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Proteomic investigation of the molecular mechanism of king cobra venom L-amino acid oxidase induced apoptosis of human breast cancer (MCF-7) cell line

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Snake venom is known for its therapeutic applications since long. Researchers have earlier demonstrated antiarthritic, anticancer, anti-inflammatory, antinociceptive activities of snake venom toxins apart from their use in the treatment of alzheimer's disease, neural trauma, parkinson's disease, stroke, etc. King cobra [*Ophiophagus hannah* (Cantor)] venom L-amino acid oxidase (OH-LAAO), a LAAO that possesses unusual thermal stability, also exhibits potent and selective antiproliferative activity against human tumorigenic cell lines. In this study, we investigated molecular mechanism of the enzyme induced apoptosis by examining the differential protein expressions in MCF-7 cell after treatment with the enzyme, using 2DE for separation and MALDI-TOF/TOF for protein identification. Proteomic analysis revealed a total of 21 differentially expressed proteins that are involved in various biological processes, of which 8 were involved in LAAO-induced cell death, including stress response, oxido-reduction, protein ubiquitination, proteolysis, and apoptosis. Upregulation of NADPH-cytochrome P450 reductase, in particular, may trigger excessive production of cellular ROS and contribute further to cellular oxidative stress and potentiate the cytotoxic action of the enzyme. These alterations of protein expression that are involved in different pathways or cellular functions were presumably caused by the non-specific oxidative modification of transcriptional factors, which may further modulate the activity of the signalling proteins that eventually lead to apoptosis and cell death. The results are consistent with earlier observations from gene expression studies that also demonstrated the involvement of non-specific oxidative modifications of signalling molecules in the apoptosis induced by OH-LAAO.

Keywords: Anticancer, Antiproliferative activity, Breast adenocarcinoma, Cytotoxicity, MALDI-TOF/TOF, *Ophiophagus hannah*, Oxidative stress, 2D-PAGE, Protein expression, ROS, Snake vernom, Transcriptional factors modification

Medicinal applications of snake venom and toxins are known in folk and traditional practice, and it has been the focus of research among toxinologists since last few decades targeting drug development[1]. Venom toxins of banded krait and cobra have been shown to possess antiarthritic[2,3], anticancer[1,2,4], anti-inflammatory[2,5] and antinociceptive[2,6] activities. Further, snake venom toxins have exhibited curative potential against stroke[7], neural trauma, alzheimer's disease and parkinson's disease[8]. Snake venom L-amino acid oxidase (LAAO, L-amino acid: O_2 oxidoreductase, EC 1.4.3.2), a flavoenzyme found in snake venom toxin catalyzes the stereospecific oxidative deamination of L-amino acid substrate to produce the corresponding α-keto acid, hydrogen peroxide and ammonia[9-11]. The enzyme exhibits a wide range of pharmacological activities including apoptosis-inducing, edema-inducing, inhibition or induction of platelet aggregation, bactericidal and antiviral activity[9]. King cobra [*Ophiophagus hannah* (Cantor)] venom L-amino acid oxidase (OH-LAAO) (<4% venom dry wt.), unlike most snake venom LAAOs, is a thermal-stable LAAO. At neutral pH, the enzyme retained its full activity even after 5 days of incubation at 37°C. This enzyme was also stable at alkaline condition and was not inactivated by freezing[12]. These favourable stability properties render OH-LAAO a suitable candidate to be used for investigations for therapeutic applications.

There have been various studies reported on the antiproliferative activities of snake venom L-amino acid oxidases on a variety of cell lines[13-17]. OH-LAAO from unknown geographical origin has shown antiproliferative activity on murine melanoma B16/F10, human fibrosarcoma HT 1080, murine epithelial cells Balb/3T3[18]. In our previous study,

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OH-LAAO (Malaysia) has been shown to exhibit extremely potent cytotoxicity against MCF-7 (breast adenocarcinoma) cells with IC_{50} value of 0.05 µg/mL after 24 h incubation. The enzyme induced apoptosis involved generation of oxidative stress, as addition of catalase (a H_2O_2 scavenger) significantly reduced the cytotoxicity of the enzyme[19]. We also demonstrated that the enzyme effectively inhibited growth of solid PC-3 tumor, implanted subcutaneously in immune odeficient NU/NU (nude) mice[20]. Nevertheless, the exact mechanisms leading to cell death induced by the enzyme are still not clear. Recently, using oligonucleotide microarray analysis, we demonstrated the potent apoptosis inducing activity of OH-LAAO was likely due to the direct cytotoxic effect of H_2O_2 generated during the enzymatic reaction, as well as the non-specific oxidation of signalling molecules[21].

