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APPENDIX I

CHEMICAL AGENTS AND INSTRUMENTS

A. Chemical substances.

Alkaline phosphatase, Sigma type VII (Sigma,Mo,USA)

Bentonite (Sigma, Mo, USA)

Bovine serum albumin (Sigma, Mo, USA)

Coomasie brilliant blue R (Sigma, Mo., USA)

Diethanolamine ($NHCH_2CH_2OH$) (E.Merck,Darmstadt,W.Germany)

Disodium hydrogen phosphate (Na_2HPO_4)(E.Merck,Darmstadt, W.Germany)

Glacial acetic acid (CH_3COOH) (E.Merck, Darmstadt, W.germany)

Glycine (NH_2CH_2COOH) (BDH, England)

Glutaraldehyde (Sigma, Mo, USA)

Hydrochloric acid (HCl) (E.Merck,Darmstadt.W.Germany)

L-Lysine (Sigma, Mo. USA)

Magnesium chloride ($MgCl_2.6H_2O$)(E.Merck,Darmstadt.W.Germany)

Methanol (CH_3OH) (E.Merck,Darmstadt, W.Germany)

Noble agar (Difco, Detroit, Michigan, USA)

p-nitrophenyl phosphate (Sigma, Mo, USA).

Potassium chloride (KCl) (E.Merck,Darmstadt, W.Germany)

Potassium dihydrogen phosphate (KH_2PO_4) (E.Merck,Darmstadt, W.Germany)

Protein A-sepharose CL-4B (Pharmacia, Uppsala,Sweden)

Sodium azide (NaN_3) (E.Merck,Darmstadt,W.Germany)

Sodium bicarbonate ($NaHCO_3$) (BDH,England)

Sodium carbonate (Na_2CO_3) (E.Merck,Darmstadt, W.Germany)

Sodium chloride (NaCl) (E.Merck,Darmstadt,W.Germany)

Sodium dihydrogen phosphate (NaH_2PO_4) (E.Merck, Darmstadt, W.Germany)

Sodium hydroxide (NaOH)(BDH, England)

Tris (Hydroxymethyl aminomethane (Tris : $C_4H_{11}NO_3$) (E.Merck, Darmstadt. W.Germany)

Tween 20 (Sigma, MO, USA)

B. Antiserum and serum

Equine antiserum to cobra venom (Thai Pharmacentical Organization,Bangkok, Thailand)

Goat antirabbit IgG (DAKO, Igs., Glastrup, Denmark)

Rabbit anticobra venom

Swine anti-rabbit serum (DAKO, Igs., Glastrup, Denmark)

C. Glasswares

Beaker (Pyrex, Corning, N.Y., USA)

Cylinder (Witeg, W. Germany)

Disposible 96 wells polystyrene microtiter plate (flat bottom) certified plate lot No 2784 (Nunc,Roskilde,Denmark)

Disposable syringe (Nipro Medical iIdustries, Tokyo, Japan)

Erlenmeyer flask (Pyrex, Corning, N.Y., USA.)

Glass tube (Pyrex, Corning, N.Y., USA.)

Microtiter plate (96 wells, U plate) (Nunc,Roskilde,

Denmark

Scalpvein infusion set (Abbott, Ireland)

D. Instruments

Analytical balance, (Precisa, Switzerland)

Automatic pipet. (Gilson, Lyon, France)

Centrifuge (Sorvall,Dupont, USA)

ELISA reader, Titertek Multiscan (Flow Labs., Helsinki, Findland)

Fraction collector, Model alpha 400 (Buchler Fractometer,USA)

Incubator (Forma Scientific, Ohio, USA)

Mixer Vortex-Genie (Scientific industries, N Y, USA.)

pH meter, model 10 (corning, N.Y.USA.)

Ultrafiltration, stirred cells (Amicon, Danver, MA, USA.)

UV-Visible spectrophotometer, model ACTA CIII(Beckman,CA, USA)

APPENDIX II

REAGENTS AND PREPARATIONS

1. Reagents for Immunoglobulin G Preparation

1.1 Phosphate buffer saline 0.05 M, pH 7.4

Stock solution A:

$NaH_2PO_4.2H_2O$	15.6	gm.
Distilled water to	1000	ml.

