actin has been reported in response to wounding, H_2O_2, UV-A and desiccation stress in *Arabidopsis* (Dunaeva and Adamska, 2001). Also, increased expression of tropomyosin was observed in response to dioxin in rats (Ishimura et al., 2002).

3.3.2. Chaprone proteins

Chaprone proteins HSP 90 (IB03), and calreticulin (IB05), a Ca^{2+} binding protein, were found to increase in the present study (Figs. 2B and 3A). It has been postulated that various physiological, developmental and environmental stress conditions causes unfolding or misfolding of proteins, thereby triggering the increased synthesis of HSP 90 and calreticulin, which protects the cells by preventing protein aggregation (Conway et al., 1995; Pratt and Toft, 2003). Thus, the increase in expression of chaprone proteins can be postulated to maintain homeostasis under stress induced by exposure to BPMC.

3.3.3. Mitochondrial proteins

Mitochondrial proteins showing differential expression in response to BPMC includes ATP synthase (IB06), β-MPP (IB07), dihydrolipoamide dehydrogenase (IB11) and acyl-coA dehydrogenase (IB15) reflecting the overall change in mitochondrial response. In the present study, an increased level of ATP synthase was observed after BPMC exposure (Fig. 3A), which is expected to be essential to maintain the high levels of ATP required by the stressed cells. Earlier, an induction of ATP synthase was observed in response to aluminium (Al) in Al-resistant cultivar of *Triticum aestivum* and it has been suggested that induction of ATP synthase could be involved in Al-resistance in wheat (Hamilton et al., 2001).

3.4. MALDI-TOF analyses

The six proteins for which the N-terminal and internal sequence were not determined were subjected to MALDI-TOF analysis. Based on a set of the observed molecular masses, a sequence database search using Mascot was carried out for protein identification. All the six proteins subjected to MALDI-TOF analysis showed homology to hypothetical proteins from different organism (Table 2). The protein score for spot IB16 was found to be significant ($p < 0.05$) as analyzed by probability based mowse score (Matrix Science, London, UK). The rest of the five proteins showed insignificant score ($p > 0.05$). This might be due to the unavailability of information for these proteins in the databases as four proteins were observed only after treatment of BPMC.

The results presented here acknowledge the possibilities to study insecticide toxicity at molecular level using proteomics technology. The study of partial amino acid sequence analysis will greatly contribute to the molecular biology for the identification of target genes and predicting their functions. As the nature of the proteins IB11 and IB18 were predicted from the EST data, it is an useful resource in proteomic analyses for not only for the confirmation of protein annotation but also identification of the molecules. Future studies will be directed towards the characterization of

Table 2
The identification of trysinated peptides of proteins subjected to MALDI-TOF

Spot no.	Experimental pI/mol wt. (kDa)	Mass of peptide sequence matched (Da)	Homologous protein	Sequence coverage (%)	Accession no.
IB10	6.24/68.5	642.42, 650.36, 684.91, 706.86, 788.11, 849.06, 954.11, 2140.00, 2385.41	Hypothetical protein (*Drosophila melanogaster*)	19	NP_572216
IB12	6.91/53.5	650.38, 727.90, 744.18, 756.13, 772.21, 787.93, 891.28, 2385.74	Hypothetical protein (*Homo sapiens*)	20	T14792
IB16	7.81/36.6	635.91, 642.50, 647.52, 650.11, 744.15, 954.19, 986.00, 1060.07, 1410.35	Hypothetical protein (*Plasmodium falciparum* 3D7)	22	NP_473026
IB17	5.12/32.5	624.09, 635.97, 650.00, 679.72, 1242.36	Putative fibronectin/fibrinogen-binding protein (*Fusobacterium nucleatum*)	15	EAA2477
IB19	5.33/29.8	612.14, 635.59, 659.78, 674.61, 763.94, 804.77, 860.24, 986.46, 1504.13, 2652.41, 2707.18, 3507.19	Sugar ABC transporter permease protein (*Mycoplasma pulmonis*)	17	NP_32644
IB21	6.55/30.7	624.41, 647.54, 684.39, 729.34, 874.48, 888.51, 1198.48	Similar to RIKEN cDNA gene (*Danio rerio*)	10	AAH453

unknown proteins and determining their role in the toxicity response.

