CHAPTER 2

MATERIALS AND METHOD

2.1) MATERIALS

Chemicals	Company
Acetonitrile	Sigma U.S.A.
Acetone	Fluka U.S.A.
Acrylamide	aMESCO U.S.A.
Acetic acid	Merck U.S.A.
Agar	Bio-rad U.S.A.
Amido Black	Sigma U.S.A.
Ammonium bicarbonate	Fisher U.S.A.
Ammonium persulfate $(NH_4)_2S_2O_6$	Fluka U.S.A.
AspN	Sigma U.S.A.
Bromophenol blue	Sigma U.S.A.
CAPS (3-(cyclohexylamino)-1-propanessulfonic acid	Sigma U.S.A.
CHCA, α-cyano-4-hydroxy cinnamic acid $C_{10}H_7NO_3$	Sigma U.S.A.

Cy3 Maleimide	Bioscience Amershame U.K.
Cy5 Maleimide	Bioscience Amershame U.K.
DTNB, 5,5'Dithiobis(2-nitrobenzoic acid)	Sigma U.S.A.
DTE, 1,4-Dithioerythritol	Bio-rad U.S.A.
DTT, Dithiothreitol	Bio-rad U.S.A.
EDTA(Ethylenediamine tetraacetic acid)	Sigma U.S.A.
Ethanol	Merck U.S.A.
Glycerol	Sigma U.S.A.
HEPES (N-2[2-Hydroxymethyl] piperazine-N'-2[-ethasulfonic acid])	Sigma U.S.A.
IPG strips	Bioscience Amershame U.K.
Iodoacetamide	Sigma U.S.A.
Low melt agarose	Bio-rad U.S.A.
Methanol	Merck U.S.A.
Mineral oil	Bio-rad U.S.A.
Nutrient broth	Difco U.S.A.
PMSF (Phenylmethylsulfonyl fluoride)	Sigma U.S.A.

Protease inhibitor cocktail	Sigma U.S.A.
Protein assay	Bio-rad U.S.A.
Protein marker	Invitrogen U.S.A.
Pro Q Diamond	Molecular Probe U.S.A.
Sodium Chloride	Sigma U.S.A.
Sodium Dodecyl Sulfate	Sigma U.S.A.
Sypro Ruby	Molecular Probe U.S.A.
TCEP, Tris-(2-carboxyethyl)-phosphine	Sigma, U.S.A.
TEMED $(CH_3)_2NCH_2CH_2N(CH_3)_2$	Sigma U.S.A.
Thiourea	Sigma U.S.A.
Trichloroacetic acid (TCA)	Fluka U.S.A.
Trifluoroacetic acid (TFA)	Fluka U.S.A.
Tris-Glycine	Sigma U.S.A.
TRIZMA® Hydrochloride	Sigma U.S. A.
Trypsin	Promega U.S.A.
Urea	Bio-rad U.S.A.
1.5 M Tris buffer pH 8.8 (4XResolving gel buffer)	aMESCO U.S.A.

37.5:1 mono-stock solution	aMESCO U.S.A.

2.2) EQUIPMENT	**Company**
Image master software	Bioscience Amershame U.K.
IPG phore	Bioscience Amershame U.K.
LC MS/MS Q-STAR (Liquid Chromatography mass/ mass spectrometer)	Applied Bioscience U.S.A.
MALDI-TOF TOF MS (matrix associated desorption ionization-time of flight time of flight mass spectrometer)	Applied Bioscience U.S.A.
Molecular Imager Fx Pro Plus™	Bio-rad U.S.A.
Multiphore	Bio-rad U.S.A.
NOvex Xcell II	NOvex U.S.A.
PDQUEST 2-D analysis software	Bio-rad U.S.A.
Protein Blotting Apparatus	Bioscience Amershame U.K.
SDS-PAGE running	Bio-rad U.S.A.
Typhoon 9400 scanner	Bioscience Amershame U.K.

2.3) METHODS

2.3.1 Bacterial culture

Bacillus stearothermophilus TLS33, isolated from a soil in a hot spring in Chiang Mai, Thailand, The bacterial colony was picked from an agar plate and inoculated into 50 mL of nutrient broth. In 250 mL flask as preculture was incubated in the water bath at 65°C with shaking at 200 rpm. After 24 hours of preculture, 1 mL of cell suspension was transferred to 100 mL of fresh nutrient broth in 250 mL flask. (Triplicate for each experiment). The bacterium was cultured at 65°C until mid-log phase (OD600 ~0.6) and subsequently changed dramatically in physical such as salt (10% w/v NaCl), ethanol (10% v/v ethanol) cold (25°C) and various concentration of hydrogenperoxide. The cell were collected every 30 min after shift to stress until 120 minutes in order to study on protein expression in different time course. In each stress condition, the bacterial viable cells were counted by using viable plate count. For oxidative stress (treat with various concentration of hydrogenperoxide), the bacterium were collected cell after 15 minutes after it were treated with hydrogenperoxide.

