

## APPENDIX

Appendix List of chemicals and materials used in this study.

The chemicals used were analytical grade unless specified.

### Chemical and reagents for synthesis, purification and analysis of sulfated chitosan

Absolute methanol	Fluka, Switzerland
Absolute ethanol	Fluka, Switzerland
Acetic acid glacial	BDH Laboratory Supplies, UK
Acrylamide	BDH Laboratory Supplies, UK
Alcian Blue*	Sigma-Aldrich, USA
(*a kind gift from Dr Siriwan Ong-chai, Department of Biochemistry, Faculty of Medicine, Chiang Mai University)	
Ammonium persulfate	Amersham Pharmacia Biotech, USA
Bisacrylamide	BDH Laboratory Supplies, UK
Blue Dextran	Amersham Pharmacia Biotech, USA
Chitosan	Sigma-Aldrich, USA
Chlorosulfonic acid	Fluka, Switzerland
Dextran sulfate 8, 40, 500 Kd	Sigma-Aldrich, USA
Dimethylmethylene Blue	Sigma-Aldrich, USA
Di-sodium hydrogenphosphate	BDH Laboratory Supplies, UK
Dimethylsulfoxide (DMSO)	BDH Laboratory Supplies, UK

Glycine	BDH Laboratory Supplies, UK
Hydrochloric acid	BDH Laboratory Supplies, UK
N, N, dimethylformamide	Fluka, Switzerland
Nitrogen gas 98%	Lanna gas supply, Chiang Mai
Phenol Red	BDH Laboratory Supplies, UK
Potassium chloride	BDH Laboratory Supplies, UK
Potassium dihydrogen phosphate	BDH Laboratory Supplies, UK
SDS	Sigma-Aldrich, USA
Sepharose CL-6B	Sigma-Aldrich, USA
Sodium acetate	BDH Laboratory Supplies, UK
Sodium carbonate	BDH Laboratory Supplies, UK
Sodium chloride	BDH Laboratory Supplies, UK
Sodium hydroxide	BDH Laboratory Supplies, UK
Sucrose	BDH Laboratory Supplies, UK
Sulfuric acid	Fluka, Switzerland
TEMED	Sigma-Aldrich Inc., USA
Trisma Base	Sigma-Aldrich Inc., USA
Urea	Fluka, Switzerland

Reagents and materials for biological assays

Accuclot™ Heptest (CRS 114)	Sigma-Aldrich, USA
Accucolor™ Heparin (CRS 106)	Sigma-Aldrich, USA
Antithrombin Accucolor™(CRS 117)	Sigma-Aldrich, USA
Accuclot™ thrombin time reagent (A8713)	Sigma-Aldrich, USA
Atroxin (845-2)	Sigma-Aldrich, USA
Accumark™ Accuclot Control I (A4089)	Sigma-Aldrich, USA
Bovine serum albumin Fraction V	Sigma-Aldrich, USA
Cytotoxicity Detection Kit (Cat. No. 1 644 793)	Roche Diagnostic, USA
Cell Proliferation ELISA, BrdU (colorimetric) (Cat. No. 1 647 229)	Roche Diagnostic, USA
Fetal calf serum	Gibco BRL, USA
Gentamycin	Roussel, UK
Goat anti human IgA, $\alpha$ -chain specific	Sigma-Aldrich, USA
Goat anti human IgM, $\mu$ -chain specific	Sigma-Aldrich, USA
Goat anti human IgE, $\epsilon$ -chain specific	Sigma-Aldrich, USA
Goat anti human IgD, $\delta$ -chain specific	Sigma-Aldrich, USA
Goat anti human polyvalent Immunoglobulins	Sigma-Aldrich, USA
Heparin Sodium (5000 IU/ml)	LEO Pharmaceutical, Denmark
HRP-Goat anti human polyvalent Immunoglobulins	Sigma-Aldrich, USA
Human Interleukin-2 ELISA	Endogen, USA
Human Interferon gamma ELISA	Endogen, USA
Ionomycin	Sigma-Aldrich, USA

Pentosan Polysulfate MW 5400\*

(\*kind gift from Professor Peter Ghosh, The Royal North Shore Hospital of Sydney, Australia)

Phytohemagglutinin (PHA) Sigma-Aldrich, USA

Pokeweed mitogen (PWM) Sigma-Aldrich, USA

Phorbol 12-myristate 13-acetate (PMA) Sigma-Aldrich, USA

Rabbit anti human IgG,  $\gamma$ -chain specific DAKO, USA

RPMI-1640 Gibco BRL, USA

Thromboplastin-HS (T6540) Sigma-Aldrich, USA

Tuberculin purified protein derivatives (PPD)\*

(\*kind gift from Dr. Chaisuree Supawilai, Research Institute of Health Sciences, Chiang Mai University)

