CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and chemicals

2.1.1 Chemicals for investigation of GABA and amino acids content

Chemical	MW	Company
Acetonitrile	41.05	LAB-SCAN, Ireland
Alanine	89.10	Fluka, Switzerland
Arginine	174.20	BDH, biochemika, England
Asparagine	132.12	Fluka, Switzerland
Aspartic acid	133.11	Fluka, Switzerland
Cystein	121.16	Fluka, Switzerland
Glutamic acid	147.13	Fluka, Switzerland
Glutamine	146.14	Fluka, Switzerland
Glycine	75.07	Fisher chemical
Histidine	155.16	BDH, biochemical, England
Isoleucine	131.18	Sigma, USA
Leucine	131.18	BDH, biochemical, England
Lysine	146.21	Sigma, USA
Methionine	149.21	BDH, biochemical, England
Proline	115.13	Fluka biochemika, Switzerland
Phenylalanine	165.20	Fluka biochemika, Switzerland
Serine	105.09	Fluka, Switzerland
Threonine	119.12	Fluka, Switzerland
Tyrosine	181.19	Fluka, China
Tryptophan	204.23	BDH, biochemical, England
Valine	117.15	Fluka, Switzerland

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Chemical	**MW**	**Company**
Boric acid	61.84	CARLO, ERBA, France
Ethanol	46.07	BDH, England
Formic acid	46.03	Fisher, England
Gamma-aminobutyic acid	103.12	Fluka, China
Hydroxy-naphaldehyde (HN)	172.18	Aldrich, Germany
Methanol	32.04	MERCK, Germany
di-Sodium tetraborate	381.37	MERCK, Germany

2.1.2 Chemicals for determination of GAD activity

Chemical	MW	Company
Acetonitrile	41.05	LAB-SCAN, Ireland
Boric acid	61.843	CARLO, ERBA, France
Formic acid	46.03	Fisher, England
Gamma-aminobutyic acid	103.12	Fluka, China
L-Glutamic acid	147.13	Fluka, France
Hydroxy-naphaldehyde (HN)	172.18	Aldrich, Germany
Liquid nitrogen	-	-
Methanol	32.04	MERCK, Germany
2-Mercaptoethanol	78.13	Fluka, China
Pyridoxyl 5´phosphate (PLP)	265.16	Fluka, Switzerland
Sodium citrate	294.10	MERCK, Germany
Sodium tetraborate	381.37	MERCK, Germany
Sodium dihydrogen phosphate-di-hydrate	156.01	Fluka, Switzerland
Di-sodium hydrogen phosphate-di-hydrate	177.19	Scharlau, Spain

2.1.3 Chemicals for protein extraction and SDS-PAGE analysis

Chemical	MW	Company
Acrylamide	71.08	Wako pure chemical, Japan
Ammonium persulphate (APS)	228.20	Biorad, USA
Coomassie Brillant Blue R-250	826.0	Sigma, USA

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Chemical	MW	Company
Coomassie Brillant Blue G-250 solution kit	854.0	Biorad, USA
2[4-(2-hydroxyethyl)-1-piperazinyl] Ethane sulfonic acid (HEPES buffer)	238.3	Dijindo, Japan
Ethylene diamine tetraacetic acid disodium salt (Na_2-EDTA)	372.44	Sigma, USA
Dithiothreitol (DTT)	154.25	Wako pure chemical, Japan
Glycerol	92.09	Wako pure chemical, Japan
Leupeptin	463.01	Wako pure chemical, Japan
Magnesium choride	95.21	Wako pure chemical, Japan
N,N' methylene bis acylamide	157.14	Wako pure chemical, Japan
Polyvinyl polypyrrolidone (PVPP)	2500	NAKARAI chemicals, Japan
Potassium hydroxide	56.10	Wako pure chemical, Japan
Phenyl methane sulphonyl fluoride	174.19	Wako pure chemical, Japan
Sodium dodecyl sulphate (SDS)	288.38	Wako pure chemical, Japan
N,N,N',N' - Tetramethylethylenediamine - (TEMED)	116.20	Wako pure chemical, Japan
Tris (hydroxymethyl) aminomethane- (Tris-HCl)	121.14	Wako pure chemical, Japan