2.3.2 Protein sample preparation

The bacterial cells were disrupted by sonication with 4 s for 10 min in preparation buffer pH 8.0 containing 10 mM Tris-HCl, 1 mM of ethylenediamine tetraacetic acid (EDTA) and 0.1 mM of phenylmethylsulfonyl fluoride (PMSF), and the supernatant was obtained by centrifugation at 4°C 12,000 rpm for 20 min. The sample solution was either precipitated by addition of 10% trichloroacetic acid (TCA) and 0.1% w/v dithioerythritol (DTE) or lyophilized. For TCA precipitation,

the mixture was stored overnight at -20°C and the pellet was obtained by centrifugation at 10000 x g 4°C for 15 min. The pellet was washed twice first with cold acetone containing 20 mM DTE and secondly with cold acetone without DTE, and centrifuged as described above. The supernatant was removed and the pellet was vacuum dried.

2.3.3 Cold Acetone/TCA precipitation

After cell was disrupted by sonication and they were separated cell debris. The proteins were concentrated and removed salt by cold acetone/TCA precipitation. Before adding into protein solution, 20% TCA in acetone were chilled in -20°C. Then, the 20% TCA in acetone was added into the protein solution by 1:1 v/v of protein/20% TCA acetone. And then the solution was thoroughly mixed by vortex. The white pellet was observed. After that it was chilled at -20°C at least 30 minutes. After then, the solution was centrifuged at 12,000 rpm for 10 minutes by bench-top centrifuge. The supernatant was discarded. The TCA was washed out from the protein pellet by using -20°C chilled acetone containing 0.1% DTE. Then it was mixed well by vortex and keep at -20°C for at least 30 minutes. This step was repeated in order to ensure TCA was completely removed out. Because TCA can interfere to isoelectric focusing electrophoresis (IEF) step. After that, the protein pellet was centrifuged at 20,000 rpm for 20 minutes and freeze dried. The protein sample was stored at -80°C before running 2DE gel in the next step.

2.3.4 Protein measurement

Protein measurement was used in sample preparation for one dimensional SDS-PAGE or sample preparation before IEF experiment in two-dimensional electrophoresis analysis. In this experiment two type of protein assay were used.

2.3.5 Bicinchonic acid (BCA) Protein Assay

BCA is used for the colorimetric detection and quantization of total protein. The method combined the reduction of Cu^{2+} to Cu^{+} by protein in alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of cuprous cation (Cu^{+}) using a unique reagent containing bicinchonic acid. The purplecolored reaction product of this assay is formed by the chelation of 2 molecules of BCA with one cuprous ion. The product exhibit strong absorbance at 562 nm that is linear with increasing protein concentration working range 20- 20,000 µg/ml.

2.3.6 Assay procedure

One hundred µl of standards and unknown samples were added into appropriately labeled test tubes. Two ml of the working reagent was added to each tube and mix well. Tubes were covered and incubated at 37°C for 30 min. After cooling all tubes to room temperature, the absorbance at 562 nm was measured with water as blank.

2.3.7 Bio-rad DC Protein Assay

It is a colorimetric assay for protein concentration following detergent solubilization. The reaction is similar to Lowry assay. The assay is base on the reaction of protein with alkaline copper tartrate solution and Folin reagent. Color development is primarily due to the amino acids tyrosine and histidine. The maximum absorbance is measured at 750 nm. SDS-PAGE sample buffer composition used for preparation was in denaturing conditions. The buffer compositions and concentration are indicated in Table 2.1

Table 2.1 SDS sample buffer composition

Compostions	**Concentration**
Tris-HCl pH 6.5	50 mM
DTT	0.1 M
Glycerol	10%
SDS	2%
Bromophenol blue	0.1%

2.3.8 SDS-PAGE gel composition

Resovling gel and stacking gel compositions used in this study are shown in Table 2.2