While microarray technology enables simultaneous measurement of global mRNA level reflecting gene expression and allows changes in expression to be measured between different cellular states[22], measurement of mRNA level (gene expression) alone is insufficient to give a complete picture of cellular activity in response to external stimuli, as each gene may give rise to multiple proteins due to the alternative splicing of mRNA, and also the post-translational protein modifications processes[23]. Therefore, alternative approaches such as proteome analysis coupled with gene expression analysis may be necessary to provide a more completed/dynamic reflection on the changes in the biological processes within the cells. Here, we examined the alterations in protein expression profile of human breast cancer (MCF-7) cell line as a result of apoptosis induced by the king cobra venom LAAO. The proteomic profiling was carried out using 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled with MALDI-TOF/TOF analysis.

Material and Methods

Isolation of king cobra venom L-amino acid oxidase (OH-LAAO) and assay of enzyme activity

King cobra (*Ophiophagus hannah*) venom was milked from several adult Malaysian king cobras obtained from Snake Valley (Seremban, Malaysia). The snakes were identified by one of the authors (NH Tan). King cobra venom LAAO (OH-LAAO) was purified using a single-step Resource Q high performance liquid chromatography (HPLC)[24]. The enzyme activity was determined using horseradish peroxidase coupled assay, as described by Bergmeyer[25], using L-leucine as substrate[24]. Protein concentration was determined by the Bradford's method, using bovine serum albumin (BSA) as standard[26]. The homogeneity of OH-LAAO was assessed by 12.5% (w/v) SDS-PAGE[27]. Specific activity of the pure OH-LAAO was 437.7 µmol/min/mg[24].

Cell culture and treatment

Human breast adenocarcinoma (MCF-7) cell line was purchased from American Type Culture Collection (ATCC), USA. Cells were cultured in RPMI-1640 medium (Biowest, France) supplemented with 10% (v/v) foetal bovine serum (Sigma, USA). After overnight seeding in 37°C humidified incubator; cells were treated with 0.04 µg/mL (IC_{50} value) of OH-LAAO for 72 h.

Preparation of protein lysate

Protein lysates for Western blotting and proteomic analysis were prepared from OH-LAAO and phosphate buffered saline (PBS)-treated (control) cells. Cells were harvested using trypsin-EDTA, washed twice with PBS, and centrifuging at 1500 rpm for 5 min. Lysis buffer (containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% v/v IPG buffer, 40 mM DTT, and protease inhibitor mix) was added to the cell pellet and incubated on ice for 30 min. After centrifugation at 10000 ×g for 30 min at 4°C, the supernatant was recovered and stored at −80°C for subsequent experiments.

Two Dimensional gel electrophoresis (2D PAGE)

Protein lysate was precipitated and the concentration was determined using 2-D clean-up kit and 2-D quant kit, respectively according to the manufacturer's guideline (GE Healthcare, Sweden). One hundred fifty microgram proteins were rehydrated overnight on 13 cm immobilized pH gradient (IPG) gel strips with linear pH 3-10 gradient (GE Healthcare, Sweden). Strips rehydrated with proteins were transferred into IPG chambers and focused using Ettan IPGphor3 Isoelectric Focusing System (GE Healthcare, Sweden). Focusing was carried out according to the following profile: constant 500 V for 90 min, gradient to 1000 V for 1 h, gradient to 8000 V for 150 min, and constant 8000 V for 75 min for a total of 17000 Vh. Prior to electrophoresis, the IPG strips were first subjected to a two-step equilibration procedure for 15 min each. Firstly, the strips were equilibrated with SDS

equilibration buffer I [6 M urea, 50 mM Tris-HCl (pH 8.8), 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) orange G, and 50 mg (w/v) DTT], followed by equilibrated with SDS equilibration buffer II [6 M urea, 50 mM Tris-HCl (pH 8.8), 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) orange G, and 125 mg (w/v) IAA]. The strips were then subjected to second dimensional SDS-PAGE on 12.5% SDS-PAGE, using SE 600 Ruby electrophoresis tank (GE Healthcare, Amersham Biosciences, Sweden). The running parameter was programmed as follows: 80 V (15 mA/gel) for 20 min, and followed by 200 V (40 mA/gel) for 6 h. The gels were stained using Pierce® silver stain for mass spectrometry kit, according to the manufacturer's instructions (Thermo Scientific Pierce, USA). Three independent gels were run for each treatment (n = 3). Image acquisition was performed using Image Scanner™ III (LabScan6.0, Swiss Institute of Bioinformatics). Differences in the protein expression were identified by ImageMaster™ 2D Platinum 7.0 software (GE Healthcare, Amersham Biosciences, Sweden). Protein spots that displaying at least 1.5-fold difference in expression (ANOVA *P*-value <0.05) were selected and excised for MALDI-TOF/TOF analysis.