2.1.4 Chemicals for western blotting analysis

Chemical	MW	Company
Acylamide	71.08	Wako pure chemical, Japan
Ammonium persulphate (APS)	228.20	Biorad, USA
Blocking agent ECL TM membrane - blocking agent kit	-	GE healthcare, USA
Coomassie Brillant Blue R-250	826.0	Sigma, USA
Coomassie Brillant Blue G-250 - solution kit	854.0	Biorad, USA

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Chemical	MW	Company
Dithiothreitol (DTT)	154.25	Wako pure chemical, Japan
2[4-(2-hydroxyethyl)-1-piperazinyl] Ethane sulfonic acid (HEPES buffer)	238.3	Dijindo, Japan
Ethylene diamine tetraacetic acid-disodium salt (Na_2-EDTA)	372.44	Sigma, USA
Extra Thick blot paper mini blot size 7×8.4 cm		GE healthcare, USA
ECL plus western blotting-detection containing kit; Limigen TM PS-3 detection reagent solution A Limigen TM PS-3 detection reagent solution B		GE healthcare, USA
Anti- GAD antibody		Amersham, Bioscience, UK
Hybond TM-P Membrane optimized for protein transfer		Amersham, Bioscience, UK
Horseradish peroxidase linked whole antibody kit		Amersham, Bioscience, UK
Magnesium choride	95.21	Wako pure chemical, Japan
N,*N*' methylene bis acylamide	157.14	Wako pure chemical , Japan
Polyvinyl polypyrrolidone (PVPP)	2500	NAKARAI chemicals, Japan
Potassium hydroxide	56.10	Wako pure chemical, Japan
Rabbit IgG from donkey kit		Amersham, Bioscience, UK
Sodium dodecyl sulphate (SDS)	288.38	Wako pure chemical, Japan
N,*N*,*N*',*N*' - Tetramethylethylenediamine - (TEMED)	116.20	Wako pure chemical, Japan
Tris (hydroxymethyl) aminomethane-(Tris-HCl)	121.14	Wako pure chemical, Japan

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2.1.5 Chemicals for proteomic analysis

Chemical	MW	Company
Acetronitrile	41.05	Wako pure chemical, Japan
Acylamide	71.08	Wako pure chemical, Japan
Ammonium persulphate (APS)	228.20	Biorad, USA
Bromophenol blue	669.96	Sigma, USA
Coomassie Brillant Blue R-250	826.0	Sigma, USA

Chemical	**MW**	**Company**
Coomassie Brillant Blue G-250 solution kit	854.0	Biorad, USA
2[4-(2-hydroxyethyl)-1-piperazinyl] Ethane sulfonic acid (HEPES buffer)	238.13	Dijindo, Japan
Ethylene diamine tetraacetic acid disodium salt (Na_2-EDTA)	372.44	Sigma, USA
Dithiothreitol (DTT)	154.25	Wako pure chemical, Japan
Glycerol	92.09	Wako pure chemical, Japan
Iodoacetamide	184.96	Wako pure chemical, Japan
IPG buffer pH 3-10	-	Amersham, Bioscience
Immobiline drystrip NL3-10	-	Amersham, Bioscience
MALDI Matrix alpha-cyano-4-hydroxy cinnamic acid	188.16	Bruker, daltonic, Germany
Magnesium choride	95.21	Wako pure chemical, Japan
N,N' methylene bis acylamide	157.14	Wako pure chemical, Japan
Polyvinyl polypyrrolidone (PVPP)	2500	NAKARAI chemicals, Japan
Potassium hydroxide	56.10	Wako pure chemical, Japan
Sodium dodecyl sulphate (SDS)	288.38	Wako pure chemical, Japan
N,N,N',N' - Tetramethylethylenediamine - (TEMED)	116.20	Wako pure chemical, Japan
Tris (hydroxymethyl) aminomethane-(Tris-HCl)	121.14	Wako pure chemical, Japan
Tri fluoro acetic acid (TFA)	114.02	Wako pure chemical, Japan
Tween 20	1227.54	Wako pure chemical, Japan
Triton-X	602	Wako pure chemical, Japan
Tributyl phosphine (TBP)	266.32	Wako pure chemical, Japan
Thiourea	76.12	Wako pure chemical, Japan
Urea	66.07	Wako pure chemical, Japan