Table 2.2 SDS-PAGE

Reagent	**Volume**			
	7%	**10%**	**12.5%**	**15%**
30% Acrylamide:Bis (29:1)	5.84 mL	8.33 mL	10.42 mL	12.5 mL
1.5 M Tris-HCl (pH 8.8)	6.25 mL	6.25 mL	6.25 mL	6.25 mL
10% SDS	0.25 mL	0.25 mL	0.25 mL	0.25 mL
H_2O	12.52 µL	10.03 µL	7.94 µL	5.86 µL
TEMED	15 µL	15µL	15µL	15µL
Total	25 mL	25 mL	25 mL	25 mL

2.3.9 SDS-PAGE running conditions

Each protein sample was dissolved in sample buffer at 0.15 mg/ml. Each solution was separately loaded into the wells. The low molecular weight calibration kit was used as standard molecular weight marker proteins. The SDS-PAGE gel was run in NOvex Xcell II (NOvex, USA) at 25 mA per gel.

2.3.10 Protein marker composition and preparation

Low molecular weight protein calibration is a lyophilized mixture of six highly purified well-characterized proteins for using in molecular weight determination in the presence of sodium dodesyl sulphate (SDS). The components were phosphorylase b, Mr 97,000; albumin Mr 66,000; ovalbumin Mr 45,000; carbonic anhydrase Mr 30,000; Trypsin inhibitor Mr 20,100; α-lactalbumin 14,400. For Coomassie blue staining, one vial of the protein mixture was dissolved SDS-PAGE sample buffer 200 µl. 7 µl of the marker solution was loaded on one well for each separation. For Sypro Ruby, reconstituted the contents of a vial as described for Coomassie blue staining, then dilute aliquots by at least 50-fold in 1x sample buffer. The reconstituted protein solution was heated for 5 minutes at 95-100°C.

2.3.11 Two dimensional electrophoresis (2DE)

Lyophilized protein samples were resolve in lysis buffer containing 7 M, 2 M thiourea, 4% 3-[(3-chloamidopropryl)-dimethylamino]-1-propanesulfonate (CHAPS), 4mM tris-(2-carboxyethyl)-phosphine (TCEP), 100 mM DTE and 0.5% IPG buffer pH 4-7 Linear. The protein sample solutions containing 150 µg protein concentrations in 350 µL total volume were applied on IPGphor strip using IPGphore. The first-dimensional isoelectric focusing electrophoresis (IEF) on IPGphore was performed under the following condition:30 V, 12 h (rehydration) 100 V 3 h 350 V, 1 h, 500 V 1h 1000 V, 1 h 5000 V 1 h, 8000V 55 kVhr. After IEF, the IPG stripes,

2.3.12 Protein staining

2.3.12.1 Sypro Ruby staining

After 2DE were completed, the gels were fixed in the fixing solution containing10% methanol, 10% acetic acid for 30 minutes before Sypro Ruby staining. After that the fixing solution was discarding and the gels were washed twice with double distilled water for 30 seconds. The 350 ml of Sypro Ruby was added into the gel-container to cover the gel. After the gels were soak in Sypro Ruby solution for overnight with shaking speed at 50 rpm. Subsequently, the gels were destained twice in fixing solution for 30 minutes and followed by washing out the fixing solution with double distilled water and shake in shaker at 50 rpm speed. After that the gel was performed for image scanning.

2.3.12.2 Pro Q Diamond phosphoprotein staining

After 2DE were completed, the gels were fixed in Pro Q Diamond containing 50% methanol/10% acetic acid in order to wash out SDS for 1 hr. After that, the used fixing solution was discarded and the remaining SDS were washed out from the gels with fixing solution containing 50% methanol 10% acetic acid for overnight. After the gels were washed SDS out for overnight, the gels further were washed out the remaining fixing solution, which contained high percentage of methanol and acetic acid, with double distilled water. Double distilled water was added into gel container until covered the 2DE gels, the gels were allowed them shake with 50 rpm for 10 minutes. This was performed for 3 times to ensure the methanol and acetic acid was

completely removed out after that remove. The 350 ml of Pro Q Diamond was added into the gel container and covered the gels, after that shake with 50 rpm for 3 hours. This step required the light-protection and performed in dark place. After completed Pro Q Diamond staining, the Pro Q Diamond must to be removed the Pro Q diamond solution, otherwise, the image gel will have the inference from the image background. 350 ml of Pro Q Diamond destaining solution were added into the gel container and shake with 50 rpm for 1 hour in the dark place. This was performed for three times and it is optional further detain the gels if the background is still high. After that the gels were removed the destaining solution and washed again with double distilled water before image acquisition. For image acquisition, the gel images were obtained from Molecular Imager Fx Pro Plus™ (Bio-Rad) with scan with 532 nm wavelength at 50 micron resolution. For viewing total protein, the gels were performed as described in Sypro Ruby staining. The image gels were saved the image as gel or tiff file in order to further analysis with image analysis software.