In-gel enzyme digestion

Protein spots of interest were manually excised from the gels and destained using 50 mM sodium thiosulphate containing 15 mM potassium ferricyanide. The gel plugs were then washed with 50% acetonitrile in 100 mM ammonium bicarbonate, incubated with 100% acetonitrile for 15 min, and dried completely using speed vacuum. The gel plugs were subsequently incubated with 150 ng trypsin (Trypsin Gold, mass spectrometry grade, Promega) in 50 mM ammonium bicarbonate overnight at 37°C. The digested samples were extracted using 50% acetonitrile and followed by 100% acetonitrile. The supernatant of the extractions were dried using speed vacuum. Prior to MALDI-TOF/TOF mass spectrometry analysis, peptides were reconstituted using 0.1% (v/v) formic acid and desalted using C18ZipTip® (Milllipore, Bedford, MA, USA).

MALDI-TOF/TOF mass spectrometry and database searching

Prior to MALDI-TOF/TOF analysis, peptide samples were mixed with an equal volume of α-cyano-4-hydroxycinnamic acid matrix [(10 mg/mL in 50% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid]. A volume of 0.7 µL of each sample was spotted onto Opti-TOF™ LC/MALDI INSERT (123 × 81 mm) (Applied Biosystems Inc., CA, USA) and allowed to air dry. MALDI-TOF/TOF analysis was performed using MDS SCIEX 4800 Plus MALDI TOF/TOF™ analyzer (Applied Biosystems Inc., CA, USA). All acquired spectra from the samples were processed using Data Explorer™ 4.0 software (Applied Biosystems Inc., CA, USA). The data was searched against SwissProt database using MASCOT search engine version 2.2 (Matrix Science Inc., MA, USA). Typical search parameters for MASCOT search engine were defined as follows: taxonomy; *Homo sapiens,* protease enzyme; trypsin, fixed modification; carbamidomethylation, variable modification; oxidation of methionine, MS/MS fragment tolerance; ±0.2 Da. Protein scores greater than 55 were considered significant (*P* <0.05) and classified according to SwissProt and Gene Ontology Databases.

Western blot analysis

Protein lysate concentration was determined using 2-D quant kit according to the manufacturer's instructions (GE Healthcare, Sweden). Twenty micrograms protein of each samples were loaded on a 12.5% of SDS-polyacrylamide gel. Electrophoresis was carried out at a constant voltage of 90 V for approximately 2 h. After electrophoresis, proteins were transferred onto iBlot™ Polyvinylidene fluoride (PVDF) membrane using iBlot™ dry blotting system, iBlot™ gel transfer device (Invitrogen™, Grand Island, USA) at 20 V for 8 min. The membrane was blocked with blocking solution (5% BSA in TBS-T) for 1 h, followed by overnight hybridization with primary antibodies: mouse-anti-Hsc 70 or mouse-anti-Grp 75 (Abcam, UK). After washing with Tris buffered saline buffer consisting 0.1% Tween 20 (TBST) thrice, the blot was incubated with secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature, 25°C. The immune-reacted bands were detected using 1-step 3,3',5,5'-tetramethylbenzidine (TMB)-blotting solution (Thermo Scientific, USA) for 3-5 min. Membrane was stripped in the stripping buffer [0.4 M Glycine, 2% (v/v) Tween-20, and 0.2% (w/v) SDS, pH 2.2], followed by re-hybridized with antibody against beta-actin, as a loading control. Calculation of intensity was done using ImageJ by NIH (https://imagej.nih.gov/ij/).

Data analysis

Differences in the protein expression were analyzed using ImageMaster™ 2D Platinum 7.0 software and ANOVA *P*-values were applied (GE Healthcare, Sweden). Changes of 1.5-fold or greater in protein expression (*P* <0.05) were considered as significant.

Results

Proteomic analysis of MCF-7 cells treated with OH-LAAO

Fig. 1 shows the representative 2D-PAGE images for control and OH-LAAO treated MCF-7 samples. A total of 823 well-defined protein spots were detected but of these, only 21 protein spots are appeared in all replicates (n=3) and exhibiting 1.5-fold or greater change in expression (Protein scores greater than 55 are significant, P <0.05). Those protein spots (as indicated by numbered circles) were subsequently excised for protein identification by mass spectrometry (Table 1). Of the 21 proteins, 10 (47.6%) were upregulated and 11 (52.4%) were downregulated. However, five of the spots were with protein score of <55. The identified proteins were further categorized into 8 groups (summarized below) based on their biological functions according to SwissProt and Gene Ontology Databases (Table 1).