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2.1.6 Instruments

Instruments	Model	Company
Autoflex MALDI-TOF Masspectrometry	-	Bruker, Germany
Centrifugal concentrator	-	TAITEC, Japan
Centrifuge	Himac CF 15D	HITACHI, Japan
Chemiluminescense detector	LAS-3000 mini	Fujifilm, Japan
High speed refrigeratored microcentrifuge	TOMY-MX 201	HITACHI, Japan
Incubator, 37 ^{o}C	-	SANYO, Japan
Liquid Chromatography - Masspectrometry (LC-MS)	1100 Binary/G1946A	HP, USA
Mild mixer	XR-36	TAITEC, Japan
PH-meter	F-22	Horiba, Japan
Spectrophotometer	U-2001	HITACHI, Japan
SDS gel electrophoresis set (18×35cm)	-	ATTO, Japan
SDS gel electrophoresis set (7×7cm)	-	EIDO, Japan
Transfer-Blot SD semi dry transfer cell	-	Biorad, USA
Weight, 4 point	2CP 3202s	Sartorius,
Water bath	WPE 45	Memmert, Germany

2.1.7 Plant Materials

The seven cultivars of the polished form of normal rice; *O. sativa* L. cv. Kawdokmali 105 (KDML105), *O. sativa* L. cv. Supan 1 (SP1), *O. sativa* L. cv. Chainat 1 (CN1), *O. sativa* L. cv. Pitsanulok2 (PL2) and *O. sativa* L. cv. Patumtanee1 (PT1). The second one is glutinous rice; *O. sativa* L. cv. Sanpatong 1 (SPT1), *O. sativa* L. cv. Korkor 6 (RD6) were purchased from a local market to be used in fermentation and the same seven cultivars of whole grain rice were obtained from Pitsanulok and Chiang Mai rice seed centers in Thailand, all of which were to be used for the germination experiments.

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2.2 Methods

2.2.1 Germination

The seven cultivars of whole grain rice were washed with distilled water three times. They were then soaked in distilled water for 72 h, while the water was changed

every 24 h and finally the water was decanted. Whole grain rice was allowed to germinate on moist paper in separate plastic boxes at 30°C. The germinated rice samples were collected every 5 days during germination. The rice germ was removed by cutting and eliminating the bran layer by machine. Germinated polished rice grains were obtained from this procedure and were dried at 70°C for 12 h, then ground by blender. (Komatsuzaki *et al,*. 2007; Xing *et al,*. 2007). Ground samples were collected for GABA extraction and further analysis.

2.2.2 Physiological measurement

The seedling growth, shoot length was measured from culms base to the tip of the longest leaf. Root length was measured from the root-shoot junction to the tip of the longest root. The rice seedling and grain were separately weighed, respectively.

2.2.3 GABA and amino acid extraction

250 mg of ground samples were vigorously mixed in 800 µl of 70% ethanol by vortex mixer. The mixture was shaken for 30 min at room temperature and then centrifuged at 13000×g at 4°C for 10 min. The supernatant in the upper layer was collected and then 800 µl of 70% ethanol was added to the pellet, the extraction process was repeated three times. The supernatant was collected and combined with the first supernatant. (Baum *et al.,* 1996; Oh and Choi 2001; Komatsuzaki *et al.,* 2007). The obtained crude extract containing GABA was adjusted volume to 4 ml and then passed through the 0.45 µm filter and analyzed by HPLC and LC-MS techniques after 2-hydroxynaphthaldehyde (HN) derivatization.

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2.2.4 Derivatization and standard calibration curve of GABA

1 ml of rice extract sample was added to 0.5 ml of borax buffer pH 8 and 0.5 ml of HN (0.3%w/v in methanol). The solution was heated in a water bath at 80°C for 10 min and was allowed to cool. The standard curve was constructed from five standard solutions of GABA and amino acid (125, 50, 25, 10 and 5 ppm of GABA) analyzed by the same procedure (Khuhawar and Rajper, 2003).

2.2.5 GABA and amino acid contents determination

GABA and amino acids were determined to be present in the sample by HPLC (JASCO, Japan) with pre-column derivatization with HN was done using the following conditions; UV detection at 330 nm with a flow rate of 1 ml/min. Mobile phase containing acetonitrile (mobile phase A):0.1% formic acid (mobile phase B) were applied. The elution gradient was evaluated using %mobile phase A; 30-40, 40-55, 55-30 at 0-5 min, 5-10 min and 10-25 min, respectively.

