CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and equipments

All chemicals, reagents and equipments used in this study are listed in Appendix

2.2 Preparation of chitosan polysulfate (CPS) by random substitution

2.2.1 Synthesis of CPS (Gamzazade *et al.*, 1997)

2.2.1.1 Preparation of the solvent including chitosan

Three grams of chitosan with degree of acetylation of 0.12 was added into 700 ml aqueous 1% v/v acetic acid and stirred until all of the chitosan dissolved. The solution was then precipitated by adding 100 ml of aqueous 10% w/v NaOH. The precipitate was collected using Buchner funnel through the filter paper (Whatman No.42) and washed until neutral with water. The filtrate was tested with lithmus paper. The precipitate was then washed three times with absolute methanol following washing twice with dried *N, N*-dimethylformamide (DMF). The gel-liked precipitate in DMF was obtained and stored at room temperature.

2.2.1.2 Preparation of sulfating complex

After the precooled DMF (300 ml) was throroughly flowed with nitrogen gas for 10 minutes, freshly distilled $HClSO_3$ (45 ml) was added dropwisely. During the addition of 2 hrs, the temperature was controlled between 0-4 °C. Then the temperature was allowed to rise up to room temperature (25°C). During which, the mixture was continuously stirred.

2.2.1.3 Sulfation reaction

The solvent including chitosan (10 gm) was added into the sulfating complex and the mixture was stirred for 5 hrs. The solid swollen polymer suspended in the solvent was diluted with distilled water and neutralized with aqueous 20% w/v NaOH (checked by lithmus paper). The solution of the polymer in form of sodium salt was then stirred for additional 30 minutes before precipitating with cold methanol (3 L). The precipitate was collected using Buchner funnel through the filter paper (Whatman No.42) and washed several times with absolute methanol. Finally the precipitate was dissolved in distilled water and dialysed against distilled water for 48 hrs using a dialysis bag cut-off 3500 daltons. Dialysed water was tested for neutrality and sulfate radicals using phenolphthalein and barium chloride (Mihai *et al.*, 2001), respectively, to confirm that all free sulfate radicals was eliminated. Finally, the precipitate was obtained by lyophilization giving 2.9 gm.

2.2.2 Isolation and purification of CPS

2.2.2.1 Setting and equilibration of the Sepharose CL-6B column

Sepharose CL-6B (Sigma-Aldrich) was washed twice with filtered Phosphate Buffered Saline pH 7.2 (PBS) and degassed in 60 °C water bath for 3 hrs. The column (diameter 1.6 cm) was packed with Sepharose CL-6B as indicated in the instruction manual to get 100 cm height and equilibrated with PBS for 3-5 void volume. Blue dextran (M_v 1000 kDa, 20 mg/ml, 75 µl) and Phenol Red (357 Da, 20 mg/ml, 3.5 µl) mixed in the PBS in the final volume of 2 ml was used to calibrate and determine for total volume (V_t) and void volume (V_o). The fraction of 2 ml was eluted with PBS pH 7.2 at flow rate of 20 ml/hr. Each fraction was determined for the absorbance at 595 and 565 nm for blue dextran and phenol Red, respectively. Calibration graph was plotted between the optical density of both calibrators and fraction numbers in Y and X axis, respectively.

2.2.2.2 Calibration of the Sepharose CL-6B column

Dextran sulfate (8_v 40 and 500 kDa) was used as the standard sulfated polysaccharides for the determination of the dissociation constant (K_d). Ten mg/ml of each of standard dextran sulfate was prepared by dissolving 100 mg of dextran sulfate in 10 ml of PBS pH 7.2. Ten ml of each standard dextran sulfate was applied onto the Sepharose CL-6B column chromatography (1.6 x 100 cm). The fraction of 2 ml was eluted with PBS pH 7.2 at flow rate of 20 ml/hr. The sulfate substitution in the polymer chains was determined by mixing 100 µl of each fraction with 100 µl of 1, 9-dimethylmethylene blue (Farndale reagent, appendix) (Farndale *et al.*, 1986). The intensity of the reaction color

was measured at wavelength 540 nm by ELISA plate reader. The O.D. of each fraction numbers were plotted for the standard calibration curve and determined the K_d.

2.2.2.3 Isolation of CPS of different molecular weights

Dried CPS was dissolved in PBS pH 7.2 at the concentration of 10 mg/ml. The colloidal suspension was vigorously mixed and centrifuged to eliminate the undissolved particle. Ten ml of suspension was applied onto Sepharose CL-6B column. Fractions of 2 ml were eluted by PBS pH 7.2 at the flow rate of 20 ml/hr. Each fraction was determined for sulfate subsitution by Farndale reagent. The positive fractions were divided into 3 peaks at the K_d of 0.16 and 0.58. Each peak was then dialysed against distilled water and lyophilized to obtain the molecular weight of 6.8×10^4, 3.6×10^4, 2.0×10^4 kDa, respectively.

2.2.2.4 Purification of CPS

Unmodified chitosan was eliminated using FPLC (MonoQ® HR5/5, Code No. 17-0546-01). Firstly, the column was equilibrated with filtered 20 mM Tris-HCl pH 8.0 before applying of 500 µL of filtered CPS (1 mg/ml in 20 mM Tris-HCl pH 8.0). The column was developed by a linear gradient of 0-2.0M NaCl in the same buffer (Pavao *et al.*, 1995). The flow rate of the column was 1 ml/min and fractions of 1 ml were collected. One hundred µl of each fraction was assayed for the sulfate substitution in the polymer chains using the Farndale reagent. Finally, the positive fractions were pooled and

dialysed against distilled water and lyophilized. This was done in every peak obtained from Sepharose CL-6B column.