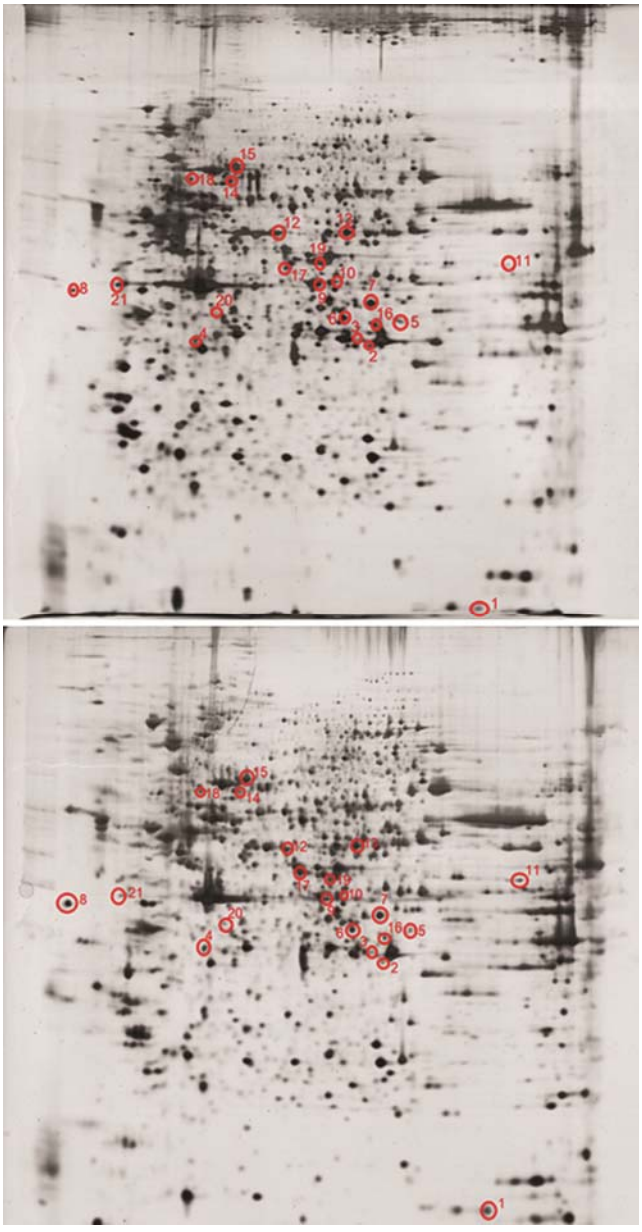

Fig. 1 — Proteomic profiling of the differentially expressed proteins in MCF-7 cells treated with OH-LAAO. [The differentially expressed proteins (indicated by circles with numeral) in control (top) or OH-LAAO (bottom) treated MCF-7 cells were excised for mass spectrometry analyses]

Regulation of proteins involved in metabolism

Two proteins that are involved in metabolism were differentially expressed: cytosolic 10-formyltetrahydrofolate dehydrogenase (upregulated), and UDP-glucose 4-epimerase (downregulated).

Regulation of proteins involved in mRNA processing and translation

Four proteins in this category were found to be differentially expressed in the LAAO-treated MCF-7 cells. Of these, three proteins were down-regulated (neuroguidin, poly(rC)-binding protein 1, and heterogeneous nuclear ribonucleoprotein H). The upregulated protein was 40S ribosomal protein SA.

Regulation of proteins involved in nucleotide and amino acid biosynthesis

Three proteins in this category were differentially expressed, including two downregulated (ribose-phosphate pyrophosphokinase 1, and mitochondrial ornithine aminotransferase) and one upregulated (nucleoside diphosphate kinase B) proteins.

Regulation of proteins involved in oxido-reduction

Three proteins involved in oxido-reduction were differentially expressed following LAAO-treatment: NADPH-cytochrome P450 reductase, and mitochondrial monofunctional C1-tetrahydrofolate synthase (both up-regulated) and D-3-phosphoglycerate dehydrogenase (downregulated).

Regulation of proteins involved in protein ubiquitination and proteolysis

A protein that is involved in ubiquitination was upregulated (E3 ubiquitin-protein ligase UBR5), whereas another protein that acts as protease inhibitor or negative regulator of proteolysis was downregulated (alpha-1-antichymotrypsin).

Regulation of stress-related proteins

Heat shock cognate 71 kDa protein, and stress-70 protein were all found to be upregulated in the LAAO-treated cells.

Regulation of proteins involved in structural integrity

Three proteins involved in structural integrity were found to be differentially expressed in the LAAO-treated cells. The upregulated proteins were lamin-B1,

Table 1 — Identification of the differentially expressed proteins by MALDI-TOF/TOF mass spectrometry

