Appendices

Appendix 1

Medium preparation

1. Potato Dextrose Agar (PDA)

Potato	200	g
Dextrose	20	g
Agar	15	g
Distilled water	1,000	ml

Method: dissolved PDA 39 gram into distilled/deionized water and bring volumn to 1,000 ml. Mixing throughly. Gently heat and bring to boiling. Distribute into tubes or flasks. Autoclaved at 15 pound/inch^2 at 121°C for 15 minutes. Pour into sterile Petri dishes or leave in tubes.

2. Potato Dextrose Agar (PDB)

Potato	200	g
Dextrose	20	g
Distilled water	1,000	ml

Method: dissolved all ingredients in distilled water, then autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

3. Sabouraud Dextrose Broth (SDB)

Peptone	10	g
Dextrose	20	g
Distilled water	1,000	ml

Method: dissolved all ingredients in distilled water, then autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

4. Malt yeast extract broth (MY)

Malt extract	3	g
Peptone	5	g
Dextrose	10	g
Yeast extract	3	g
Agar	15	g
Distilled water	1,000	ml

Method: dissolved all ingredients in distilled water, then autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

5. Peptone Yeast Glucose Medium (PYGM)

Peptone	5	g
Yeast extract	20	g
Dextrose	10	g
KH_2PO_4	1	g
$MgSO_4.7H_2O$	0.5	g
Distilled water	1,000	ml

Method: dissolved all ingredients in distilled water, then autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

6. Minimal Salt Medium (MM)

NH_4NO_3	3	g
Dextrose	20	g
KH_2PO_4	0.5	g
NaH_2PO_4	0.5	g
$MgSO_4.7H_2O$	0.5	g
$CaCl_2$	0.5	g
Yeast extract	1	g
Distilled water	1,000	ml

Method: dissolved all ingredients in distilled water, then autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

Appendix 2

Buffer preparation

1. Citrate buffer

Stock solutions

A : 0.1 M solution of citric acid (21.01 g of $C_6H_8O_7.H_2O$ in 1,000 ml)

B : 0.1 M solution of sodium citrate (29.41 g of $C_6H_5O_7Na_3.2H_2O$ in 1,000 ml)

X ml of A + Y ml of B, diluted to a total of 100 ml

X	Y	pH
46.5	3.5	3.0
43.7	6.3	3.2
40.0	10.0	3.4
37.0	13.0	3.6
35.0	15.0	3.8
33.0	17.0	4.0
31.5	18.5	4.2
28.0	22.0	4.4
25.5	24.5	4.6
23.0	27.0	4.8
20.5	29.5	5.0
18.0	32.0	5.2
16.0	34.0	5.4
13.7	36.3	5.6
11.8	38.2	5.8
9.5	41.5	6.0

2. Tris-HCl

Stock solutions

A : 0.1 M solution of tris (hydroxymethyl) aminomethane (12.114 g in 1,000 ml)

B : 0.1 M HCl

Contains 50 ml of A and X ml of B, diluted to 100 ml

pH	X
7.00	46.6
7.10	45.7
7.20	44.7
7.30	43.4
7.40	42.0
7.50	40.3
7.60	38.5
7.70	36.6
7.80	34.5
7.90	32.0
8.00	29.2
8.10	26.2
8.20	22.9
8.30	19.9
8.40	17.2
8.50	14.7
8.60	12.4
8.70	10.3
8.80	8.5
8.90	7.0
9.0	5.7

3. Phosphate buffer

Stock solutions

A: 0.2 M solution of monobasic sodium phosphate (27.8 g of $NaH_2PO_4.H_2O$ in 1,000 ml)

B : 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ or $Na_2HPO_4.7H_2O$ in 1,000 ml)

X ml of A + Y ml of B, diluted to a total of 200 ml :

X	Y	pH
87.7	12.3	6.0
85.0	15.0	6.1
81.5	18.5	6.2
77.5	22.5	6.3
73.5	26.5	6.4
68.5	31.5	6.5
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5
13.0	87.0	7.6
10.5	90.5	7.7
8.5	91.5	7.8
7.0	93.0	7.9
5.3	94.7	8.0