Stock solution B:

Na_2HPO_4	14.2	gm.
Distilled water to	1000	ml.

0.05 M PBS, pH 7.4

Solution A	250	ml.
Solution B	750	ml.
NaCl	8.0	gm.
Distilled water to	2000	ml.

Stored at 4°c.

1.2 1 g/l Tween 20 in 0.05 mol/l PBS, pH 7.4

Add 1 gm of Tween 20 to 1000 ml of PBS, pH 7.4

Stored at 4°c.

1.3 0.1 mol/l glycine/HCl-1 mol/l NaCl, pH 3.0

Glycine	7.507	gm.
NaCl	58.44	gm.

Dissolve the glycine and NaCl in 500 ml of distilled water; then add the 2M HCl until pH 3.0 and add ditilled water to 1000 ml. Store at 4°c.

1.4 NaOH, 1M

NaOH	40	gm.
Distilled water to	1000	ml.

2. Reagents for the preparation of enzyme-labeled anticobra venom (IgG).

2.1 Phosphate buffer saline (PBS) 0.15 M, pH 7.4

NaCl	8.0	gm.
KH_2PO_4	0.2	gm.
$Na_2HPO_4 .12H_2O$	2.9	gm.
KCl	0.2	gm.
$MgCl_2 .6H_2O$	2.03	gm.
distilled water	1000	ml.

Adjust the pH to 7.4 and store at 4°C

2.2 Tris-(hydroxymethyl) aminomethane (Tris) buffer 0.05 M, pH 8.0

Stock solution A (0.2 M Tris solution)

$C_4H_{11}NO_3$ (Tris)	24.2	gm.
Distilled water	1000	ml.

Stock solution B (0.2 M HCl)

conc. HCl (37%)	16.5	ml
Distilled water	983.5	ml

Stock solution A 250 ml were mixed with 134 ml of

stock solution B and distilled water was added to 1 litre. Adjust the pH to 8.0 with 1 N HCl.

3. Reagents for the detection of cobra venom by ELISA test

3.1 Coating buffer, pH 9.6

Na_2CO_3	1.59 gm.
$NaHCO_3$	2.93 gm.
NaN_3	0.2 gm.

Make up to 1 litre with distilled water and adjust pH to 9.6 with 1 M NaOH.

Store at 4°C or room temperature for not more than 2 weeks.

3.2 Phosphate buffer saline-Tween (PBS-Tween), pH 7.4

NaCl	8.0 gm.
KH_2PO_4	0.2 gm.
$Na_2HPO_4.12H_2O$	2.9 gm.
KCl	0.2 gm.
NaN_3	0.2 gm.
Tween 20	0.5 ml.

Make up to 1 litre with distilled water and adjust the pH to 7.4

Store at 4°c.

3.3 Phosphate buffer saline Tween-albumin (0.5%)

Dissolve 0.5 gm. of bovine serum albumin (BSA) in 100 ml of PBS-Tween, pH 7.4. Store at -20°C until use.

3.4 Conjugate

Alkaline phosphatase labeled anticobra venom IgG, store in concentrate form at 4° C with sodium azide as preservative. Dilute stock solution in PBS-Tween albumin immediatly before use.

3.5 Diethanolamine buffer (10%), pH 9.8

Diethanolamine	97 ml.
Distilled water	800 ml.
NaN_3	0.2 gm.
$MgCl_2 .6H_2O$	100 mg

1 M HCl was added until the pH is 9.8

The total volume is made up to 1 litre with distilled water. Store at room temperature of 4°C in an amber bottle.

Substrate solution is p-Nitrophenyl phosphate (1 mg/ml). Tablets (5 mg) are stored at -20° C in the dark until used. Immediately before use, one (5 mg) tablet is dissolved in each 5 ml of 10% diethanolamine buffer at room temperature. The substrate solution must be used in the same day.

3.6 Reaction stopping solution, 3M NaOH

NaOH	12 gm.
Distilled water to	100 ml.