Spot no	Accession no	Protein description	Peptide mass	MS/MS-derived sequence	MW (Da)	Protein scores	% coverage	Fold-change
Function: Metabolism								
4	O75891	Cytosolic 10-formyltetrahydrofolate dehydrogenase	1029.33 1126.56 1196.65 1265.42 1287.87 1747.52 1307.62	MKVGNPLDR SVWQRILPK AKGQALPDVVAK DINKALYVSDK FAELTLKAGIPK AGLILFGNDDKMLLVK ECEVLPDDTVST	99621.9	49	9	3.3
16	Q14376	UDP-glucose 4-epimerase	1624.90 1858.49 851.95 1178.84	AVGESVQKPLDYYR FFIEEMIRDLCQADK PQGIPNNL HIAALRKLKE	38656.4	64	13	-1.8
Function: mRNA processing and translation								
6	Q8NEJ9	Neuroguidin	1088.50 1199.86 1082.64	DARHPHVTR LVEIRTVLEK ELKEQYSDA	35872	35	9	-1.8
7	Q15365	Poly(rC)-binding protein 1	1086.57 1231.66 1388.88 1385.99 1615.92 1247.82	IANPVEGSSGR INISEGNCPER IITLTGPTNAIFK LVVPATQCGSLIGK AITIAGVPQSVTECVK QVTITGSAASISL	37987.1	137	22	-1.5
12	P31943	Heterogeneous nuclear ribonucleoprotein H	956.56 1013.43 1092.53 1340.68 1447.90 1544.90 1684.95 1158.80	IQNGAQGIR THYDPPRK VHIEIGPDGR AEVRTHYDPPR GLPWSCSADEVQR DLNYCFSGMSDHR HTGPNSPDTANDGFVR FFSDCKIQNG	49483.5	313	20	-1.6
21	P08865	40S ribosomal protein SA	912.52 1200.86 1203.58 1291.81 1542.93	LLVVTDPR TWEKLLLAAR FAAATGATPIAGR SDGIYIINLKR FTPGTFTNQIQAAF	32947.5	195	19	2.0
Function: Nucleotide and amino acid biosynthesis								
1	P22392	Nucleoside diphosphate kinase B	984.60 994.65 841.99	GLVGEIIKR GDFCIQVGR NIIHGSDS	17401	70	15	1.6
2	P60891	Ribose-phosphate pyrophosphokinase 1	1388.85 1184.82 1336.94 1685.47 1106.56	WIRENISEWR IADRLGLELGK VTAVIPCFPYAR IQVIDISMILAEAIR THNGESVSYL	35325.1	108	18	-1.6
17	P04181	Ornithine aminotransferase, mitochondrial	1237.82 1247.42 1736.96 1982.56 2100.84 814.94	IVFAAGNFWGR SQVDKLTLTSR YGAHNYHPLPVALER TVQGPPTSDDIFEREYK WLAVDYENVRPDIVLLGK VAIAALEE	48846.3	84	18	-1.7

(*Contd*.)

Table 1 — Identification of the differentially expressed proteins by MALDI-TOF/TOF mass spectrometry

Spot no	Accession no	Protein description	Peptide mass	MS/MS-derived sequence	MW (Da)	Protein scores	% coverage	Fold-change
Function: Protein ubiquitination and proteolysis								
8	O95071	E3 ubiquitin-protein ligase UBR5	900.56	SLRAAGLGR	312351.6	42	10	3.2
			905.93	SSAGARDSR				
			913.48	ANAHFILK				
			921.45	VDGAYVAVK				
			947.11	FAQLALER				
			990.49	TTEAKPESK				
			1007.48	AGSSSSRSLR				
			1030.48	GTHTSLMQR				
			1044.63	AAVIIMAVEK				
			1063.42	EVVFVEDVK				
			1106.58	SARLDLLYR				
			1163.44	MTAREEASLR				
			1177.89	VQAMQPAFASK				
			1175.39	REEAIAVTMR				
			1409.91	VFVILSVEMASSK				
			1823.85	WSSGVGGSGGGSSGRSSAGA				
			1893.52	R				
			1927.45	TEILAVNVDSKGVHAVLK				
			1895.58	LTYQDAVNLQNYVEEK				
			2009.53	NCLEVLPTKMSYAANLK				
			2063.83	TASPEDSDMPDHDLEPPR				
			1705.49	CLMEVTVDRNCLEVLPTK				
			1766.43	LHAMRKGLLDVLPKN				
				GENAEKLLQFKRWF				
19	P01011	Alpha-1-antichymotrypsin (SERPINA3)	1029.57	QSPRWSIR	10911.5	70	15	-2.6
			1067.84	LCLMYLRR				
			1277.88	AQKHLLPQQSK				
			2225.49	GTHVDLGLASANVDFAFSLYK				
			981.34	DRFTEDAK				
			828.95	NLAVSQVV				
Function: Oxidoreduction								
5	P16435	NADPH-cytochrome P450 reductase	914.53	LEQLGAQR	77097.4	64	12	1.5
			916.44	GVATNWLR				
			923.52	ALVPMFVR				
			1029.43	EELAQFHR				
			1111.22	QDREHLWK				
			1115.59	GVATNWLRAK				
			1473.96	LQARYYSIASSSK				
			1175.80	VPEFTKIQTL				
			1070.52	RLEQLGAQR				
11	Q6UB35	monofunctional C1-tetrahydrofolate synthase, mitochondrial	1240.64	TDLSLSHQPDK	12435.6	58	10	2.3
			1244.85	DIELCPEAQVK				
			1439.91	GGWWLPIVDKIR				
			1643.96	MWRGGWWLPIVDK				
			2055.83	IDRYTQQGFGNLPICMAK				
			1029.42	AIIQAGDDNL				
			1107.53	TRVHGLALQI				
			1043.59	GGGGGGGREGLLG				

(*Contd*.)