All samples containing GABA and amino acid were analyzed by LC-MS (HP 1100 Binary/G1946A) to confirm the HPLC results. The mass spectrometer with an electrospray ionization (API-EI) source was operated in the positive ion mode. Mass spectrometry experiments were performed to isolate and fragment the targeted ions. The operation conditions of the MS detector were optimized with a solution of GABA standard with an abundance of *m*/*z* 258 $[M+H]^+$ which was determined as follows; Fragmentation range:70, Mass range: 100-1000, Drying gas flow:12 l/min, Neubulizer pressure:32 psig, Drying gas temperature: 350^{o}C, and Capillary voltage: 3000 V. Data were processed with data analysis software.

2.2.6 GAD enzyme activity determination

2.2.6.1 Preparation of crude GAD

0.5 g of the rice germ and germinated grains were separately ground with liquid nitrogen in a mortar. Transfer the ground sample in eppendorf and added 2 ml of extraction buffer containing 0.2 mM PLP, 2 mM 2-mercaptoethanol (ME), 2 mM Na-EDTA, and 1 mM PMSF, immediately. Centrifuge at 10000 rpm for 10 min, at 4^{o}C. The supernatant was called the crude GAD and used for further step.

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2.2.6.2 Effect of factors influencing the GAD activity

(1) Optimal pH

This assay was investigated using two buffer systems (0.2 M citrate buffer pH 4, 5 and 0.2 M sodium phosphate buffer of pH 5.8, 6, 7, 8). GAD activity was determined after mixing crude GAD with buffers at various pHs as shown in Table 2.1. Reaction mixture was incubated at 40^{o}C. After stop reaction, the reaction solution was derivatized with HN and injected into LC-MS instrument, immediately.

The activity of GAD enzyme was calculated according to the peak area of reaction tube and the control.

One unit of GAD activity was defined as release of 1 µmol of GABA produced from L-glu per 30 min at 40^{o}C.

(2) Optimal enzyme

Influence of amount of enzyme before catalysis L-glu was investigated. The constant of 200 µl substrate was incubate with varying amount of enzyme at phosphate buffer pH 6 as show in Table 2.2, then the remaining enzyme activity was measured as those described above.

Table 2.1 The reaction mixture of crude GAD enzyme at different pH values

Solution	Volume (µl)											
	1		2		3		4		5		6	
	1	1c	2	2c	3	3c	4	4c	5	5c	6	6c
Crude enzyme	200	200	200	200	200	200	200	200	200	200	200	200
100 mM L-glu	200	-	200	-	200	-	200	-	200	-	200	-
0.2 mM PLP	100	100	100	100	100	100	100	100	100	100	100	100
Citrate buffer pH 4	300	500	-	-	-	-	-	-	-	-	-	-
Citrate buffer pH 5	-	-	300	500	-	-	-	-	-	-	-	-
0.2 M Phosphate buffer pH 5.8	-	-	-	-	300	500	-	-	-	-	-	-
0.2 M Phosphate buffer pH 6	-	-	-	-	-	-	300	500	-	-	-	-
0.2 M Phosphate buffer pH 7	-	-	-	-	-	-	-	-	300	500	-	-
0.2 M Phosphate buffer pH 8	-	-	-	-	-	-	-	-	-	-	300	500

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Table 2.2 The reaction composition for determination of GAD activity at varying amount of crude enzyme.

Solution	Volume (µl)									
	1		2		3		4		5	
	1	1c	2	2c	3	3c	4	4c	5	5c
Crude enzyme	200	200	400	400	600	600	800	800	1000	1000
100 mM L-glu	200	-	200	-	200	-	200	-	200	-
0.2 mM PLP	100	100	100	100	100	100	100	100	100	100
0.2 M Phosphate buffer pH 6	1000	1200	800	1000	600	800	400	600	200	400

c = control tube

(3) Optimal substrate concentration and incubation time

1 ml of crude enzyme was incubate with different amount of L-glu at constant reaction buffer pH 6 and PLP as cofactor of this enzyme as shown in Table 2.3. Total reaction solution was incubated in water bath at 40^{o}C. The reaction was sampling at varying activation time 0, 10, 20, 30, 40 and 60 min respectively for analyzing by LC-MS as described above.

