APPENDIX

APPENDIX A

REAGENTS PREPARATION

1. Reagents for isozyme electrophoresis.

1.1 Reagents for extraction buffer (isozyme)

1.1.1 0. 2 M. tris-HCl buffer pH 8.2

Stock A

0.2 M tris-hydroxymethyl aminomethan (24.2 gm in 1,000 ml)

Stock B

0.2 M hydrochloric acid (37% cone. HCl 16.5617 ml in water and adjust to 1,000 ml)

To prepare 0.2 M tris-HCl buffer pH 8.2 by combine stock A 50 ml with stock B 21.9 ml and make up the volume to 200 ml after adjust pH to 8.2 with NaOH or HCl. Store tris-HCl buffer in dark bottle and cold temperature (+4 °C)

1.2 Reagents for gel electrophoresis

1.2.1 acrylamide/bis (30% T, 2.67% C)

Dissolve 29.2.00 g of acrylamide and 0.80 g of N,N-methylene bisacrylamidde in water and make up the volume to 100 ml, filtrated with Whatman#1, store in dark bottle and cold temperature (+4 °C)

1.2.2 3 M. tris-HCl buffer pH 8.8 (for separating gel)

Dissolve 36.60 g of tris-hydroxymethyl aminomethan in water when it dissolved add 1 M hydrochloric acid 48.0 ml and make up the volume to 100 ml. Adjust pH to 8.8 with NaOH or HCl. Store in dark bottle and cold temperature (+4 °C)

1.2.3 0.5 M. tris-HCl buffer pH 6.7 (for spacer gel)

Dissolve 5.98 g of tris-hydroxymethyl aminomethan in water when it dissolved add 1 M hydrochloric acid 48.0 ml and make up the volume to 100 ml. Adjust pH to 6.7 with NaOH or HCl. Store in dark bottle and cold temperature (+4 °C)

1.2.4 10% ammonium persulphate

Dissolve 0.10 g of ammonium persulphate in water and make up the volume to 1 ml. It

1.2.5 5x electrode buffer

Dissolve 45.0 g of tris-hydroxymethyl aminomethan in water when it dissolved add 216.0 g of glycine and make up the volume to 3,000 ml.

1.2.6 0.5% dye marker

Dissolve 0.05 g of bromophenol blue in 10 ml of water.

1.3 Reagents for enzyme staining and condition of incubation

1.3.1 peroxidase isozyme (modified by Soltis *et al* , 1983)

3-amino-9-ethyl carbazole 0.04 g

N.N'-dimethylformamide 2.5 ml

add in

0.05 M sodium acetate buffer pH 5.0 5.0 ml

0.1 M calcium chloride 1.0 ml

3% hydrogen peroxide

Incubate the gel slice in refrigerator, usually for 30-60 min.

1.3.2 esterase isozyme (Brewer, 1970)

0.2 M tris-HCl pH 7.0 50 ml

α-naphthyl acetate solution (1% in 50% acetone) 3 ml

fast blue BB salt 0.05 g.

Incubate at ambient temperature and dark not require.

1.3.3 shikimic dehydrogenase isozyme (Soltis *et al*, 1983)

0.2 M Tris-HCl pH 8.0 50 ml

shikimic acid 0.05 g

nicotinamide adenine dinucleotide phosphate (NADP) 0.01 g

5 mg /ml nitro blu tetrazolium (NBT) 1.0 ml

5 mg /ml phenazine methosulfate (PMS) 1.0 ml

Incubate at ambient temperature.

1.3.4 malate dehydrogenase isozyme (Harris and Hopkinson, 1978)

0.2 M tris-HCl pH 8.0 50 ml

2.0 M DL-malic acid 5.0 ml

10 mg/ml nicotinamide adenine dinucleotide (NAD) 1.0 ml

5 mg /ml NBT 1.0 ml

5 mg /ml PMS 1.0 ml

Incubate at ambient temperature.

1.3.5 glucose-6-phosphate dehydrogenase isozyme (Brewer, 1970)

0.2 M tris-HCl pH 8.0 50 ml

0.1 M $MgCl_2$ 3.0 ml

D-glucose-6-phosphate 0.3 g

NADP 0.03 g

5 mg /ml NBT 1.0 ml

5 mg /ml PMS 1.0 ml

Incubate at ambient temperature.