4. Reagents for immunodiffusion test

4.1 Sodium barbital buffer (0.05 M), pH 8.2

Barbital sodium	47.6	gm.
1 N HCl	69	ml.
10% NaN_3	4.2	ml.
Distilled water to	4200	ml.

Adjust the pH to 8.2

4.2 Protein staining

4.2.1 Protein staining solution

Coomassie brilliant blue R	5	gm.
destaining solution	1000	ml.

stirred overnight until dissolved.

4.2.2 Destaining solution

Distilled water	1000 ml.
Glacial acetic acid	2000 ml.
Methanol	1000 ml.

Mix and store at room temperature.

4.3 Agar gel (1.5%)

Special agar Noble	1.5	gm.
Distilled water	100	ml.

The agar was heated in a double boiling water until dissolved and aliquot into 20 ml test tube, allowed to cool and stored in 4°C until use.

APPENDIX III

FIGURES

APPENDIX

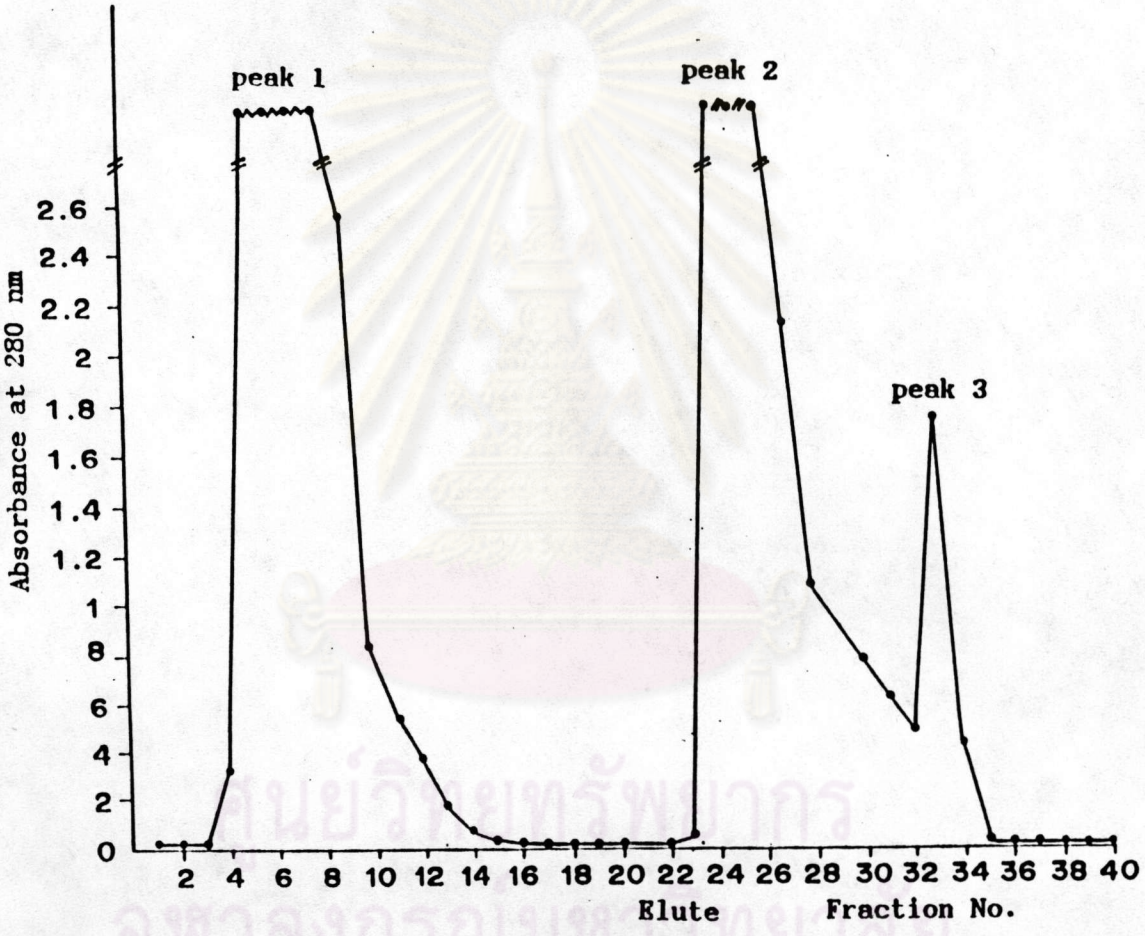

Figure 1. Affinity chromatography of rabbit anticobra venom on protein-A sepharose CL-4B column. The column was eluted with 0.1 mol/l glycine/HCl-1 mol/l NaCl,pH 3, flow rate 50 ml/hr; volumn of fractions 3 ml.

APPENDIX.

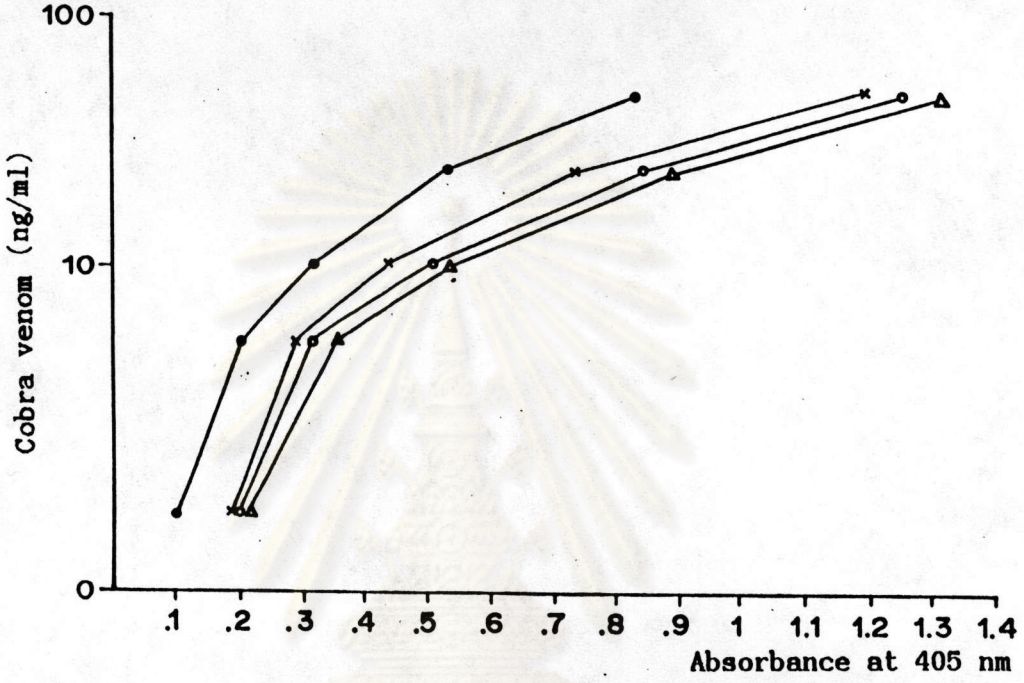

Figure 2. Temperatures and reaction times of anticobra venom in coating plate. Five concentrations of cobra venom (50,25,10,5,1 ng/ml) were tested.

- ●——● 4°C overnight
- ×——× 37°C 1 hour
- ○——○ 37°C 1 hour and kept at 4°C overnight
- △——△ 37°C 3 hours