Table 2.3 The varying amount of substrate for GAD enzyme activity assay

Solution	Volume (µl)									
	1		2		3		4		5	
	1	1c	2	2c	3	3c	4	4c	5	5c
Crude enzyme	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
100 mM L-glu	200	-	400	-	600	-	800	-	1000	-
0.2 mM PLP	100	100	100	100	100	100	100	100	100	100
0.2M Phosphate buffer pH 6	1000	1200	800	1200	600	1200	400	1200	200	1200

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2.2.6.3 GAD activity determination in germinated rice grain and young leaves

Because the optimal for GAD enzyme results obtained from step 2.2.6.2 The optimal data will be applied for determination of GAD activity in germinated rice grain and young leave as following condition; 200 µl of 0.2M sodium phosphate, pH 6,

400 µl of 100 mM L-glu, 100 µl of 0.2 mM PLP, and 1000 µl of rice crude extract. The reaction solution was incubated at 40^{o}C for 10 min, and then terminated by boiling in water for 5 min. The suspension was derivatized-HN solution, filtered through a 0.45 micron filter. The filtrate was analyzed for GABA content by LC-MS, immediately.

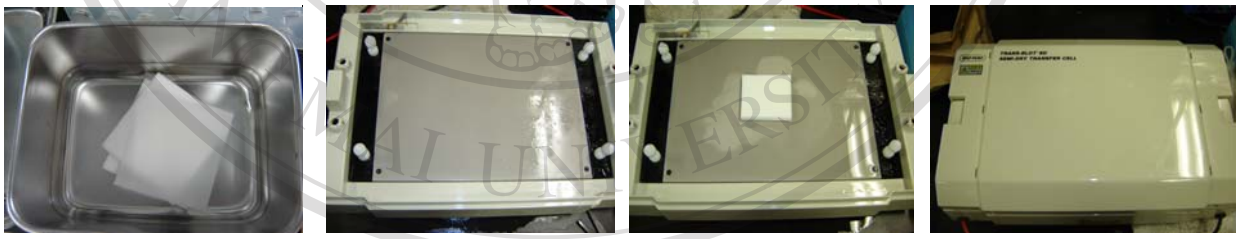
One unit of GAD activity was defined as release of 1 µmol of GABA produced from glutamate within 30 min at 40^{o}C as describe above.

2.2.6.4 Western blotting analysis GAD protein in germinated rice grain and young leave

This method begins with a SDS gel has already been run to separate the protein of interest from other protein in sample. It have to run 2 gels, staining one directly and using the other for western blotting.

(1) Preparing transfer cell and membrane

Washed membrane sheet (6×6cm) in methanol for 3 min and rinse with distillated water. Soaked in blotting solution pH 8.3 until use as shown in Figure. 2.1a.



(**a**) (**b**) (**c**) (**d**)

Figure 2.1 Membrane preparation and gel sandwich setting of western blotting technique. (**a**) Filtered paper and blotting membrane soaked in blotting solution before used. (**b**) Open the transfer machine (**c**) Place the filter paper, SDS-PAGE gel and transfer membrane (d) covered with the transfer magnetic cassette.

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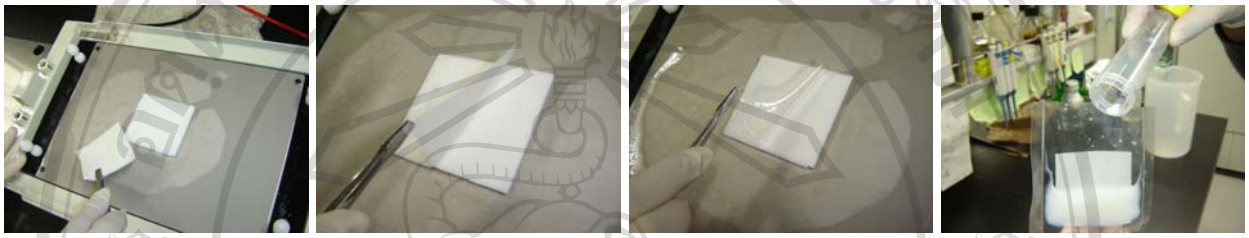
(2) SDS-PAGE gel membrane sandwich setting