Table 1 — Identification of the differentially expressed proteins by MALDI-TOF/TOF mass spectrometry

Spot no	Accession no	Protein description	Peptide mass	MS/MS-derived sequence	MW (Da)	Protein scores	% coverage	Fold-change
13	O43175	D-3-phosphoglycerate dehydrogenase	901.34 1099.45 1384.95 1320.87 1488.58 2431.92 1150.89	IGREVATR GGIVDEGALLR EVATRMQSFGMK VLISDSLDPCCR AGTGVDNVDLEAATR ALQSGQCAGAALDVFTEEPPR DR VQFVDMVKGK	57355.7	104	17	-1.5
Function: Stress-related protein								
14	P11142	Heat shock cognate 71 kDa (Hsc70)	1081.53 1199.67 1228.53 1253.79 1252.82 1450.99 1480.95 1481.97 1487.83 1982.59 1423.85	LLQDFFNGK DAGTIAGLNVLR VEIIANDQGNR FEELNADLFR MKEIAEAYLGK IQKLLQDFFNGK ARFEELNADLFR SQIHDIVLVGGSTR TTPSYVAFTDTER TVTNAVVTVPAYFNDSQR GGGTFDVSILTIED	71082.3	100	21	1.9
15	P38646	Stress-70 protein, mitochondrial (Grp75)	900.48 958.54 972.59 1029.57 1242.69 1290.84 1341.69 1361.87 1446.99 1450.89 1568.93 1592.96 957.32 1920.53 1893.45 1522.84 2004.87 632.88 1169.65 840.94	HIVKEFK VLENAEGAR NVPFKIVR LFEMAYKK DAGQISGLNVLR VQQTVQDLFGR ASNGDAWVEAHGK AQFEGIVTDLIR SDIGEVILVGGMTR TTPSVVAFTADGER QAVTNPNNTFYATK LLGQFTLIGIPPAPR RLVGAAASRG NGLSHEAFRLVSRRDY NEPTAAALAYGLDKSEDK GQFTLIGIPPAPRGV LPADECNKLKEEISKMR SASRAAA PEVQKDIKNV HVSAKDKG	73919.9	508	35	6.6
Function: Structural integrity								
9	P60709	Actin, cytoplasmic 1	1132.63 1198.85 1516.83 1790.96 1854.51	GYSFTTTAER AVFPSIVGRPR QEYDESGPSIVHR SYELPDGQVITIGNER MDDDIAALVVDNGSGMCK	42051.9	79	18	-1.5
18	P20700	Lamin-B1	999.62 1044.69 1045.56 1076.63 1215.68 1251.88 1293.85 1397.82 803.98 1468.97	AKLQIELGK LQEKEELR DAALATALGDK LAVYIDKVR LLEGEEERLK ALYETELADAR LREYEAALNSK HETRLVEVDSGR LALDMEI CQSLTEDLEFRK	66652.7	95	17	1.6

(*Contd*.)

Table 1 — Identification of the differentially expressed proteins by MALDI-TOF/TOF mass spectrometry

Spot no	Accession no	Protein description	Peptide mass	MS/MS-derived sequence	MW (Da)	Protein scores	% coverage	Fold-change
20	P05787	Keratin, type II cytoskeletal 8	1555.86 1532.74 1204.83 1510.94 1052.56 1358.85 788.93 1007.56	QKSYKVSTSGPRAF LLQQQKTARSNMD NEFVLIKKDV YMNKVELESRLE LSSAYGGLTSP GKLVSESSDVLPK AKLSELE YRKLLEGE	53704	76	16	2.0
Function: Others								
3	Q3SY17	Solute carrier family 25 member 52	1613.84 1522.96	SLISWGIINATYEF MIDSEAHEKRPPI	34042	52	9	-2.2
10	O14793	Growth/differentiation factor 8	1732.58 1221.65 903.55	EQIIYGKIPAMVVDR ANYCSGECEFV SSRIEAIK	43520.8	43	9	-2.6

[A positive fold-change indicates up-regulated, while a negative fold-change indicates down-regulated in protein expression in relative to the control. Protein scores greater than 55 are significant ($P < 0.05$). Data was obtained from tri plicate analyses (n = 3)]

and keratin type II cytoskeletal 8. The downregulated protein was actin cytoplasmic 1.