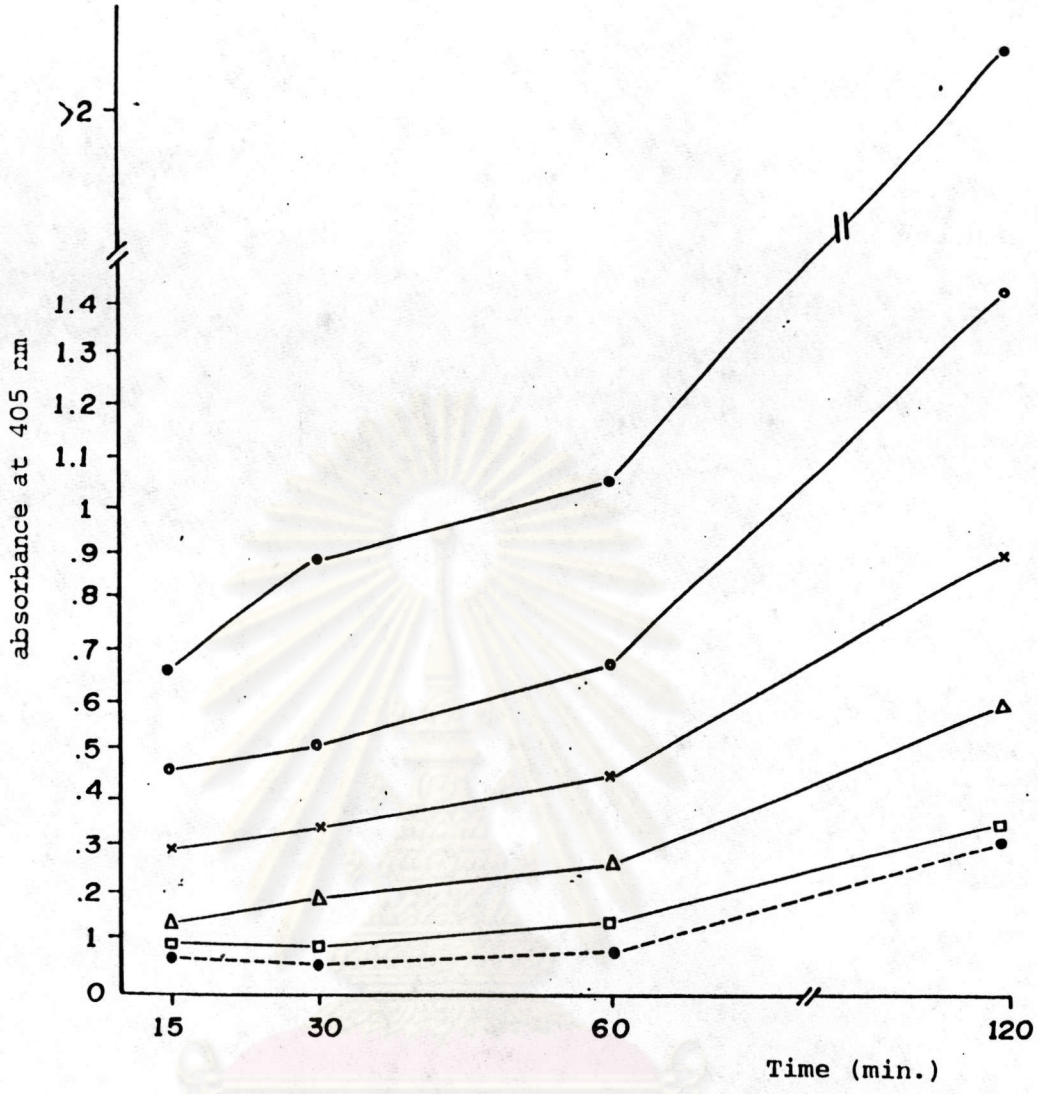

Figure 3. Time course of coated anticobra venom and cobra venom reaction. Five concentrations of cobra venom (50,25,10,5,1 ng/ml) and normal rabbit serum (1:5) were tested.