Open the transfer cassette (Figure. 2.1b) and place a wetted sheet of filer paper. Carefully place the SDS-PAGE gel on the wet filter paper and blotting solution should be used for soaking. After wetting gel, carefully lay a wetted membrane sheet on the top of

SDS-PAGE gel. Place a wetted sheet of filter paper over the wetted membrane (Figure. 2. 1b-c) and roll a small pipette over the sandwich like rolling pin to remove any air bubbles. Cover with the second well soaked pad, close the transfer magnetic cassette (Figure. 2. 1 d). Attracted the electrodes, set the power supply to 45 V and current 72 mA for 1.30 h.

(3) Membrane blocking



(a) **(b)** **(c)** **(d)**

Figure 2.2 Transferred membrane was transfer to blocking solution in heat-sealable plastic bag (**a**) Open the transfer cassette and remove filter paper on the top of gel. (**b**) Removed SDS-PAGE gel (**c**) Keep transferred membrane into (**d**) blocking solution.

Disconnect transfer apparatus, remove transfer cassette, and peel filter paper and SDS-PAGE (Figure 2.2 a-b) from the membrane. Using forcep for remove membrane (Figure 2.2 c) from transfer apparatus to heat-sealable plastic bag. Add 10 ml 5% BSA blocking solution (Figure 2. 2 d) and keep in refrigerator 4^{o}C, overnight.

(4) Wash membrane

Pour off blocking solution and rinse with TBS-T for 15 min, 3 times on the shaker.

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(5) First antibody fixing

Pour off TBS, place the membrane in a heat-sealable plastic bag, add 10 ml GAD antibody dilution 1:5,000 in TBS-T and seal. Be careful to remove air bubbles in this step before seal. Air bubbles prevent antibody-epitope contact and can result undetectected band. Rock gently for 1.30 hours incubation.

(6) Wash membrane

Pour off the first antibody solution from membrane and wash twice for 15 min with TBS-T in aluminum container.

(7) Second antibody wash

Pour off TBS-T and add 10 ml anti rabbit antibody-coupled with horseradish peroxides dilution 1:100,000 in TBS-T. Rock the membrane gently for 2 hours.

(8) Wash membrane

Pour off second antibody solution from membrane. Rinse for 15 min with TBS-T for three times in aluminum container.

(9) Develop membrane

Pour off TBS-T from membrane, add 1 ml developing reagent (1 ml solution A: 25 µl Solution B; ECL plus western blotting-detection kit) within 5 min. Store the membrane protected from light and the atmosphere. Photograph with a LAS-3000 mini chemiluminescence's detector, immediately.

2.2.7 Investigation of protein profile of GABA enriched-rice using SDS-PAGE

2.2.7.1 Protein extraction

Total protein from 1 g of sample were grounded in a cooled mortar with 2 ml extraction buffer containing 50mM HEPES-KOH pH 7.4, 10 mM $MgCl_2$ pH 7.0, 1 mM EDTA-Na_2, 10% (w/v) glycerol, 5% (w/v) insoluble PVP, 5mM DTT, 0.01 mM leupeptin and then 200 mM PMSF was added during extraction. Transfer the extraction to eppendrof and was centrifuge at 15,000 rpm at 4^{o}C for 10 min. The supernatant was collected and the protein concentration determined using ready to use Coomassie BioRad reagent by Bradford method.

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2.2.7.2 Protein determination by Bradford method

Crude extracted was determined the total protein concentration before used the protein in the further step. BSA standard was prepared in five concentration of 0, 2, 4, 6 and 8 µl. Pipette BSA solution amount of 0, 20, 40, 60, 80 µl. Add water, crude extracted and Coomassie BioRad as shown in Table 2.4. The total volume of mixture solution 1 ml was measured by spectrophotometer at 595 nm.