2.2.3 Chemical analysis and molecular weight determination

2.2.3.1 IR spectra

IR measurement was carried out as follows. The CPS (1 mg.) was dried overnight at 60 °C under reduced pressure. The dried sample was mechanically well-blended with 100 mg. of potassium bromide (KBr) and pressed on a vacuum-connected disk instrument at 10 ton/inch^2 for 5 minutes. The thickness of the KBr disc was 0.5 mm. The IR spectrum was recorded using an air as a reference. The same experiment was done with the starting material, chitosan for comparison.

2.2.3.2 Specific rotation

Chitosan and CPS were prepared to 0.5 gm% by dissolving 0.125 gm in the final volume of 25 mL of 2% (v/v) acetic acid or distilled water, respectively. The sample was stirred until well dissolved and detected for the optical rotation in the polarize light by polarimeter at 28 °C. The recorded rotation (α) was used for calculating the specific rotation, ($[\alpha]^{28}_{D}$) by the equation

$$[\alpha]^{28}_{D} = \frac{\alpha \text{ (from experiment)}}{c \times l}$$

where $c = 0.005$ g/cm^3

$l = 2.1$ dm^2

2.2.3.3 ^{1}H-NMR and ^{13}C-NMR

The analysis of NMR spectra of the CPS was kindly performed by the National Metal and Materials Technology Center (MTEC). ^{1}H-NMR were recorded. Chemical shifts (δ) were reported in parts per million (ppm) using D_2O (except otherwise mentioned) as solvent and 3-(trimethylsilyl)propanesulfonic acid sodium salt (DSS) as an internal standard. CP-MAS ^{13}C-NMR spectra of chitosan and CPS were recorded in comparison.

2.2.3.4 X-ray diffraction

Five hundred milligrams each of chitosan and CPS were mechanically pressed on a vacuum-connected disk instrument at 10 ton/inch^2 for 5 minutes. The thickness and diameter of the KBr disk was 0.2 cm and 2.0 cm, respectively. The disk was transferred to the X-ray diffractometer. The diffraction pattern was analyzed at 35 kV, 30 mA.

2.2.3.5 CHNS/O analysis

Chitosan, CPS, and standard L-cysteine with accurate weight (1.5 mg) were wrapped in the pieces of aluminum foil and placed into well plate before analysis. The composition of C, H, N and S atom in the CPS was analyzed by the CHNS/O analyzer. The data was reported in percentage of each atom with reference to the standard L-cysteine. The analysis was performed at the Service Center of Chemical Analysis, Faculty of Science, Chiang Mai University.

2.2.3.6 Determination of the degree of deacetylation (Prochazkova *et al.*, 1999)

To determine the degree of deacetylation in the starting material, free amino group has to be investigated. Briefly, various amount of chitosan with accurate weight (0.01-0.05 gm) was dissolved with 40 ml of 2% (v/v) acetic acid. The sample solution was stirred until well dissolved and filtered through Whatman No.42 to eliminate the trace of undissolved particle. The sample solution was then made up to 50 ml with 2% (v/v) acetic acid.

Determination of free amino group was performed by the reaction of 2 ml of sample solution or various concentrations of standard phenylalanine (10-80 mg/L), 1 ml of 4M acetate buffer (appendix), and 2 ml of 2% (w/v) ninhydrin solution in the screw cap tube. The reaction was done in duplicate. The tubes were immediately capped, briefly shaken by hand, and heated in a covered boiling water bath for 20 minutes. The tubes were then cooled below 30 $^{\circ}$C in a cold water bath. The content was diluted with 50% (v/v) ethanol/water and added up to the final volume of 25 ml. The optical density was recorded by the UV visible spectrophotometry at 570 nm using 2% (v/v) acetic acid as a blank. Free amino group in sample was determined from standard curve set from phenylalanine standard solution. Taken together with total nitrogen obtained from CHNS/O analysis, the degree of deacetylation was calculated in percentage.

2.2.3.7 Determination of the average molecular weight by viscosity measurement

(Nishimura *et al.*, 1986)

CPS (0.4 gm% (w/v) in 0.1M NaCl) was prepared by dissolving 0.4 gm. of CPS in 80 ml of 0.1M NaCl and mixed well. Trace particle was discarded by filtering through filter paper (Whatman No. 42) and then made up to 100 ml with 0.1M NaCl. The sample solution was diluted to 0.1, 0.2 and 0.3 gm/dl using 25-ml volumetric flask by mixing 6.2, 12.5, and 18.7 ml of sample solution and made up to 25 ml with the 0.1M NaCl.

Calibration of the viscometer system was done before the determination of tested samples. The 0.1M NaCl (15 ml) of was transferred into the Oswald type viscometer embedded in the temperature-controlled water bath (25 ± 0.1 °C). The solution was raised up to the indicated level A and let release to indicated level B by gravity (Figure 8). The time of decreasing from level A to level B was recorded in seconds. Each experiment was done for 5 times and mean of all data recorded was calculated.