Regulation of proteins involved in other functions

The differentially expressed proteins that were grouped under this category are solute carrier family 25 member 52, and growth/differentiation factor 8. Both were downregulated, and with protein score <55.

Validation of differential expression of proteins by Western blot analysis

Western blot analyses were performed to validate the observed alterations in protein expression by 2D-PAGE proteomic analysis. The heat shock cognate 71 kDa (Hsc70) and stress-70 proteins (Grp75) were selected for Western blot validation, using beta-actin as a loading control (intensities range from 58-67). As shown in Fig. 2, both proteins examined were upregulated in the LAAO-treated samples (intensities 47 for Hsc70, 37 for Grp75) when compared with the control (intensities 25 and 18, respectively). The results were consistent with the observations in the 2D-PAGE proteomic analysis.

Discussion

In this study, we investigated the alterations of protein expression profile of MCF-7 cells as a result of OH-LAAO treatment. Proteomic approach allows identification of differentially expressed proteins in response to the specific treatment. The present proteomic analysis revealed that the LAAO treatment of the MCF-7 cell resulted in differential expression of 21 proteins. The proteins are involved in various biological processes, and only those proteins that are related to cell death process were considered in greater detail.

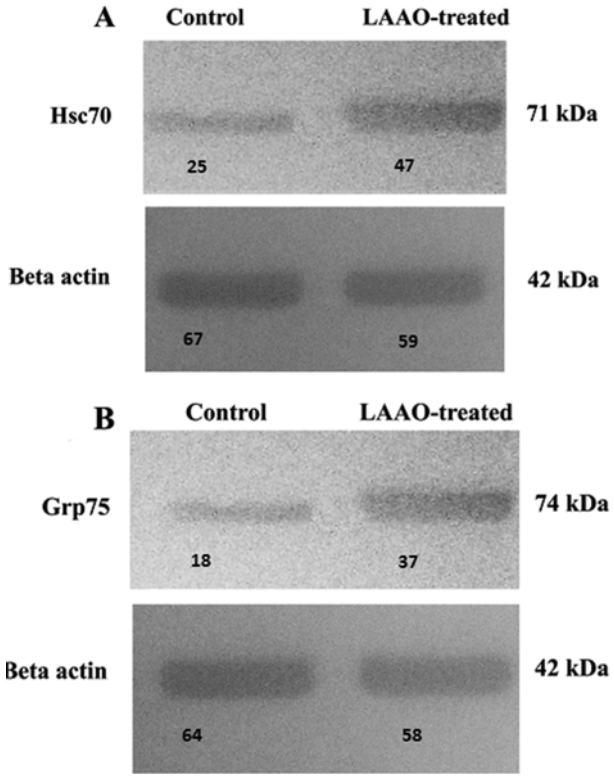

Fig. 2 — Western blot analysis of Hsc70 and Grp75 in LAAO-treated MCF-7 cells. [The expression level of (A) Hsc70 and (B) Grp75 in the LAAO-treated cells was compared to the control, using beta-actin as an equal loading control. The numbers indicate intensities of the bands. Molecular weights are indicated on the right. The images are representative of three independent experiments]

Modulation of proteins involved in stress response

King cobra venom LAAO (OH-LAAO) has been shown to induce oxidative stress through the production of H_2O_2 during the enzymatic reaction[19]. Synthesis of stress proteins is a ubiquitous characteristic for cells in response to various environment insults including oxidative stress[28]. Increase expressions of stress proteins confer cellular resistance against oxidative stress condition.

In the present work, the increase in the expression of heat shock cognate 71 kDa (Hsc70) and mitochondria stress-70 protein (Grp75) provided evidence for the role of oxidative stress in king cobra venom LAAO-mediated cell death. The Hsc70, and Grp75 function to regulate cell survival and confer cytoprotection against oxidative stress[29-31] through the modulation of the activity of ROS scavenger[32]. The increase in the expression of these two proteins was also validated by Western blot analysis.

The observed increased expression of the stress-related proteins confirms the earlier suggestion that the primary event leading to cell death as a result of the OH-LAAO treatment is due to oxidative stress induced by H_2O_2[19]. Since the amount of enzyme used in the study (0.04 µg/mL) is so low, the net amount of H_2O_2 released by the enzymatic action must be very limited, yet the enzyme exhibits extremely potent cytotoxic activity (IC_{50} = 0.04 µg/mL). It has been suggested that the potent cytotoxic effect induced by OH-LAAO may be due to its ability to bind to cancer cells, and thereby releasing high local levels of H_2O_2 on the surface of cancer cells[19]. Nevertheless, gene expression studies indicated that there is also the possibility of involvement of other molecular mechanisms that can trigger excessive production of cellular ROS[21]. Such mechanism may involve cellular oxido-reductases that regulate the balance between cellular oxidant and reductant levels, as discussed below.