Table 2.4. The amount of solution for the Bradford assay

Solution	Volume (µl)							
	1	2	3	4	5	6	7	8
BSA 100 µg/µl	0	20	40	60	80	-	-	-
Water	800	780	760	740	720	798	798	798
Crude extracted	-	-	-	-	-	2	2	2
Coomassie BioRad	200	200	200	200	200	200	200	200

2.2.7.3 Protein separation by polyacrylamide gel electrophoresis

Assemble gel sandwich according to the manufacture's instructions in the case of commercial apparatus. Carefully introduce separating gel solution (Table 2.5) into gel sandwich using a pipette until appropriate amount of separating gel solution (about 1.5 cm from top of front plate or 0.5 cm below level where teeth of comb will reach). Gently added 100 µl of water on the top of separating gel solution for keep gel surface to flat. Allow gel to polymerize for 60 min.

Table 2.5 10% separating gel and stacking gel solution preparation for gel sandwich (7×7cm)

Chemical	Separating gel (µl)	Stacking gel (µl)
30% acrylamide-bis	2500	0.325
acrylamide	-	-
4X Tris-Cl pH 6.8	1875	0.625
4X Tris-Cl pH 8.8	3125	-
Steriled water	10	1.525
TEMED	10	5
10% APS	50	25

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When the gel has polymerized, the interface will appear between separating gel and water. Pour off water covering the separating gel (the small droplets remaining will not disturb the stacking gel at the lower layer).

Introduced stacking gel solution onto separating gel until the solution reaches top of front plate. Carefully insert comb into gel sandwich until the bottom of teeth reach top of front plate. Be sure no bubbles are trapped on ends of teeth. Allow stacking gel to polymerized about 30 min.

2.2.7.4 Preparing and loading sample

After stacking gel has polymerized, remove comb carefully, making sure not to touch the well. Place gel into electrophoresis chamber and added electrophoresis running buffer to inner and outer reservoir (making sure that the both top and bottom of gel are immersed in buffer). Air bubbles, unpolymerized acrylamide and any contaminants at the bottom of gel should be removed. Sample solution was mixed with loading buffer (2X) and heat at boiling water for 5 min. Introduce sample solution into well using micropipette (Be careful to avoid introducing air bubbles)

2.2.7.5 Running

Attached electrode plugs to proper electrode as shown in Figure 2.3. Turn on power supply to 200 V and current will be about 50 mA for 2 gels. The dye front should migrate to 1-5 mm to bottom of gel within 40-60 min.

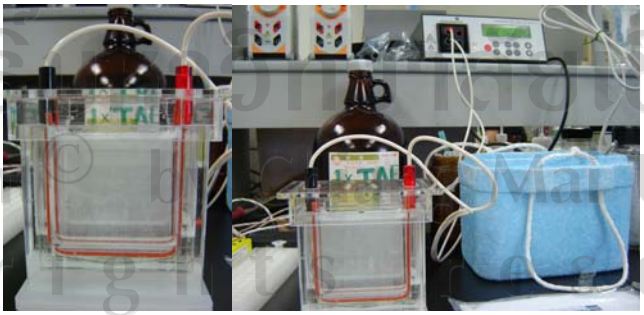

Figure 2.3 SDS-PAGE setting machine attached with the power supply for protein separation

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2.2.7.6 Staining and destining

Turn off power supply and remove electrode plug from electrode. Pick up the gel and transfer it to a small container containing 20 ml of Coomassie Brilliance Blue R stain (CBB R-250). Agitate on slow rotary shaker with plastic wrap covering during staining for overnight. Pour out stain and rinse the gel with a few change of water. Added destining solution about 50 ml into stained gels with 1-2 pieces of kimwise paper to adsorb CBB R-250 stain which diffuse from gel. Agitate for 4 h, stong bands protein can be visible during this step.

2.2.8 Proteomic analysis

2.2.8.1 First dimension gel electrophoresis

470 µg of crude protein extract was precipitated with cold acetone at -30±2 ^{o}C for 2 h in the ratio of acetone to crude extract (3:1v/v). Precipitated protein was solubilized in 100 µl of rehydration buffer containing 6M urea, 2M thiourea, 20 mM DTT, 2% (w/v) Triton-X, 0.001% bromophenol blue, Tributyl phosphine (TBP) and 1% (v/v) pH 3-10 IPG buffer. Prior to the second-dimension gel electrophoresis, the protein solution was separated by loading 340 µl protein solution into NL 18 cm non-linear pH 3-10 Immobiline Drystrip gel (Figure 2.4 a) which was used for the first dimension separation according to IEF value of protein. IEF was then performed by holding the rehydration step for 12 hours, ramping to 500 V 1 h, 1000 V 1 h, 3500 V 4 h, 5500 V 3 h and the final step was 8000 V for 3 hours (Figure 2.4 b-c).