For the determination of average molecular weight of CPS, each of various concentration of the sample solution was applied on the Oswald type viscometer embedded in the temperature-controlled water bath (25 ± 0.1 °C). The method was run as previous described.

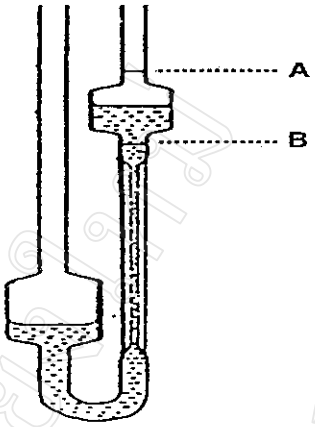

Figure 8 Oswald type viscometer

Data in seconds recorded from each experiment were used to determine the reduced viscosity (η_{red}) and inherent viscosity (η_{inh}) which then was converted to intrinsic viscosity (η) by plotting the curve between reduce viscosity (η_{red}) and inherent viscosity (η_{inh}) in the Y axis, and sample concentration (g/dl) in the X axis (Figure 9). The average molecular weight (M_v) was calculated by applying the Mark-Houwink equation $[\eta] = 1.75 \times 10^{-5} Mv^{0.98}$ (0.1M NaCl, 25 °C) (Nishimura *et al.*, 1987).

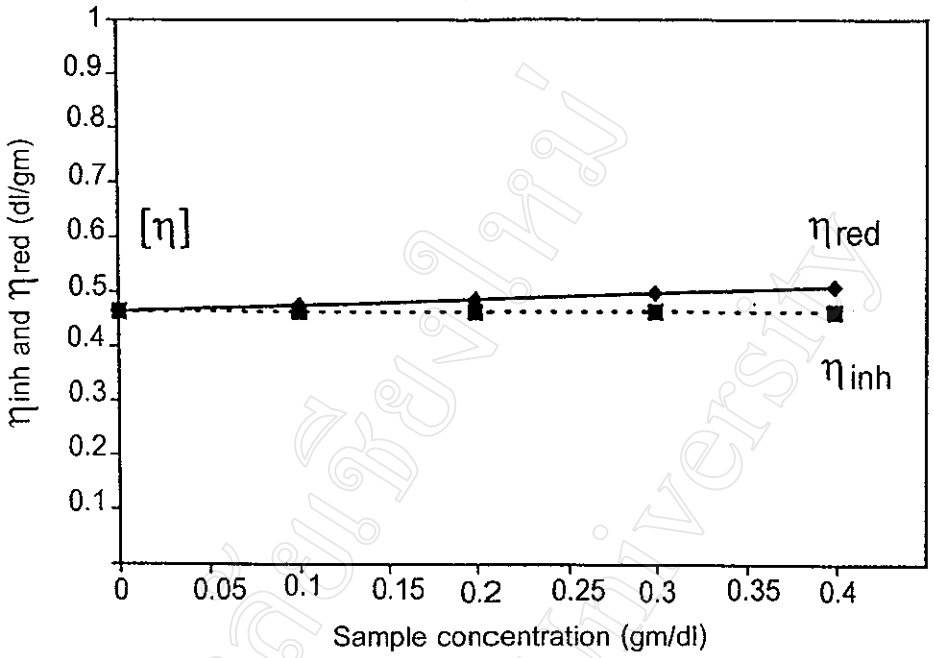

Figure 9 Graph plotted between η_{inh} and η_{red} for determination of [η]

2.3 Biological assay of CPS

2.3.1 Anticoagulant activity

2.3.1.1 Determination of anticoagulant activity

A. Preparation of normal human plasma (NHP)

NHP was prepared from five healthy adults who were not applied on any medication or oral contraceptive agents. Moreover, these volunteer had no history of liver disease or thrombosis. Whole blood was collected in 3.8% sodium citrate in a ratio of blood: anticoagulant of 9:1. The whole blood was immediately centrifuged at 2500g for

20 minutes at 4 °C to obtain platelet-poor plasma. The plasma was pooled and aliquoted to 1 ml each. The NHP was stored at –20 °C.

B. Preparation of heparin standards and a standard heparin calibration curve

The standard therapeutic heparin (5000 IU/ml) was used for the preparation of the assay standards. Standard therapeutic heparin was diluted with NHP to a various final concentrations as described in Table 6. Each concentration was assayed by the Accuclot™ Heptest and clotting time was recorded. The clotting time was plotted against heparin concentration (IU/ml) to set the standard calibration curve.

Table 6 Preparation of heparin standards for the determination of Factor Xa activity

Tube No.	Volume (ml)	NHP (ml)	Heparin standard (IU/ml)
1	0.1 ml of heparin (100 IU/ml)	0.9	10
2	0.1 ml of tube 1	0.9	1.0
3	0.4 ml of tube 2	0.4	0.5
4	0.4 ml of tube 3	0.4	0.25
5	0.4 ml of tube 4	0.4	0.125
6	0.4 ml of tube 5	0.4	0.062
7	0.4 ml of tube 6	0.4	0.031
8	0	0.4	0

C. Inhibition effect on Factor Xa activity

Factor Xa solution and Recalmix® was prepared as indicated in the instruction manual (Accuclot™ Heptest. Recalmix® was prewarmed at 37 °C for 5 minutes before use. Two mg/ml of chitosan and CPS was prepared by dissolving 2.0 mg of sample

in 1 ml of PBS pH 7.2. Glass test tubes contained 100 µl of test sample (1 µl of 2 mg/ml sample in 99 µl of NHP) or various concentrations of heparin standard were prewarmed in 37 °C water bath for 10 minutes. One hundred µl of Factor Xa was added and mixed well. First timer was simultaneously started. The reaction was then incubated for exactly 120 seconds before adding of 100 µl of Recalmix.® Second timer was simultaneously started and clotting time was recorded in second. The assay was performed in duplicate. The activity of CPS was determined by converting the clotting time to heparin IU/ml using standard heparin calibration curve.