1.3.6 superoxidase dismutase isozyme (Healy and Mulcahy, 1976)

0.2 M tris-HCl pH 9.0 50 ml

0.1 M $MgCl_2$ 1.0 ml

NADP 0.03 g

5 mg /ml NBT 1.0 ml

5 mg /ml PMS 1.0 ml

5 mg /ml MTT (tetrazolium salt) 1.0 ml

Incubate at ambient temperature.

1.4 Reagents for stain-fixing solution

1.4.1 1:5:5 glacial acetic:methanol:water

This fixative may be use for general gel strain but do not use for gel stained with MTT because it will be fading.

1.4.1 50% glycerol (in water)

It may be preferred for gels stained using MTT as a dry to reduce fading. This fixative may result in the resolution of faint LDH isozyme.

2. Reagents for protein assay

2.1 Reagents for extraction buffer (protein assay)

2.1.1 0. 5 M. tris-HCl buffer pH 7.0

Dissolve 5.98 g of tris-hydroxymethyl aminomethan in water when it dissolved add 1 M hydrochloric acid 48.0 ml and make up the volume to 100 ml. Adjust pH to 7.0 with NaOH or HCl. Store in dark bottle and cold temperature (+4 °C)

2.2 Reagents for protein assay

2.2.1 0.1 M phosphate buffer saline pH 6.0

0.2 M monobasic stock

Dissolve 13.9 g sodium phosphate monobasic in 500 ml dH_2O

0.2 M dibasic stock

Dissolve 53.65 g sodium phosphate dibasic heptahydrate (or 28.4 g of anhydrous form) in 1 L dH_2O

Combine of 600 ml dH_2O , 263.1 ml monobasic stock and 36.9 ml of dibasic stock.

2.2.2 Bradford reagent:

Dissolve 100 mg coomassie brilliant blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman #1 paper just before using.

3. Reagents for protein analysis by SDS-PAGE

3.1 Reagents for protein extraction buffer (for SDS-PAGE)

0.1 M Tris-HCl pH 8.8

Dissolve 1.196 g of tris-hydroxymethyl aminomethan 100 ml dH_2O and adjust pH to 8.8 with HCl. Add with:

0.4% 2-mercaptoethanol

10 % SDS

0.9 M sucrose

Add with proteases inhibitors:

1 mM phenylmethylsulphonyl fluoride (PMSF)

5 mM ethylenediamine tetraacetic acid (EDTA)

0.3 M dithiothreitol (DTT)

Store in dark bottle and cold temperature (+4 °C)

3.2 Reagents for gel electrophoresis

3.2.1 acrylamide/bis (30% T, 2.67% C)

Dissolve acrylamide 146.0 g and N,N'-Methylene-bis Acrulamide 4.0 g in distilled water and add to 500 ml. Filer and store at 4 °C in the dark. Maximum shelf life under this condition is 30 days.

3.2.2 1.5 M tris-HCl, pH 8.8

Dissolve 54.45 g tris base in distilled water. Adjust to pH 8.8 with HCl and adjust volume to 300 ml with distilled water. Store at 4 °C.

3.2.3 0.5 M tris-HCl, pH 6.8

Dissolve 6.0 g tris base in distilled water. Adjust to pH 6.8 with HCl and adjust volume to 100 ml with distilled water. Store at 4 °C.

3.2.4 10% (w/v) SDS

10.0 g sodium dodecylsulfate (SDS) in 60 ml of distilled water. Diluted by distilled water to 100 ml.

3.2.5 10 % ammonium persulfate (w/v)

Dissolve 100 mg in 1 ml of distilled water.

3.2.6 Sample Buffer

Distilled water 3.0 ml

0.5 M tris-HCl, pH 6.8 1.0 ml

glycerol 1.6 ml

10% SDS 1.6 ml

β-mercaptoethanol 0.4 ml

0.5% (w/v) bromophenol blue (in water) 0.4 ml

4. Reagent for Elution.

4.1 Elution buffer (48 mM Tris, 39 mM glycine, 20 % methanol, 0.00375 % SDS)

Dissolve 5.82.03 g tris base and 2.93 g, glycine and 1.875 ml of 20% SDS in distilled water and add 200 ml of methanol; adjust volume to 1 liter with distilled water.