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References

Antelman, H., Scharf, C., Hecker, M., 2000. Phosphate starvation-inducible proteins in *Bacilus subtilis*: proteomics and transcriptional analysis. J. Bacteriol. 182, 4478–4490.

Chen, G., Gharib, T.G., Huang, C.-C., Thomas, D.G., Sheddan, K.A., Taylor, J.M.G., Kardia, S.L.R., Misek, D.E., Giordano, T.J., Iannettoni, M.D., Orringer, M.B., Hanash, S.M., Beer, D.G., 2002. Proteomic analysis of lung adenocarcinoma: identification of a highly expressed set of proteins in tumors. Clin. Cancer Res. 8, 2298–2305.

Cleveland, D.W., Fischer, S.G., Kirschner, M.W., Laemmli, U.K., 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel. J. Biol. Chem. 252, 1102–1106.

Conway, E.M., Liu, L., Nowakowski, B., Steiner-Mosonyi, M., Ribeiro, S.P., Michalak, M., 1995. Heat shock-sensitive expression of calreticulin in vitro and in vivo up-regulation. J. Biol. Chem. 270, 17011–17016.

Damodaran, T.V., Mecklai, A.A., Abou-Donia, M.B., 2002. Sarin causes altered time course of mRNA expression of alpha tubulin in the central nervous system of rats. Neurochem. Res. 27, 177–181.

Dunaeva, M., Adamska, I., 2001. Identification of genes expressed in response to light stress in leaves of *Arabidopsis thaliana* using RNA differential display. Eur. J. Biochem. 268, 5521–5529.

Dyck, V.A., Thomas, B., 1979. Brown Planthopper; Threat to Rice Production in Asia. International Rice Research Institute Los Banos, Philippines.

Hamilton, C.A., Good, A.G., Taylor, G.J., 2001. Induction of vacuolar ATPase and mitochondrial ATP synthase by aluminium in aluminium-resistant cultivar of wheat. Plant Physiol. 125, 2068–2077.

Heinrichs, E.A., 1994. Impact of insecticides on the resistance and resurgence of rice planthoppers. In: Denno, R.F., Perfect, T.J. (Eds.), Planthoppers: Their Ecology and Management. Chapman and Hall Press, New York, pp. 571–614.

Hemingway, J., Karunaratne, S.H.P.P., Claridge, M.F., 1999. Insecticide resistance spectrum and underlying resistance mechanisms in tropical populations of the brown planthopper (*Nilaparvata lugens*) collected from rice and wild grass *Leersia hexandra*. Int. J. Pest Mgmt. 45, 215–223.

Ishimura, R., Ohsako, S., Kawakami, T., Sakaue, M., Aoki, Y., Tohyama, C., 2002. Altered protein profile and possible hypoxia in the placenta of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-exposed rats. Toxicol. Appl. Pharmacol. 185, 197–206.

Komatsu, S., Rakwal, R., Li, Z., 1999. Separation and characterization of proteins in rice suspension cultured cells. Plant Cell Tissue Organ Culture 55, 183–192.

Konishi, H., Ishiguro, K., Komatsu, S., 2001. A proteomics approach towards understanding blast fungus infection in rice grown under different levels of nitrogen fertilization. Proteomics 1, 1162–1171.

Natera, S.H., Guerreiro, N., Djordjevic, M.A., 2000. Proteome analysis of differentially displayed proteins as a tool for investigation of symbiosis. Mol. Plant Microbe Interact. 13, 995–1009.

Pratt, W.B., Toft, D.O., 2003. Regulation of signaling protein function and trafficking by hsp90/hsp70-based chaprone machinery. Exp. Biol. Med. 228, 111–133.

Robertson, J.L., Russell, R.L., Savin, N.E., 1980. POLO: a user's guide to probit or logic analysis. United States Forestery Service Technical Report PSW.

Sogorb, M.A., Vilanova, E., 2002. Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. Toxicol. Lett. 128, 215–228.

Suh, S.-O., Noda, H., Blackwell, M., 2001. Insect symbiosis: derivation of yeast-like endosymbionts within an entomopathogenic filamentous lineage. Mol. Biol. Evol. 18, 995–1000.

Wellner, D., Paneerselvam, C., Horecker, B.L., 1990. Sequencing of peptides and proteins with blocked N-terminal amino acids: N-acetylserine or N-acetylthreonine. Proc. Natl. Acad. Sci. USA 87, 1947–1949.