2.3.1.2 Effect of CPS on the inhibition of Factor Xa by antithrombin III (ATIII)

A. Preparation of heparin standards and standard heparin calibration curve

An eighty IU/ml of standard therapeutic heparin was prepared by diluting 16 µl of heparin (5000 IU/ml) with 984 µl of normal saline solution (NSS). Heparin (80 IU/ml) was then ten-fold diluted into 8 IU/ml. This concentration of heparin was used to prepare the heparin standards using NHP as described in Table 7. Each concentration was then assayed in duplicate by the Accucolor™ Heparin. Measurement of the residual factor Xa activity with a factor Xa-specific chromogenic substrate, was recorded in the intensity of absorption at 405 nm. The Absorption (O.D.) was plotted against heparin concentration (IU/ml) to set the standard heparin calibration curve.

Table 7 Preparation of heparin standards for the determination of the inhibition of Factor Xa by ATIII

Heparin solution	NHP	Heparin standard (IU/ml)
100 µl of 8 IU/ml	900 µl	0.8
500 µl of 0.8 IU/ml	500 µl	0.4
500 µl of 0.4 IU/ml	500 µl	0.2
0	500 µl	0

B. Determination of the effect of CPS on the inhibition of factor Xa by ATIII

Using the microplate method, firstly, Bovine Factor Xa, human antithrombin III (ATIII) and Factor Xa substrate were prepared as indicated in the instruction manual. Test sample (2 µg/25 µl NHP) and various concentrations of heparin standards was 2-fold diluted in normal saline prior to assay. Seventy-five µl of human ATIII was dispensed into individual wells of a 96-well microtiter plate following with 25 µl/well of pre-warmed diluted sample test or heparin standards. The reaction was incubated for exactly 120 seconds. The 75 µl of bovine factor Xa was simultaneously dispensed into each well and incubated for additional 60 seconds. Finally, factor Xa substrate was dispensed 75 µl/well and the reaction was incubated for another 10 minutes before stopping with 75 µl/well of glacial acetic acid. The reaction was read for absorbance at 405 nm using the reagent blank consisted of 75 µl/well acetic acid, 75 µl/well human ATIII, 25 µl/well diluted NHP, 75 µl/well bovine factor Xa and 75 µl/well factor Xa substrate. The residual factor Xa activity in each test sample was determined by the inversed proportion to the heparin standard concentration. The activity of CPS in the inhibition of factor Xa by ATIII was reported in the equivalent to standard heparin (IU/ml).

2.3.1.3 Determination of antithrombin activity

A. Preparation of antithrombin standards and a typical antithrombin standard curve

Antithrombin standard (Accuclot™ Reference Plasma Normal) was used to prepare the antithrombin standards as described in Table 8 and perform the antithrombin standard curve by plotting the absorbance against the % of antithrombin activity (%AT).

Table 8 Preparation of antithrombin standards

Dilution buffer	Reference plasma normal (A7432)	% AT
1000 µl	25 µl	100
500 µl	500 µl of 100%AT	50
1000 µl	0	0
1000 µl	25 µl test sample	-

B. Endpoint method for the assay of antithrombin activity

Using the test tube method, firstly, heparin/thrombin reagent, thrombin substrate, and standard dilution were prepared as indicated in the instruction manual (Antithrombin™). Two hundred µL of each antithrombin standards or test sample (1 µl of 2 mg/ml sample in 199 µl NHP) were pre-warmed in the 37 °C water bath for 5 minutes. The Heparin/thrombin reagent was added 200 µl to each tube and mixed well. The reaction was incubated for exactly 120 seconds before adding of 200 µl of thrombin substrate. The reaction was incubated for additional 120 seconds and subsequently stopped reaction with 200 µl glacial acetic acid. The reaction was read for absorbance at 405 nm using the reagent blank consisted of 200 µl acetic acid, 200 µl dilution buffer,

200 µl Heparin/thrombin reagent, and 200 µl thrombin substrate. Measurement of the residual thrombin activity with a thrombin-specific chromogenic substrate, was recorded in the intensity of absorption at 405 nm. The absorption (O.D.) was plotted against %antithrombin activity. The AT level could be determined by interpolation from the standard curve. In case of a commercial AT control was used, the AT concentration could be adjusted for the AT concentration in the control.