4. Glycine-NaOH

Stock solutions

A : 0.2 M solution of glycine (15.0 g in 1,000 ml)

B : 0.2 M NaOH (0.8 g in 1,000 ml)

50 ml of A + X ml of B, diluted to a total of 200 ml

X	pH
4.0	8.6
6.0	8.8
8.0	9.0
12.0	9.2
16.8	9.4
22.4	9.6
27.2	9.8
32.0	10.0
38.6	10.4
45.5	10.6

Appendix 3

Analytical methods and $(NH_4)_2SO_4$ fractionation

1. Quantitation of protein was conducted by the method of Lowry *et al.* (1951)

Reagents

1. 2% Na_2CO_3 in 0.1 N NaOH solution
2. 0.5% $CuSO_4.5H_2O$ in 1% sodium patassium tartrate solution
3. Alkaline copper solution was prepared by mixing 50 ml of solution 1. And 1 ml of solution 2. Immediately before used.

Procedures

1. A 0.5 ml of appropiated dilution of sample was placed into the tube.
2. A 3.0 ml of alkaline copper was added and incubated at room temperature for 10 minutes.
3. Added 0.3 ml of Folin-ciocalteaus reagent, vortexed immediately and incubated at room temperature for 30 minutes.
4. Measured the absorbanced at 750 nm.

Standard curve of protein

1. Bovine serum albumin (BSA) was prepared in various concentrations of 25, 50, 100, 150, 200 and 250 μg/ml.
2. Plotted graph of standard curve of BSA concentration and optical density at 750 nm (Figure-Appendix 3.1).

2. Bradford method for protein quantitation (with a slight modified Bradford, 1976)

Reagents

The assay reagent is made by dissolving 100 mg of Coomassie blue G250 in 50 ml of 95% ethanol. The solution is then mixed with 100 ml of 85% *ortho*-phosphoric acid and made up to 1 liter with distilled water.

Standard: Bovine serum albumin (BSA) at concentration of 0.5 μg/μl

Sample volumn: 20 μl

Procedures

1. Pipette 20 μl of sample which containing 1-5 μg of protein into a test tube. Each sample do duplicate. If an appropriate sample concentration is not known, assay a range of dilutions (1/10,1/100, 1/1000).

2. Add 1 ml of assay reagent to each tube, and mix gently, but throughtly. Measure the absorbance of each sample between 2 and 60 min after addition of the assay reagent.

3. Measure the OD_{595} value of the samples and standards against the reagent blank between 2 min and 1 h after mixing (Figure-Appendix 3.2).