●—● 50 ng/ml

○—○ 25 ng/ml

×—× 10 ng/ml

△—△ 5 ng/ml

□—□ 1 ng/ml

●---● Normal rabbit serum

APPENDIX

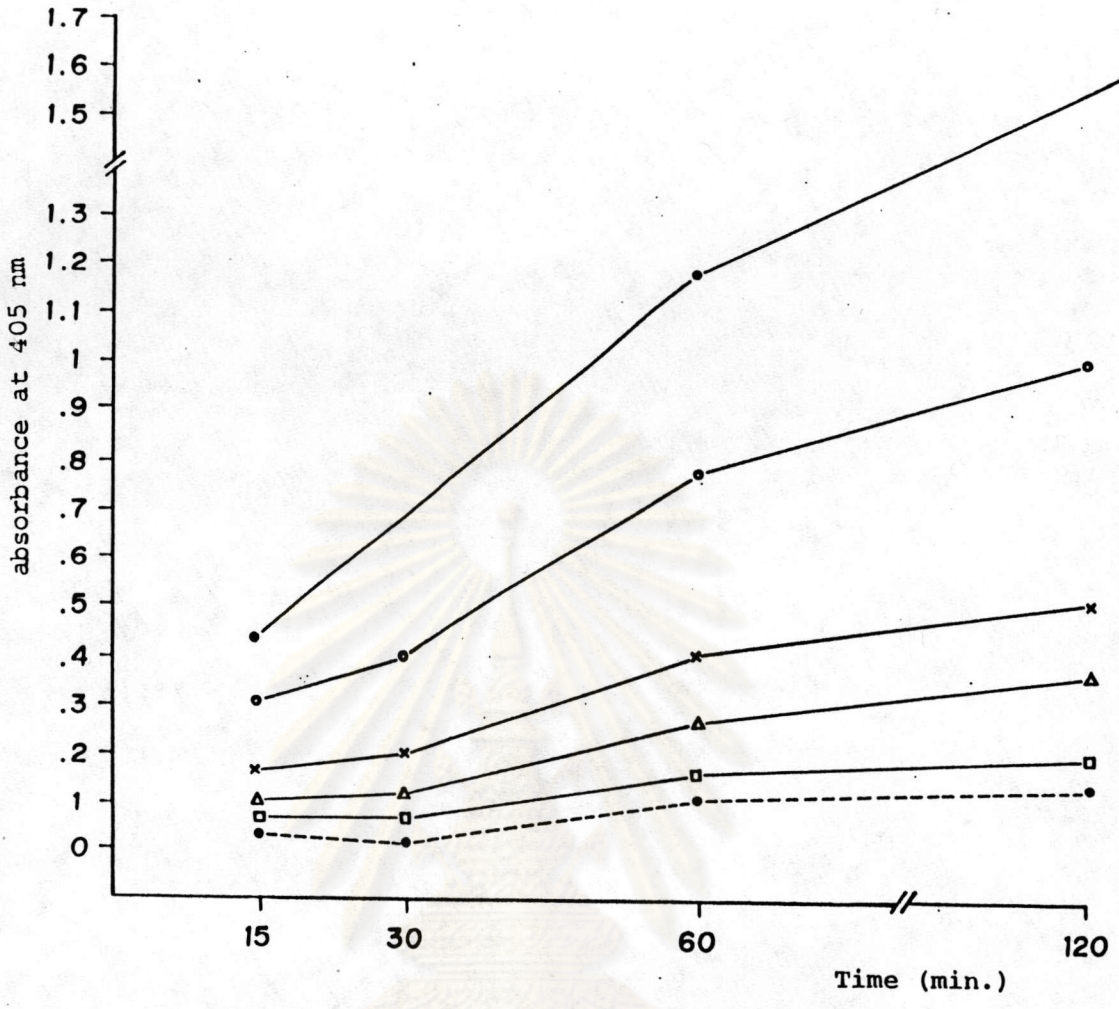

Figure 4. Time course of immobilised cobra venom with enzyme-conjugated anticobra venom. Five concentrations of cobra venom (50,25,10,5,1 ng/ml) and normal rabbit serum (1:5) were tested.