Triton X-100 Sigma-Aldrich, USA

Tween-20 Fluka, Switzerland

## Appendix B List of instruments used in this study

<u>Instrument-model</u>	<u>Source</u>
Atomic absorption spectrophotometry (AA-274)	Varian Tectron, Australia
Autobalance (AD-4)	Perkin Elmer, USA
<sup>13</sup> C NMR Spectrophotometer (DPX-300)	Bruker, Switzerland
Centrifuge with adaptor for 96 well culture plate	Kokusan H-103N Series, Japan
CHNS/O analysis (PE 2400 Series 2)	Jusco, Japan
CO <sub>2</sub> incubator (Model IR2424)	Shel Lab, USA
Dialysis bag (cut-off 3500 dalton, Spectra Pore R1)	Fisher Chemicals, UK
Electrophoresis Power Supply, ECPS 3000/150	Amersham Pharmacia Biotech, USA
FibrinTimer II	Behring, Germany
FPLC MonoQ (HR 5/5)	Amersham Pharmacia Biotech, USA
Fraction collector	Amersham Pharmacia Biotech, USA
Glass column chromatography (1.6 x 120 cm)	Amersham Pharmacia Biotech, USA
<sup>1</sup> H-NMR spectrometer (JNM-A500)	Jeol, Japan
Hot plate with magnetic stirrer	Kika Labortechnik, Germany
Infrared spectrometer (IR-810)	Jusco, Japan
Laminar air flow (SC 1200)	Astec, USA
Lyophilizer (FOC-1)	Martin Christ, Germany
Microplate Reader (EL340)	BIO-TEK Instruments, USA
Oswald viscometer size C	Sigma-Aldrich, USA

Phase contrast microscope	Olympus, Japan
Polarimeter (ADP 220)	Bellingham & Stanley, UK
RT 6000D refrigerated centrifuge	Sorvall, USA
UV 160 Spectrophotometer	Shimadzu, Japan
UV-visible spectrophotometry (Spectronic Genesy 5)	Milton Roy, USA
Water bath with shaker	Memmert, USA
X-ray diffractometer (RAD-2R)	Siemen, Sweden

#### Appendix C Reagents and buffers preparation

##### Reagents for synthesis, purification and analysis of sulfated chitosan

##### 1. Preparation of dried N, N-dimethylformamide

N, N-dimethylformamide	300 ml
NaOH	2.0 gm

Reflux for 3 hours following with distillation at 152-154 °C and stored over a molecular sieves (3 Å) for 3 days before use.

##### 2. 4M Sodium acetate buffer

Sodium acetate	3.497 gm
Conc. acetic acid	9.0 ml

-Dissolve sodium acetate in 50 ml distilled water.

-Add 9.0 ml of conc. acetic acid and add up to 100 ml with distilled water.

## 3. Standard phenylalanine solution (1 mg/ml)

Phenylalanine	1.18	gm
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1% (v/v) acetic acid	up to	100	ml
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-Dissolve phenylalanine in 50 ml of 1% (v/v) acetic acid

-Add up to 100 ml and store at room temperature

## 4. Distillation of chlorosulfonic acid

-Set the distillation set in a round bottle flask.

-Add 300 ml of  $\text{HClSO}_3$  and distill at 148-150 °C

-Store at room temperature.

## 5. Farndale reagent

Glycine	0.76	gm
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NaCl	0.59	gm
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1, 9-dimethylmethylene blue	0.04	gm
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-Mix glycine and NaCl and dissolve in 150 ml of distilled water.

-Adjust the pH to 3.0 with 0.1M HCl.

-Dissolve 1, 9-dimethylmethylene blue in 200 ml of glycine-NaCl mixture and stirred until well dissolved.

-Add up to 250 ml with glycine-NaCl mixture.

-Store at room temperature.

## 6. Phosphate Buffered Saline pH 7.2

NaCl	16.00 gm
KCl	0.40 gm
Na <sub>2</sub> HPO <sub>4</sub>	2.30 gm
KH <sub>2</sub> PO <sub>4</sub>	0.40 gm

-Dissolve all reagents in 800 ml of distilled water.