Table-Appendix 3.1 Experimental set up for the Bradford's method

BSA (μg)	Water (μl)	0.5 μg/μl BSA (μl)
0	20	0
1	18	2
3	16	4
4	14	6
5	12	8

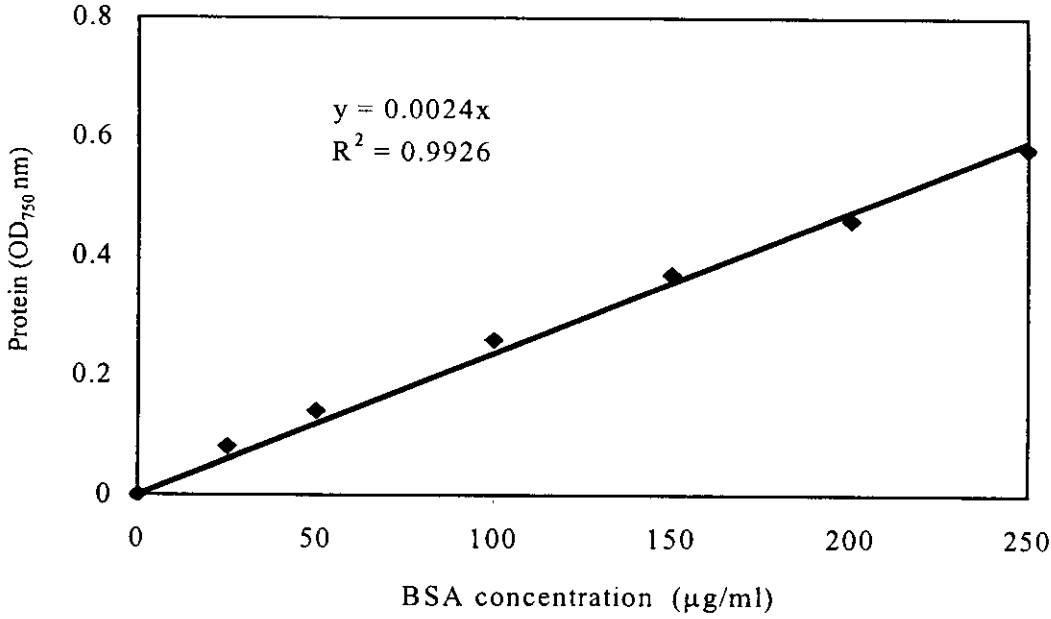

Figure-Appendix 3.1 Standard curve of BSA at the absorbance of 750 nm.

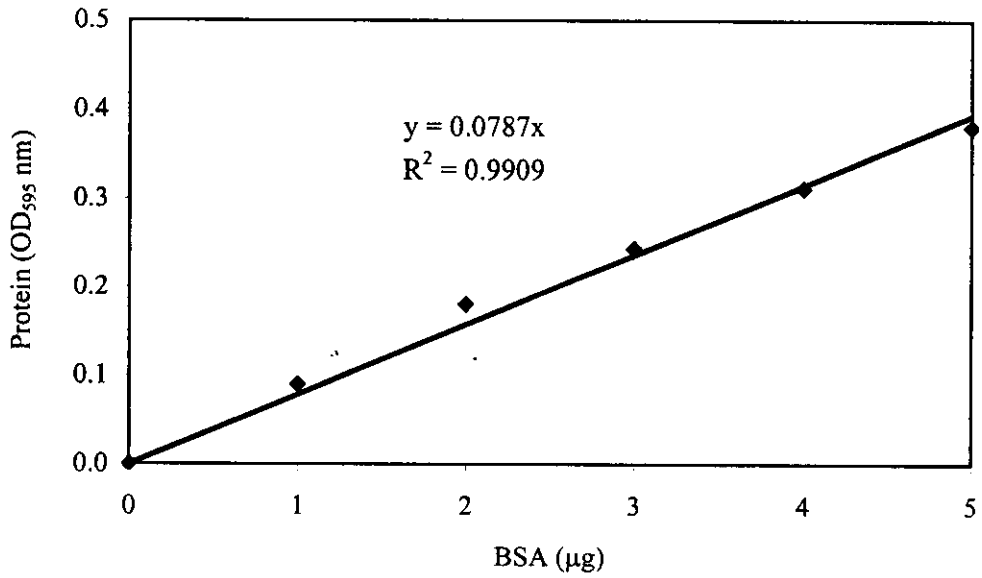

Figure-Appendix 3.2 Standard curve of BSA at the absorbance of 595 nm.

3. Calculation of parameters of an enzyme

3.1 Specific activity

$$\text{Units/mg} = \frac{\text{Activity of enzyme (units/ml)}}{\text{Protein concentration (mg/ml)}}$$

3.2 Total activity

Units = Volume of enzyme solution (ml) x enzyme activity (units/ml)

3.3 %Yield

$$\%\text{Yield} = \frac{\text{Total activity of enzyme}}{\text{Total activity of initial enzyme}} \times 100$$

3.4 Purification factor

$$= \frac{\text{Specific activity of enzyme}}{\text{Specific activity of initial enzyme}}$$

4. Native polyacrylamide gel electrophoresis (Native-PAGE)

Stock solution

4.1 Acrylamide/bis (30 %T, 2.67 %C)

The 29.2 g of acrylamide and 0.8 g of N'N'-bis-methylene-acrylamide were dissolved in 100 ml of distilled water, and stored in an amber bottle about one month after preparation.