$$\%AT\ (test,\ adjusted) = \frac{\%AT\ (control) \times \%AT\ (test)}{100}$$

when %AT control (A7432) = 113%

2.3.1.4 Determination of thrombin time (TT)

Bovine thrombin (approximately 3-4 NIH units/ml) was prepared as indicated in the instruction manual (Accuclot™ Thrombin time. Thrombin time was assayed using the FibriTimer II. Normal human citrated plasma was obtained by the method described previousely. Test assay was performed in citrated human plasma containing various final concentrations of CPS (1, 5 and 8 µg/reaction) or heparin (0.1-0.4 IU/ml/reaction). Accuclot™ Control I, (A4089) was used as the plasma control. Bovine thrombin was kept in room temperature during assay. Prior to each test, cuvets with a magnetic ball was placed in the unheated preparation area. Seventy-five µl of tested sample, normal control or heparin was dispensed into the cuvet and flap was closed immediately. The cuvet was then moved to the rotating test position and kept stirring for 5 minutes. Seventy-five µl of

Bovine thrombin was dispensed into the cuvet and flap was closed immediately which then electronically started the timer. The clotting time was recorded in second. Each test sample was individually performed in duplicate. The mean of clotting time of each test sample was reported. The thrombin time was assayed individually in different day for 3 days.

2.3.1.5 Determination of prothrombin time (PT)

Thromboplastin-HS contained tissue factor from rabbit brain and calcium chloride was reconstituted with distilled water as indicated in the instruction manual (Thromboplastin-HS with calcium). One hundred µl of citrated normal human plasma containing 2 µg of P1-P3, PPS or heparin was prewarmed in 37 °C water bath for 5 minutes. The Thromboplastin-HS was dispensed 200 µl and the clotting time was simultaneously recorded. Each assay was done in duplicate. The mean of clotting time of each assay was used for calculation of R value. Prothrombin time (PT) was reported in INR (International normalized ratio) which could be calculated from the following equation:

$$INR = R^{ISI}$$

INR = International normalized ratio

$$R = \frac{\text{Sample/NHP PT}}{\text{Mean normal PT}}$$

ISI = 1.25 (for Thromboplastin-HS, Lot number 031K6145)

2.3.1.6 Inhibition of fibrin polymerization

Atroxin reagent which is purified enzyme extracted from snake venom (*Bothrops atrox*) was reconstituted with distilled water as indicated in the instruction manual (ATROXIN 845-2). The reagent was kept at room temperature during assay. Two hundred µl of normal human plasma containing 10 µg of P1-P3 or PPS or 0.5 IU/ml heparin were assayed. Control I (A4089) was used as the normal control. Each tested sample and normal control were prewarmed in the 37 °C water bath for 5 minutes assay. One hundred µl of the atroxin reagent was added and clotting time was recorded in second.

2.3.2 Involvement in the immune response *in vitro*

2.3.2.1 Effect on cell proliferation

A. Preparation of peripheral blood mononuclear cells (PBMCs)

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by Ficoll-Hypaque gradient centrifugation. Briefly, a 20 ml of heparinized whole blood was diluted with 20 ml of sterile phosphate buffered saline pH 7.2 (PBS) in a 50 ml centrifuge tube. Forty ml of diluted whole blood were overlayered on 10 ml of Ficoll-Hypaque and tube was centrifuged at 400g at room temperature. The PBMCs rich interphase layer was collected. Prior to any experiment, the cells were washed three times with RPMI-1640 medium supplemented with gentamycin (final concentration 40 mg/L) and amphotericin B (final concentration 2.5 mg/L). The washed cells were resuspended in RPMI-1640 supplemented with 10% fetal calf serum (complete culture media).

B. Determination of the optimal conditions for cell proliferation assay

Washed PBMCs isolated from 3 healthy donors resuspended in complete culture media were cultured in a 96-well culture plate at a final concentration of 5 x 10^4 cells/well in the presence or absence of various final concentrations of tuberculin purified protein derivatives (PPD) (0-10 ug/mL) or phytohemagglutinin (PHA) (0-10 ug/ml) at 37 °C, 5% CO_2 and 95% humidity. Cells were incubated for 5 days or 2 days for PPD or PHA stimulation, respectively. After exactly incubation time, 10 µl of BrdU labeling reagent was subsequently added to the cells (10 µM/well) and reincubated for additional various times (2-24 hrs). Background control and blank were also done as indicated below. The final volume of reaction mixture was 200 /well. All assays were done in triplicate. Finally, the plates were centrifuged at 300g for 10 minutes. Cell culture supernatant was removed by suction using a canulla. Plates were dried using hair-dryer for 15 minutes.

Overview of the controls

Well controls	Blank	Background control
Culture medium	100 µl	-
Cells	-	100 µl
BrdU	10 µl	-
Anti-BrdU-POD	100 µl	100 µl

For the detection of cell proliferation , dried culture plates were dispensed with 200 µl/well of FixDenat ® and incubated for 1 hr at room temperature. The plates were flicked off and tapped onto towel pad to discard FixDenat ®. Peroxidase conjugated anti-BrdU (anti-BrdU-POD) was subsequently dispensed 100 µl/well and incubated for additional 90-120 minutes at room temperature or 37 °C. Plates were flicked off and tapped to remove

anti-BrdU-POD following with washing solution for 3 times. Plates were tapped before dispensing with 100 µl/well of TMB. Plates were incubated for 5-30 minutes at room temperature before stopping the reaction with 50 µl/well of 1M H_2SO_4. Finally, the absorbance was measured in ELISA Reader at 450 nm with reference wavelength at 630 nm.