-Adjust the pH to 7.2 with 1N NaOH and add up to 1L with distilled water

-Filter through Whatman No.1 and keep at room temperature

## 7. 1N NaOH

NaOH	4.00 gm
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-dissolve and add up to 100 ml with distilled water

-store at room temperature

## 8. 1N HCl

conc. HCl (12M)	10 ml
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-Dilute and add up to 100 ml with distilled water.

-Store at room temperature.

9. 2M H<sub>2</sub>SO<sub>4</sub>

conc. H <sub>2</sub> SO <sub>4</sub> (36M)	10 ml
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-Dilute and add up to 180 ml with distilled water.

-Store at room temperature.



Reagents for the study of biological activity

## 1. 3.8% sodium citrate

Sodium citrate (dihydrate)	3.80	gm
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-Dissolve in distilled water and add up to 100 ml

-Store at room temperature

## 2. Carbonate-bicarbonate buffer pH 9.6

$\text{Na}_2\text{CO}_3$	4.24	gm
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$\text{NaHCO}_3$	5.04	gm
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-Dissolve 2 reagents in distilled water

-Adjusted the pH to 9.6 with 1N NaOH and add up to 1L with distilled water

-Store at 4°C.

## 3. 5% BSA-PBS azide

Bovine serum albumin	5.0	gm
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(BSA, Fraction V, Sigma)

PBS pH 7.2	up to	100	ml
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-Dissolve BSA in 80 ml of PBS pH 7.2 and stir on hot plate (40-50 °C)

-Add 0.2 ml of 10% sodium azide (final 0.02%)

-Add up to 100 ml with PBS pH 7.2 and store at 4 °C

4. 2.5 NH<sub>2</sub>SO<sub>4</sub>

conc. H <sub>2</sub> SO <sub>4</sub>	35	ml
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Distilled water up to	500	ml
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-Dilute conc. H<sub>2</sub>SO<sub>4</sub> with 300 ml of distilled water

-Let the temperature cool down

-Add up with distilled water to 500 mL.

## 5. RPMI-1640

RPMI-1640 (Gibco BRL)	1	sacchet
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NaHCO <sub>3</sub>	2	gm
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-Dissolve medium powder and NaHCO<sub>3</sub> together in distilled water 800 ml.

-Add 1 mL of gentamycin (80 mg/2 ml) to a final 40 mg/L

-Adjust the pH to 7.2 with 10% acetic acid and add up to 1L with distilled water

-Add amphotericin B to the final concentration of 2.5 mg/L after sterilize by filtration and store at 4 °C.

## 6. 10% FCS RPMI-1640

Heat inactivated FCS	10	ml
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Sterile RPMI-1640	90	ml
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-Mix together by the sterile technique in a laminar air flow

-Store at 4 °C.

## 7. 2% Triton X-100 in RPMI-1640

Triton X-100	2	ml
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RPMI-1640	98	ml
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-Mix together and store at room temperature.

## PUBLICATIONS FOR THESIS

1. Vongchan, P., Sajomsang, W., Subyen, D. and Kongtawelert, P. (2002) Anticoagulant of sulfated chitosan. *Carbohydr. Res.*, 337, 13,1239-1242.
2. Vongchan, P., Kasinrerk, W. and Kongtawelert, P. (2002) Chitosan polysulfate synthesized from marine crab shell inhibited PPD stimulated human peripheral blood mononuclear cell proliferation. *Chiang Mai Med. Bull.* 4, (in press)
3. Vongchan, P., Sajomsang, W., Kasinrerk, W., Subyen, D. and Kongtawelert, P. (2002) Anticoagulant activities of the chitosan polysulfate synthesized from the marine crab shell by semi-heterogeneous conditions. *Science Asia* (submitted).
4. Vongchan, P., Sajomsang, W., Kasinrerk, W., Subyen, D. and Kongtawelert, P. Synthesis and anticoagulant effect of chitosan polysulfate. *Proceeding in the 27th Congress on Science and Technology of Thailand: International Polymer Science*, Songkla, Thailand, October 16-18.
5. Vongchan, P., Sajomsang, W., Subyen, D. and Kongtawelert, P. (2002) Structure and anticoagulant activity of chemically modified chitosan derived from the shell of rice-field crabs (*S. dugasti*). *Proceeding in 5th Asia pacific Chitin-Chitosan Symposium & Exhibition*, Bangkok, Thailand, March 13-15.

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## Publication

1. Vongchan, P., Sajomsang, W., Subyen, D. and Kongtawelert, P. (2002) Anticoagulant of sulfated chitosan. *Carbohydr. Res.*, 337,13,1239-1242.
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