4.2 1.5 M Tris-HCl, pH 8.8

Tris-base about 18.17 g was dissolved in 60 ml of distilled water and adjusted to pH 8.8 with 1 N HCl, then added to 100 ml with distilled water, kept at 4 °C.

4.3 0.5 M Tris-HCl, pH 6.8

A 6.06 g of Tris-base was dissolved in 60 ml of distilled water, then adjusted pH to 6.8 by 1 N HCl and added to 100 ml with distilled water, kept at 4°C.

4.4 5x-electrode (Running) buffer, pH 8.3

consist of

Tris-base	9.0 g
Glycine	43.2 g

: diluted to a total of 500 ml by distilled water, kept at 4°C, warm to room temperature before used if precipitation occurred. Diluted 50 ml 5x-stock with 450 ml deionized water for one electrophoretic run.

4.5 Catalyst

consist of

- Ammonium persulfate (APS) 10%, was prepared before used.
- N,N,N',N',-tetramethyl ethylenediamine (TEMED)

4.6 Staining solution

Protocol of gel silver staining was followed by the method of Toxicology Unit, Department of Pathology Faculty of Medicine, Prince of Songkla University.

4.6.1 Soak gel in 10% ethanol for 10 min.

4.6.2 Soak gel in 1% nitric acid for 10 min (3 ml of 65% HNO_3 in 200 ml of deionized water).

4.6.3 Wash gel with deionized water (3 times).

4.6.4 Soak gel in 0.012 M silver nitrate solution (0.4 g of silver nitrate in 200 ml of deionized water) for 35 min.

4.6.5 Wash gel with deionized water for about 20 seconds.

4.6.6 Develop gel in 0.28 M solution carbonate and 0.019% formalin (11.8 g of Na_2CO_3 in 390 ml of deionized water and add 205 µl of 37% formalin solution).

4.6.7 Stop reaction with 10% glacial acetic acid.

Preparation of slab gel

1. The glass plate sandwiches was assembled.
2. Prepared 5-15% separating gel which consisting of

Composition	Separating gel	
	5% (2 ml)	15% (2 ml)
30% Acrylamide-0.8% bisacrylamide	0.33	1.0
1.5 M Tris-HCl, pH 8.8	0.5	0.5
10% Ammonium persulfate	0.02	0.02
TEMED	0.002	0.002
Distilled water	1.15	0.48

3. Poured gel solution into the assembled gel sandwich and immediately overlayed the solution with water, then allowed to polymerize for 45 minutes to 1 hour.
4. Prepared the stacking gel (4%) which consist of

30% acrylamide-0.8% bisacrylamide	0.50 ml
0.5 M Tris-HCl, pH 6.8	0.63 ml
10% Ammonium persulfate	50 µl
TEMED	5 µl
distilled water	3.82 ml

5. Dried the area above the separating gel with filter paper before pouring the stacking gel.
6. Placed a comb in the gel sandwich and poured stacking gel solution down the spacer nearest the upturned side of the comb, poured until all the teeth have been covered by solution.

7. Allowed the gel to polymerized for 30-40 minutes and removed the comb by pulling it straight up slowly and gently.
8. Rinsed the wells completely with running buffer.

Sample preparation

1. Mixed the sample at least 1:4 with sample buffer before loading into the gel
2. Assembled the electrophoresis apparatus.
3. Prepared 500 ml of 5X-electrode buffer.
4. Added approximately 150 ml of buffer to the upper buffer chamber. Filled until the buffer reached a level haft way between the short and long plates.
5. Poured the remainder of the buffer into the lower buffer chamber so that at least 1 cm of the gel bottom was covered.
6. Loaded the sample into the wells under the electroded buffer with pipettor using tips.
7. Placed the lid on top of the lower buffer chamber to fully enclose the cell. The correct orientation was made by matching the colors of the plugs on the lid with the jacks on the inner cooling core.
9. Attached the electrical leads to a suitable power supply (250 volts) with the proper polarity. The usual running times was approximately 45 minutes or until marker dye was 1 cm from anodic end of gel.
10. After electrophoresis was completely, turned off the power supply and disconnected the electrical leads.
11. Placed the gel into staining solution.
12. Determined the relative mobility (R_f) of a protein by

$$R_f = \frac{\text{distance of protein migration}}{\text{distance of tracking dye migration}}$$

The R_f values were plotted against the know molecular weights on semi-logarithmic paper and estimated the molecular weight of unknown protein from calibration curve of standard.