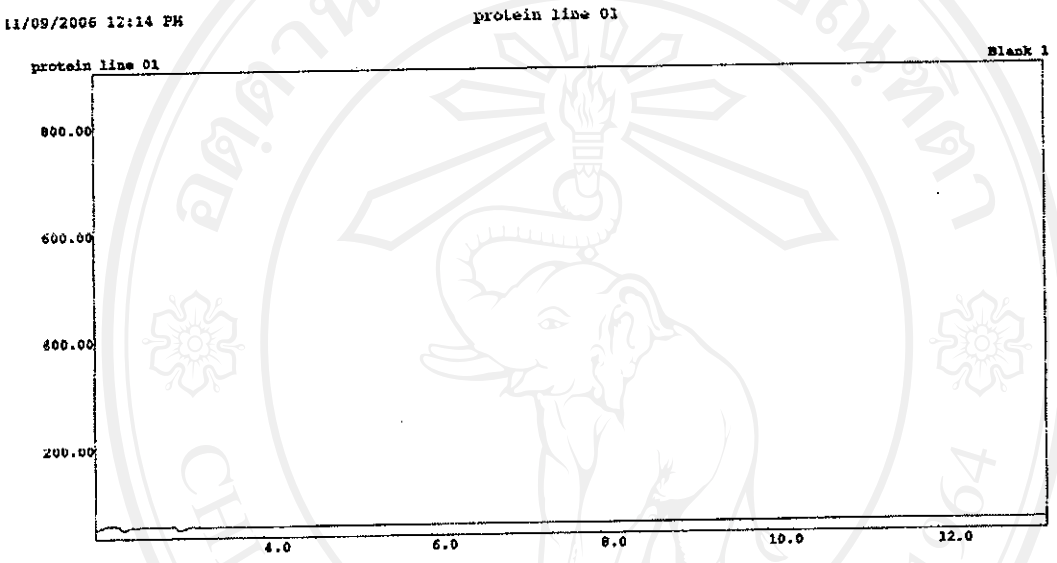
5. Reagent for semi-dry blotting

5.1 Towbin transfer buffer (25 mM Tris, 192 mM glycine (20% methanol), pH 8.3)

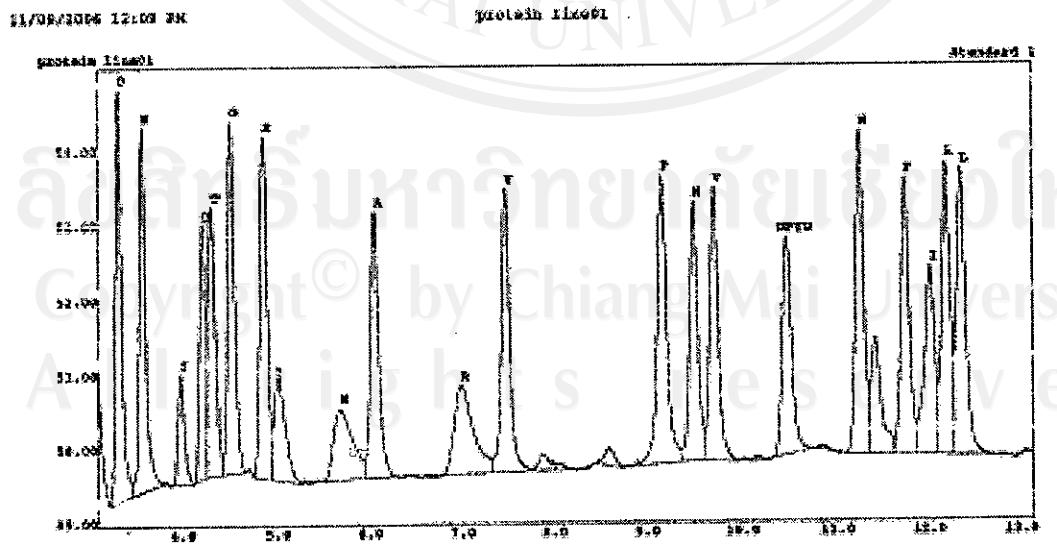
Dissolve 3.03 g tris and 14.4 g glycine in dd H_2O (add 200 ml of methanol); adjust volume to 1 liter with distilled water.