Modulation of proteins involved in oxido-reduction

Among the differentially expressed proteins that are involved in oxido-reduction, NADPH-cytochrome P450 reductase is the enzymatic component of ROS producing system in the cells[33]. Cytochrome P450 reductase (CPR) facilitates the transfer of electrons from NADPH to cytochrome P450 isoenzymes[34], and as a result increases the production of ROS during the reaction cycle and hence, the upregulation of the protein contribute further to cellular oxidative stress[35] and potentiate the cytotoxic action of OH-LAAO. The upregulation of the cytochrome P450 enzyme is consistent with the gene expression studies that demonstrated a substantial up-regulation of CYP1A1 and CYP1B1 genes[21].

Modulation of proteins involved in ubiquitination and proteolysis

The E3 ubiquitin-protein ligase confers specificity to the ubiquitination process and directs the conjugation of ubiquitin (a polypeptide co-factor) to specific target proteins. The ubiquitinated proteins lead to the recognition by the 26S proteasome that degrades ubiquitinated proteins into small peptides[36]. Numerous studies have demonstrated that the ubiquitination-mediated degradation plays an important role in controlling the levels of various cellular proteins including many molecules that regulate cell death machinery, such as p53, caspases, and Bcl-2 family members. Ubiquitination-degradation of ring finger-containing members of the IAP (inhibitor of apoptosis) family proteins is required for apoptosis to occur, indicating that apoptosis proceeds through death pathways as well as ubiquitin-proteasome pathway[37].

Thus, the upregulation of E3 ubiquitin-protein ligase UBR5 (albeit with protein score of <55) and down-regulation of alpha-1-antichymotrypsin (SERPINA3), a serine-protease inhibitor, suggest that there was likely to be an overall increase in ubiquitination mediated degradation and proteolysis, which contributed to cell death. Again, this is consistent with the observations of upregulation of genes involved in proteolysis in oligonucleotide microarray analysis[21].

Modulation of proteins involved in cell proliferation and apoptosis

The three proteins; keratin type II cytoskeletal 8 (grouped under structural integrity), cytosolic 10-formyltetrahydrofolate dehydrogenase (group under metabolism), and 40S ribosomal protein SA (grouped under mRNA processing and translation) are all involved in the regulation of apoptosis. Studies have suggested that keratin type II cytoskeletal 8 (K8) was involved in modulating of cellular responses to pro-apoptotic stimuli by desensitizing the cells to pro-apoptotic signaling mediated by tumour necrosis factor-α (TNF-α) or by Fas ligand, by binding to their receptors[38]. Cytosolic 10-formyltetrahydrofolate dehydrogenase has been shown to induce G1 cell cycle arrest and p53-dependent apoptosis[39]. The 40S ribosomal protein SA (67-kDa laminin receptor), functions as a cancer-specific death receptor induced apoptosis via cGMP-mediated pathway[40]. The

upregulation of these proteins support the earlier report that OH-LAAO causes cell death by inducing apoptosis[19].

Comparison with the results from oligonucleotide microarray studies

In the present proteomic studies, 21 proteins were found to be differentially expressed as a result of OH-LAAO treatment. However, only a few of the altered proteins are related to the genes that were found to be expressed differentially in microarray analysis[21], including alpha-1-antichymotrypsin (SERPINA3, gene expression downregulated 1.9-fold, protein expression downregulated 2.6-fold) and cytochrome P450 reductase. Apart from that, an increased in the expression of Hsc70 and Grp75 proteins were also partly associated with the upregulation of glutathione peroxidase 2 (GPX2) gene. The lack of congruence between the results of gene expression studies (oligonucleotide microarray studies) and protein expression studies (proteomic studies), however, are expected. While the former investigated the alterations at transcriptional level, the proteomic studies investigated the alterations at translational level. Also, even though samplings of lysate proteins (for proteomic studies) and RNA (for microarray studies) were both done after 72 h OH-LAAO treatment, the proteome was more a reflection of the transcriptome of the cells existed sometime earlier before the harvest of the cellular RNA. It is reasonable to expect the cell's transcriptome to change as time progress, as the H_2O_2 and ROS generated as a result of OH-LAAO treatment continue to cause oxidative damages to the cellular proteins. Another complicating factor is that the dose of OH-LAAO used in the two studies differs.

Conclusion

On the mechanism of LAAO-induced apoptosis from this proteomic analysis, with the results of gene expression (microarray), it can be postulated that the alterations of gene expression observed were largely caused by non-specific oxidative modifications of signalling proteins, such as transcriptional factors, which further modulate the activity of the signalling proteins in redox-sensitive signal transduction pathways as well as in transcriptional regulatory networks. A total of 21 proteins involved in different/unrelated pathway and cellular functions were differently expressed, indicating that it is likely due to the result of non-specific oxidative modifications of signalling proteins caused by the cellular ROS generated.

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