•—• 50 ng/ml

○—○ 25 ng/ml

×—× 10 ng/ml

△—△ 5 ng/ml

□—□ 1 ng/ml

•---• Normal rabbit serum

APPENDIX

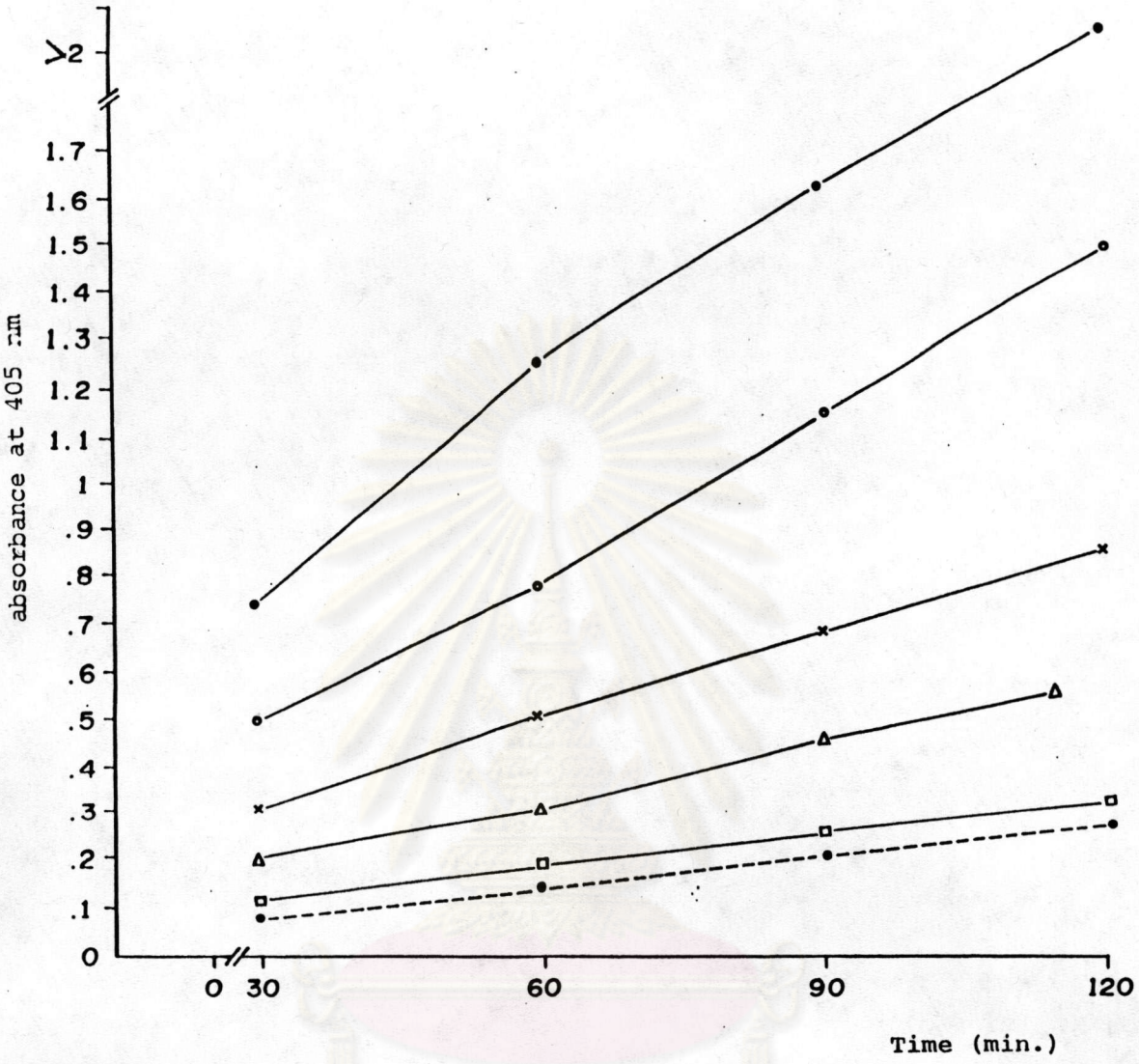

Figure 5. Time course of color development at 5 concentrations of cobra venom (50,25,10,5,1 ng/ml) and normal rabbit serum (1:5).

•—• 50 ng/ml

o—o 25 ng/ml

×—× 10 ng/ml

△—△ 5 ng/ml

□—□ 1 ng/ml

•---• Normal rabbit serum

CURRICULUM VITAE

Mrs. Orrawadee Hanvivatvong was born on April 3, 1949, in Songkhla, Thailand. She graduated with the Bachelor degree of Science in Medical Technology from Mahidol University in 1972. Her acadamic position is Faculty member of the Immunology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University.