2.3.12.3 Fluorescence labeling

2.3.12.3.1 Cy3 Maleimide and Cy5 Maleimide labeling

The intracellular protein 150 µg was dissolved in 50 µL of rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS but no adding DTT or primary NH_2 group-containing chemical. The protein was denatured for breaking disulfide bond by adding 10 µL of 18 mg/ml of TCEP and further stand at room temperature for 30 minutes. Subsequently, 10 µL of 50 fold stock solution was added into rehydration

buffer and stand it for 2 hours while it was gently mixed every 30 minutes. After that it was further incubated in 4°C overnight for completely dye labeling. Subsequently, the dye labeling solution was added DTT for making final concentration at 65 mM and incubated in room temperature for 30 minutes. The dye labeling solution subsequently added Ampholyte corresponding required pH and subjected to IEF.

2.3.12.3.2 Cy3 Maleimide labeling on thiol oxidized proteins

150 µg of protein was dissolved in rehydration buffer containing 7 M urea, 2 M thiourea and 4% CHAPS and no containing DTT. To blocking free disulfide group, 1 M iodoacetamide (IAM) was added into the protein solution with final concentration is 100 mM. After that it was incubated in room temperature for 30 minutes. For reducing sulfenic acid in protein molecule, subsequently, the 10 µL of 18 mg/ml of TCEP was added and further stand at room temperature for at least 30 minutes. Then, the 10 µL of Cy3 Maleimide from 50-fold dilution stock was added and 10 µL of 50 fold stock solution was added into rehydration buffer and was gently mixed every 30 minutes until 2 hours. After that, it was incubated in 4°C overnight for completely dye labeling. Subsequently, the dye labeling solution was added DTT with final concentration at 65 mM and incubated in room temperature for 30 minutes. The dye labeling solution subsequently added Ampholyte corresponding required pH and subjected to IEF.

2.3.13 Image analysis

After image gels were obtained from Image Scanners, the image gels were analyzed with Image analysis software. In this study, PDQUEST 2-D analysis software (Bio-rad) and Image Analysis software (Bioscience Amersham) were used for image analysis. For PDQUEST, briefly, first step, the images were acquired from documentary folder in gel file. After opened the gel images, the software selected control experiment as a reference gel image. Second step, the protein spots in image gel were detected and matched analysis with the software as optional setting. Third step, some protein spots the spot were manual detected and matched, optional some spots were not detected and the spot intensity were normalized in order to average and subtracted the background for obtain accuracy spot intensity. The data analysis also generated by the software and exported to Microsoft Excel, Microsoft Word or image file. For Image Analysis software, the gel images were performed as same as PDQUEST but there was background subtraction after manual spot detection and matching. The 3D viewing also obtained from this software. Spot quantity was analyzed by Microsoft excel.

2.3.14 Protein identification, database search and bioinformatics

2.3.14.1 Tryptic in-gel digestion

The interesting protein spots in 2DE gel were excised out by spot cutter machine and soaked in 25 mM ammonium bicarbonate buffer in 96 well. Subsequently, the each gel piece was transferred from 96-well to each new 250 µL-

micro centrifuge tube. After that the gel spots were washed the color reagent i.e. Sypro Ruby or Coomassie blue by soaking in 100 mL of 100 mM ammonium bicarbonate for 10 minutes and the buffer was removed out. The gel pieces were further soaked in the 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate for 10 minutes and the buffer was removed. 100 µL of 100 % ACN was added into the 250 µL-microcentrifuge tube for 5 minutes, subsequently, was discarded. The gel pieces were allowed air drying.

For reduction disulfide bond, the gel pieces were incubated in 50 µL of 10 mM DTT in 25 mM ammonium bicarobonate at 60 °C for 1 hour and then allowed temperature cool down. The 50 µL of 50 mM iodoacetamide (IAM) in ammonium bicarbonate was added into the solution and further incubated in 45 °C in the dark place in order to alkylate the free sulfydryl group and remove the excess DTT.