2.2.8.2 Second dimension gel electrophoresis

After IEF was carried out, the strips were separately equilibrated twice with DTT and Iodoacetamide solution for 15 min and subsequently applied to the top of vertical SDS-PAGE gel in the second dimension. Protein Marker was loaded beside the strip before being sealed with agarose. Electrophoresis was carried out at the following conditions; 100V, 20 mA for 4 h and 100 V, 15 mA for 24 h under SDS–PAGE buffer pH 8.3 (Figure 2.5). After electrophoresis, gels were stained with CBB R-250 overnight and distained.

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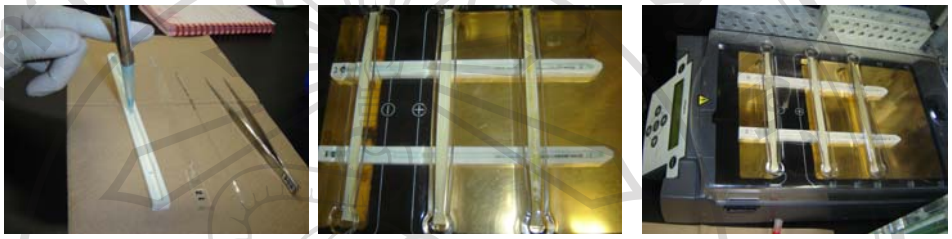
2.2.8.3 Image interpretation

CBB R-250 stained gels were analyzed with Image Master 2D platinum software version 5. Spot detection, spot measurement and spot matching were performed. One gel image was selected as a reference followed by automated spots matching among the gels. A protein was considered to be up or down-regulated when its normalized volume was at least a 1.6-fold increase or decrease, respectively.



(**a**) (**b**) (**c**)

Figure 2.4 IEF instrument setting for first dimension separation protein from rice. The instrument separate protein according to their PI of proteins. (**a**) Introduced crude protein extract into Immobiline strip gel pH 3-10 in the strip holder. (**b**) Place the strip holder (normally 2 strip; The first is the sample and the other is the control) on the magnetic field of IEF. (**c**) IEF machine was operated for 24 hours

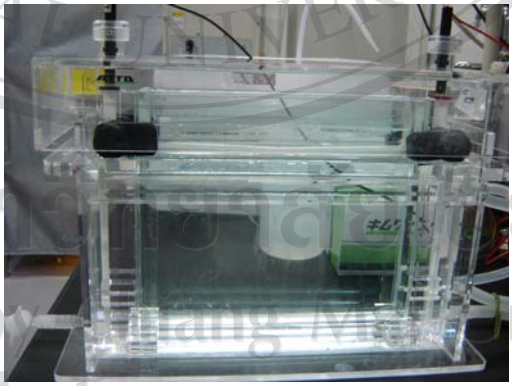


Figure 2.5 SDS-PAGE (18×35 cm) setting for protein separation in second dimension base on the molecular weight. The top of separating gel layer was covered with the strip which absorbed separated protein from the one dimension in the prior step.

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2.2.8.4 Protein identification by MALDI-TOF Mass spectrometry

Protein spots which showed difference were excised from the gels. Each small piece of the proteins were distained with distaining solution (25 mM NH_4HCO_3 in 50%ACN) until gel spots become colourless. The gel pieces were frozen at -30^{o}C, and then digested with 10%Trypsin-0.1M NH_4HCO_3 at 37^{o}C, overnight. After digestion, the peptides were collected and washed with 0.1% TFA in 50% acetonitrile to collect the remaining peptides. The peptide solution was concentrated to 5 µl using the centrifugal concentrator and 1 µl of peptide solution was co-cystallized with 1 µl of CHCA in 50% acetonitrile. Peptide masses were measured with Autoflex MALDI-TOF Masspectrometry. Peptide mass fingerprint was obtained and the NCBI database was searched using MASCOT software available at (http://www.matrixscience.com). The searching parameters were set as following; 500 ppm was used as the mass error tolerance, *O. sativa* was used for the taxonomic category. The results were chosen according to a minimum of 40 score, sequence coverage of the protein should not less than 15% by the matching peptides.

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