APPENDIX B

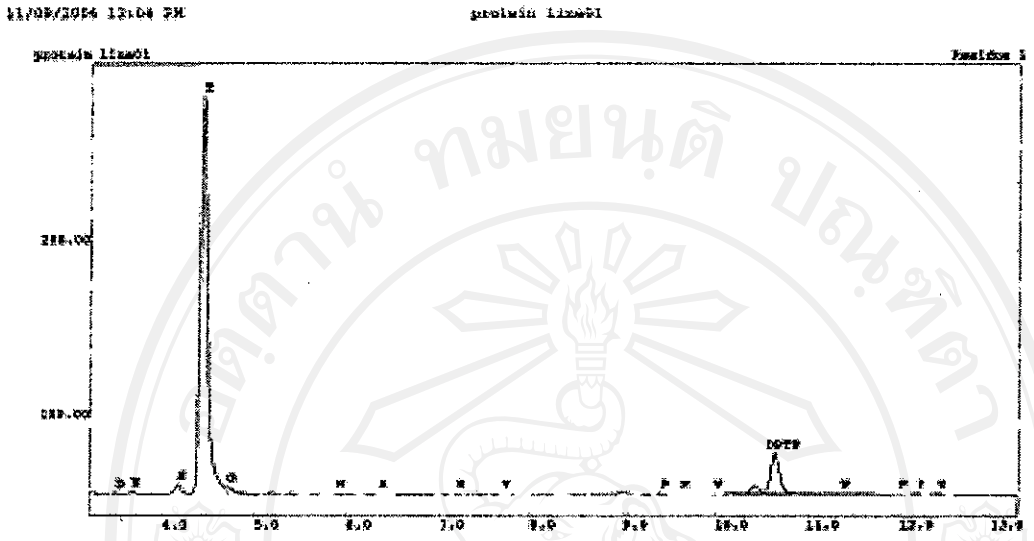
PROFILES OF PEPTIDES SEQUENCE



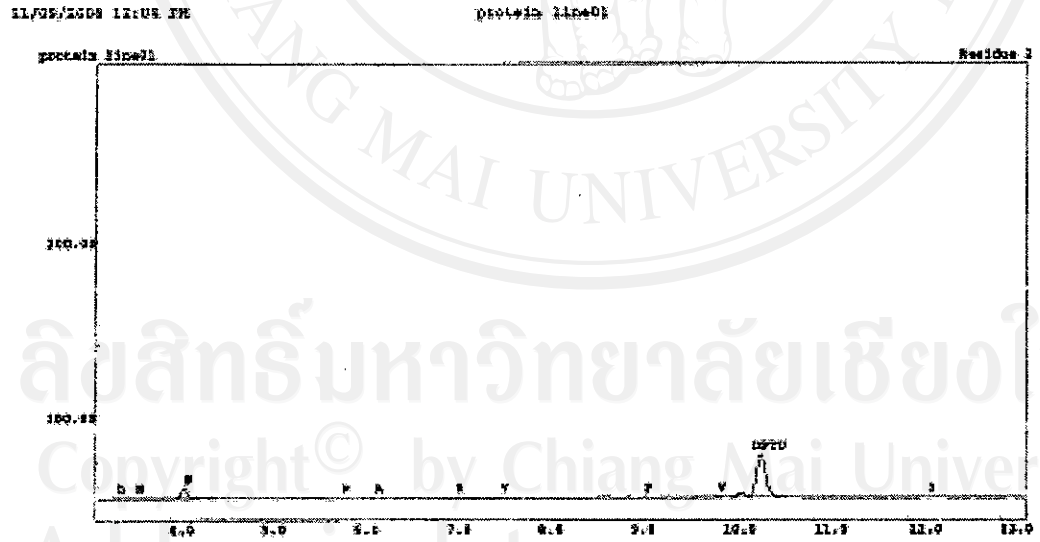
Appendix figure 1 HT profiles of the blank sample.



Appendix figure 2 HT profiles of the standard protein.



Appendix figure 3 HT profiles of the 17.18 kDa protein sample (residual 1).



Appendix figure 4 HT profiles of the 33.88 kDa protein sample (residual 1).

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