C. Determination of the sub-optimal concentration of PPD

Cell culture was set up in the 96-well tissue culture plates in a final volume of 200 µl/well. Triplicate aliquots of 5 x 10^4 PBMC from 3 healthy donors were cultured with various concentrations of PPD (0-10 µg/ml) in complete culture media. The cultures were incubated in a CO_2 incubator at 37 °C, 95% humidity for 5 days. Controls of the assay were done as described previous in the overview of the controls. After exactly incubation time, 10 µl of BrdU labeling reagent was subsequently added to the cells (10 µM/well) and reincubated for 21 hrs. Plates were then centrifuged at 300g for 10 minutes. Cell culture supernatant was removed and plates were dried. Cell proliferation was assayed by the optimal conditions which was studied. Briefly, plates were incubated for 2 hrs at 37 °C with 100 µl/well of anti-BrdU reagent and the washed out. TMB was added 100 µl/well and incubated for 15 minutes at room temperature. The reaction was stopped with 50 µl/well of 1M H_2SO_4 and measured for absorbance at 450 nm with reference wavelength of 630 nm.

D. Determination of the sub-optimal concentration of PHA

Cell culture was set up in the 96-well tissue culture plates at a final volume of 200 µl/well. Triplicate aliquots of 5 x 10^4 PBMC from 3 healthy donors were cultured with PHA (0-10 µg/ml) in complete culture media. The cultures were incubated in a CO_2 incubator at 37 °C, 95% humidity for 2 days. After exactly incubation time, 10 µl of BrdU labeling reagent was subsequently added to the cells (10 µM/well) and reincubated for 21 hrs. Plates were then centrifuged at 300g for 10 minutes. Cell culture supernatant was removed and plates were dried. Cell proliferation was assayed by Cell proliferation BrdU, ELISA (colorimetric) with the optimal conditions that has been studied as described.

E. Effect of CPS, PPS and heparin on PPD stimulated cell proliferation

Cell culture was set up in the 96-well tissue culture plates in a final volume of 200 µl/well. Triplicate aliquots of 5 x 10^4 PBMC from 3 healthy donors were cultured with PPD (0.31 and 0.62 µg/ml) in complete culture media. The cultures were incubated in the absence or presence of various concentrations of P1-P3, PPS (0-8 µg/ml) and heparin (0-0.8 IU/ml) in the CO_2 incubator at 37 °C, 95% humidity for 5 days. Controls of the assay were done as described previous in the overview of the controls. After exactly incubation time, 10 µl of BrdU labeling reagent was subsequently added to the cells (10 µM/well) and reincubated for 21 hrs. Plates were then centrifuged at 300g for 10 minutes. Cell culture supernatant was removed and plates were dried. Cell proliferation was assayed by Cell proliferation BrdU, ELISA (colorimetric) with the optimal conditions described above.

F. Effect of CPS, PPS and heparin on PHA stimulated cell proliferation

Cell culture was set up in the 96 well culture plates in a final volume of 200 µl/well. Triplicate aliquots of 5 x 10^4 PBMC from 3 healthy donors were cultured with PHA (0.5 µg/ml) in complete culture media. The cultures were incubated in the absence or presence of various concentrations of P1-P3, PPS (0-8 µg/ml) and heparin (0-0.8 IU/ml) in the CO_2 incubator at 37 °C, 95% humidity for 2 days. Controls of the assay were done as previously described. After exact incubation time, 10 µl of BrdU labeling reagent was subsequently added to the cells (10 µM/well) and reincubated for 21 hrs. Plates were then centrifuged at 300g for 10 minutes. Cell culture supernatant was removed and plates were dried. Cell proliferation was assayed by Cell proliferation BrdU, ELISA (colorimetric) with the optimal conditions that has been studied as described.

2.3.2.2 Effect on cell mediated cytotoxicity

A. Determination for the optimal target cell concentration

By the advantage of Cytotoxicity detection Kit (LDH), cell mediated cytotoxicity assay was performed instead of [^{51}Cr] release assay. Erythroid/myeloid cell line (K562) was used as target cell for the study of cell mediated cytotoxicity. Cells were collected, washed twice and resuspended in RPMI-1640 supplemented with 1% FCS (incomplete culture media) prior to do experiment. Various final concentrations of cells were prepared (1 x 10^3 – 2 x 10^5 cells/well). Two experiments were performed in parallel: low control (spontaneous LDH release) and high control (maximum LDH release) as described below in overview of the controls. Cell control and blank were also studied as

indicated and each assay was done in triplicate. Cell cultures were incubated in a CO_2 incubator at 37 °C, 95% humidity for 4 hrs. At the end of incubation, culture plates were centrifuge at 300g for 10 minutes and cell culture supernatant was collected 100 µl/well for further analysis.

Overview of the controls

Contents of the well	Background control	Low control	High control	Substance control I	Substance control II	Experimental set up
Assay medium	200 µl	100 µl		100 µl		
Cells		100 µl	100 µl			100 µl
Triton X-100 solution (2% in assay medium)			100 µl			
Test substance or effector cells				100 µl	50 µl	100 µl
LDH standard					50 µl	

B. Detection of cell mediated cytotoxicity by using Cytotoxicity Detection Kit (LDH)

One hundred µl of cell culture supernatant from each well was carefully collected and transferred into corresponding wells of an optically clear 96-well flat bottom microtiter plate. The reaction mixture consisted of catalyst and dye solution was prepared immediately before use and subsequently dispensed at 100 µl/well. The plate was incubated at room temperature for 30 minutes in a container with light protection. Measurement of the absorbance of the samples at 490 nm was performed by the ELISA reader. The reference wavelength was 630 nm.