After the alkylation was completed, the gel pieces were soaked in 100 µL of 50 mM ammonium bicarbonate for 10 minutes and then the solution was discarded. They were further incubated in 50% ACN/50 mM ammonium bicarbonate for 10 minutes and then the solution was discarded. The 100 µL of 100% ACN was added in the 250 µL-microcentrifuge tube and stand at room temperature until the gel pieces changed to white. ACN was removed. The step from adding 50 mM ammonium bicarbonate until adding 100% ACN was repeated more twice. And the gel pieces was allowed to completely dry in the air dry.

After the gel pieces was dried completely. The 5 µL of 20 ng/µL stock trypsin was added into each tube and they were let to stand in 4°C for at least 45 minutes. And then they were subjected to heat block with further incubated at 37°C for 16

hours. After in gel-tryptic digestion was completed, the supernatant in each sample was transferred to new tube. The tryptic-digested peptides were extracted by adding 50% ACN/1% triofluoroacetic acid (TFA) 50 μL and sonicated for 10 min. After that the supernatant was collected with the previous supernatant. This step was repeat for twice. The collected tryptic-digested peptide solution was evaporated by speed vac until them dried. Before subjecting to MALDI-TOF MS or LC MS/MS, the solid particles were dissolved in 0.1% TFA or 0.1% formic acid respectively.

2.3.14.2 Matrix Associated Desorption/Ionization–Time of Flight mass/mass spectrometry (MALDI-TOF MS/MS)

MALDI-TOF MS/MS were performed on a dedicated Q-Tof Ultima™ MALDI instrument (Micromass) with fully automated data directed acquisition using predefined probe motion pattern and peak intensity threshold for switching over from MS survey scan to MS/MS, and from one MS/MS to another. At a laser firing rate of 10 Hz, individual spectra from 5 s integration period acquired for each of the MS/MS performed were combined, smoothed, deisotoped (fast option) and centroided using the Micromass PGS 2.0 data processing software. All individual MS/MS data thus generated from a particular sample well were then output as a single MASCOT-searchable peak list file and all peak list files generated from each the 1-D mLC fractions as deposited by Probot on the corresponding MALDI sample well were manually combined into a single .pkl file

2.3.14.3 Liquid Chromatography mass/mass spectrometer (LC MS/MS)

The tryptic digested peptides were analyzed by 1-D LC nanoESI MS/MS. The 1-D LC-nanoESIMS/ MS analysis was performed on an integrated nanoLCMS/MS system (Mircomass) comprising a three-pumping

2.3.14.4 N-terminal protein sequencing

After running 2DE gel was completed, the 2DE SDS-PAGE was incubated in transferring buffer for 5 minute. The filter paper was soaked in transferring buffer. For optimal results with this method, however, the gel was equilibrated with 6 transferring buffer prior to transfer. The 6 sheets of filter papers were placed on the cathode (negative, usually black), followed by the gel, the PVDF membrane, 6 sheets of the filter papers, and finally the anode (positive, usually red). In this research, the current of 400 mA and the transferring time for 2 hours were according to the manufacturer's instructions.

2.3.14.5 Procedure for staining proteins transferred on the membrane

After the protein transferring was completed, the orientation of the membrane on the gel was marked and immerse in staining solution containing 0.1% (w/v) solution of Naphtol Blue Black in 10% (v/v) methanol and 2% (v/v) acetic acid with gentle shaking for 2 min. The membrane was rapidly destained in 50% (v/v) methanol, 7% (v/v) acetic acid for 5-10 minutes or until the protein bands can be seen

clearly. The membrane was cut at the desired protein and then determined the N-terminal amino acid sequence by amino acid analyzer. N-terminal amino acid sequencing was performed by Edman degradation which followed the protocol from Proteomics Core Facility, Institute of Biological Chemistry, Academia Sinica at Taipei, Taiwan.

2.3.14.6 Protein Identification and Database searching

The both of mass spectrum from MALDI-TOF TOF MS and LC MS/MS are used for protein identification through MASCOT software from www.matrixscience.com. The searching options were set as oxidation at Methionine (M), Carbamidoethyl at cysteine (C). And the mass tolerance was set as 0.5 kDa. For MS/MS or based on protein, the mass tolerance was set on 0.5 kDa and variable protein modification option was set as oxidation at Methionine (M) and Carbalmidoethyl at Cysteine (C). The reliability was based on P-value from probability scoring. For some the proteins that were identified as "hypothetical protein" were used their sequences compared to other species via BLAST program. For N-terminal protein sequencing, the protein sequence was compared with other species by BLAST-P software.