5. Ammonium sulfate fractionation

Table-Appendix 3.2 Final concentrations of ammonium sulfate: percentage saturation

From % ↘	To % →	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0		27	55	84	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
	5		27	56	85	115	146	179	212	246	282	319	357	397	439	481	526	572	621	671	723
	10			28	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
		15			28	58	88	119	151	185	219	255	292	331	371	413	456	501	548	596	647
			20			29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
				25			29	60	91	123	157	191	227	265	304	344	386	429	475	522	571
					30			30	61	92	126	160	195	232	270	309	351	393	438	485	533
						35			30	62	94	128	163	199	236	275	316	358	402	447	495
							40			31	63	96	130	166	202	241	281	322	365	410	457
								45			31	64	97	132	169	206	245	286	329	373	419
									50			32	65	99	135	172	210	250	292	335	381
										55			33	66	101	138	175	215	256	298	343
											60			33	67	103	140	179	219	261	305
												65			34	69	105	143	183	224	266
													70			34	70	107	146	186	228
														75			35	72	110	149	190
															80			36	73	112	152
																85			37	75	114
																	90			37	76
																		95			38

Source: Scopes (1978)

Appendix 4

Data analysis

Table-Appendix 4.1 Effect of cultural medium (initial pH = 6) on fibrinolytic enzyme production of *S. commune* BL 23 cultivated at 30°C, 150 rpm for 7 days

Medium	Dry cell weight (g/l)	Fibrinolytic activity* (U)
PYGM	7.82	486.27[a]
MY	7.16	245.38[b]
SDB	6.92	226.23[b]
PDB	5.23	119.99[c]
MM	4.99	108.17[c]

Table-Appendix 4.2 Effect of incubation period on growth and fibrinolytic enzyme production of *S. commune* BL 23 cultivated in PYGM medium (initial pH = 6) at 30°C and 150 rpm

Time (day)	Dry cell weight (g/l)	Fibrinolytic activity (U)
3	3.94	450.79[b]
7	7.14	506.91[a]
10	7.62	443.80[b]
12	7.56	344.73[c]
14	7.49	206.59[d]

Table-Appendix 4.3 Effect of initial pH of PYGM medium on growth and fibrinolytic enzyme production of *S. commune* BL 23 cultivated at 30°C, 150 rpm for 7 days

Initial pH	Dry cell weight (g/l)	Fibrinolytic activity (U)
5	7.61	434.13[b]
6	7.51	489.46[a]
7	9.00	441.95[b]

Table-Appendix 4.4 Effect of temperature on growth and fibrinolytic enzyme production of *S. commune* BL 23 cultivated in PYGM with initial pH of 6.0 at 150 rpm for 7 days

Temperature (°C)	Dry cell weight (g/l)	Fibrinolytic activity (U)
25	9.70	343.99[c]
30	8.79	441.75[b]
35	9.74	575.22[a]

Table-Appendix 4.5 Effect of shaking speed on growth and fibrinolytic enzyme production of *S. commune* BL 23 cultivated in PYGM with initial pH of 6.0 at 35°C for 7 days

Shaking speed (rpm)	Dry cell weight (g/l)	Fibrinolytic activity (U)
150	8.62	492.06[a]
200	9.79	259.04[c]
250	7.65	290.03[b]

Table-Appendix 4.6 Time course of growth and fibrinolytic enzyme production of *S. commune* BL 23 cultivated in PYGM medium wiyh initial pH of 6.0 at 35°C and 150 rpm

Time (day)	Dry cell weight (g/l)	Fibrinolytic activity (U)
3	5.50	492.64[b]
5	7.13	516.94[b]
7	8.93	576.73[a]
10	8.41	245.69[c]
12	6.96	159.93[d]
14	5.94	91.63[e]

*the same superscript-letter means not significantly different ($p<0.05$)