C. Determination of the cytotoxicity potential of CPS

K562 was cultured in 1% incomplete medium at a final concentration of 2 x 10^4 cells/well in the presence of various concentrations of P1-P3, PPS (0-8 μg/ml) or heparin (0-0.8 IU/ml) for 4 hrs in a CO_2 incubator at 37 °C, 95% humidity. Two experiments were performed in parallel: low control (spontaneous LDH release) and high control (maximum LDH release). Cell control and blank were also studied as was described previously and each assay was done in triplicate. At the end of exactly incubation, culture plates were centrifuged at 300g for 10 minutes and cell culture supernatant was collected 100 μl/well for further analysis as described. The cytotoxicity (%) was calculated by the equation below.

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental value} - \text{low control}}{\text{High control} - \text{low control}} \times 100$$

D. Optimization of the Effector : Target cell ratio (E:T)

PBMCs was isolated from 5 healthy volunteers by the Ficoll-Hypaque gradient centrifugation. Cells were washed with RPMI-1640 and resuspended in incomplete culture media at the concentration of 4 x 10^7 cells/ml. Cells were then 2-fold serial diluted with incomplete culture media to get the 6 various concentrations in the final volume of 100 μl /well. K562 was collected, washed twice and resuspended with incomplete culture media. These target cells were adjusted to 2 x 10^5 cells/ml and dispensed 100 μl/well. The effector : target (E:T) ratio ranged from 200:1 – 6.3:1, respectively. Cell cultures were

incubated for 4 hrs in a CO_2 incubator at 37 °C, 95% humidity. Two experiments were performed in parallel: low control (spontaneous LDH release) and high control (maximum LDH release). Cell control and blank were also studied as previously described and each assay was done in triplicate. After incubation, culture plates were centrifuged at 300g for 10 minutes and cell culture supernatant was collected 100 µl/well for further analysis as described. The cytotoxicity (%) was calculated employing the equation below.

$$\text{Cell mediated cytotoxicity (\%)} = \frac{\text{[E:T mix – Effector control] – low control}}{\text{High control – low control}} \times 100$$

E. Study of the effect of CPS, PPS and heparin on cell mediated cytotoxicity

Various final concentrations of P1-P3 and PPS (0-8 µg/ml) or heparin (0-0.8 IU/ml) were prepared. PBMCs from 5 healthy volunteers were isolated from leukocyte rich whole blood by Ficoll-Hypaque gradient centrifugation as described. Cells were washed twice and adjusted to the final concentration of 1×10^7 and 2×10^7 cells/ml with incomplete culture media. Cell culture was performed in two different E:T ratio of 100:1 and 50:1. Briefly, various concentrations of tested materials were dispensed 50 µ l/well for each condition. Two concentrations of PBMCs (2×10^7 cell/ml) and (1×10^7 cell/ml) were dispensed 100 µl/well individually into the set of E:T ratio 100:1 and 50:1, respectively. K562 (4×10^5 cell/ml) was dispensed 50 µl/well to every well of each experiment. Two experiments were performed in parallel: low control (spontaneous LDH release) and high control (maximum LDH release). Controls were performed as described earlier. In addition, toxicity potential of the tested materials to K562 was also performed in

parallel. The system consisted of K562 (2 x 10^4 cell/well), various concentrations of the tested materials (50 µl/well), and incomplete culture media (50 µl/well) which was used instead of PBMCs. All assays were done in triplicate. Subsequently, culture plates were centrifuged at 300g for 10 minutes and cell culture supernatant was collected 100 µl/well for further analysis as described. The cytotoxicity (%) was calculated from the equation.

2.3.2.3 Effect on immunoglobulin production

A. Determination of the optimal conditions for the immunoglobulin detection

To determine the optimal concentration of the first antibody (anti-human immunoglobulin G, A, M,) and second antibody (Horse-radish peroxidase conjugated anti-human immunoglobulins, polyvalent), the conditions were determined. Various dilutions of rabbit anti-human IgG, goat anti-human IgA and goat anti-human IgM (1:1000-1:4000) were diluted with carbonate-bicarbonate buffer pH 9.6. The first antibodies were then coated 100 µl/well onto the 96-well microtiter plates at 4 °C overnight and washed 4 times with 0.05% Tween in PBS pH 7.2 (PBST). Non-specific sites were blocked with 5% bovine serum albumin (BSA) in PBST 200 µl/well for 1 hr at 37 °C and washed 4 times with PBST, respectively. Standard human immunoglobulins was 2-fold serially diluted with PBST from 1:1000-1:512000 and dispensed 100 µl/well into correspondent well as was designed. Plates were then incubated for another 2 hrs at 37 °C and washed 4 times with PBST. Horse-radish peroxidase conjugated rabbit anti-human Igs was diluted with PBST to 1:20000 and added 100 µl/well into an assay series of rabbit anti-human IgG. Horse-radish peroxidase conjugated goat anti-human Igs was diluted with PBST to 1:2500 and

1:5000 and added 100 µl/well into an assay series of goat anti-human IgA and –IgM. Plates were then incubated for 2 hrs following with washing for 4 times. One hundred µl/well of OPD substrate was dispensed and plates were incubated for 30 minutes at room temperature in a light protected container. 2.5N H_2SO_4 was added 50 µl/well to stop the reaction. Finally, the absorbance was determined at 490 nm by the ELISA reader.

B. Determination of the sub-optimal concentration of pokeweed mitogen (PWM)

B1. Stimulation of PBMCs with PWM

PBMCs from 3 healthy volunteers were isolated by the Ficoll-Hypaque gradient centrifugation as described. Cells were washed 4 times with RPMI-1640 to eliminate plasma immunoglobulins and resuspended in complete RPMI. Cells were culture at a final concentration of 5 x 10^5 cells/ml in the presence of various concentrations of PWM (0-10 µg/ml) for 10 days in a CO_2 incubator at 37 °C and 95% humidity. Finally, cell culture supernatant was collected and centrifuged at 500g for 10 minutes. Cell-free supernatant was stored at 4 °C before further analysis for the immunoglobulin production.

B2. Detection of PWM stimulated immunoglobulin production

Rabbit anti-human IgG and goat anti-human IgA, IgM, IgD and IgE were diluted to 1:1000 with carbonated-bicarbonate buffer pH 9.6. These first antibodies were coated onto 96-well microtiter plates. The plates were incubated overnight at 4 °C and washed 4 times with PBST. Non-specific sites were blocked with 5% BSA in PBST 200 µl/well for 1 hr at 37 °C and the plates were washed 4 times with PBST, respectively.

PWM stimulated cell-free supernatant was diluted 1:10 with PBST and dispensed 100 µl/well into correspondent well as was designed. Plates were then incubated for another 2 hrs at 37 °C and washed 4 times with PBST. Horse-radish peroxidase conjugated rabbit anti-human Igs (1:20000) was added 100 µl/well into an assay series of rabbit anti-human IgG. Horse-radish peroxidase conjugated goat anti-human Igs (1:2500) was added 100 µl/well into an assay series of goat anti-human IgA, IgM, IgD and IgE. Plates were then incubated for 2 hrs following with 4 times washing. One hundred µl/well of OPD substrate was dispensed and the plates were incubated for 30 minutes at room temperature in a light protected container. Fifty µl of 2.5N H_2SO_4 were added into each well to stop the reaction. Finally, the absorbance was determined at 490 nm by the ELISA reader.

C. Study of the effect of CPS on the immunoglobulin production

PBMCs from 3 healthy volunteers were isolated by the Ficoll-Hypaque gradient centrifugation as described. Cells were washed 4 times with RPMI-1640 to eliminate plasma immunoglobulins and resuspended in complete culture media. Cells were culture at a final concentration of 5×10^5 cells/ml in the presence or absence of P1-P3, PPS (0-8 µg/ml) and heparin (0-0.8 IU/ml). Cell cultures were incubated for 4 hrs in a CO_2 incubator at 37 °C, 95% humidity before adding of PWM (2.5 µg/ml). The cultures were incubated for another 10 days. Finally, the cell culture supernatant was collected and centrifuged at 500g for 10 minutes. Cell-free supernatant was stored at 4 °C before subjected to the analysis of immunoglobulin production by sandwich ELISA described above.

2.3.2.4 Effect on cytokine production

A. Stimulation of PBMCs in the presence of CPS, PPS and heparin

PBMCs from 3 healthy volunteers were isolated by the Ficoll-Hypaque gradient centrifugation as described. Cells were washed 4 times with RPMI-1640 and resuspended in complete culture media. Cells were culture at a final concentration of 1 x 10^6 cells/ml in the presence or absence of P1-P3, PPS (0-8 μg/ml) and heparin (0-0.8 IU/ml). Positive control was performed using cell cultivation with Phorbol myristate acetate (PMA) and Ionomycin at the final concentration of 10 ng/ml and 1 μg/ml, respectively, 24 hrs in a CO_2 incubator at 37 °C, 95% humidity (Asemissen, *et al.*, 2001). Finally, cell culture supernatant was collected and centrifuged at 500g for 10 minutes. Cell-free supernatant was analyzed for the changes in IL-2 and IFN-γ production by sandwich ELISA.

B. Detection of IL-2 and IFN-γ in the cell culture supernatant

Various dilutions of PMA/Ionomycin stimulated supernatant in RPMI-1640 were used as a positive control. The standard IL-2 and IFN-γ were prepared by 2.5 fold serial dilution with the provided diluent following the manufacturer's recommendation. Various concentrations of IL-2 and IFN-γ standards positive control and tests were dispensed at 50 μl/well. The plates assayed for IFN-γ and IL-2 were incubated individually for 2 and 3 hrs at room temperature, respectively. Washing was performed 4 times with

the provided washing reagent. Freshly prepared Streptavidin-HRP was dispensed at 100 µl/well and incubated for 30 minutes at room temperature. Plates were washed 4 times, flicked off and tapped onto towel pad. Premixed TMB was then added 100 µl/well and incubated for 30 minutes at room temperature in the light protected container. Reaction was stopped with 100 µl/well of the stop solution and read the absorbance at 450/550 nm.

2.4 Analytical statistics

Data from the anticoagulant assays were analyzed and reported in mean ± S.D. MANOVA analysis and Pearson's test were used to analyse the data from immunological assays. They were appropriate since the independent variables were nominal and categorical and the outcomes were numerical.