CHAPTER 3

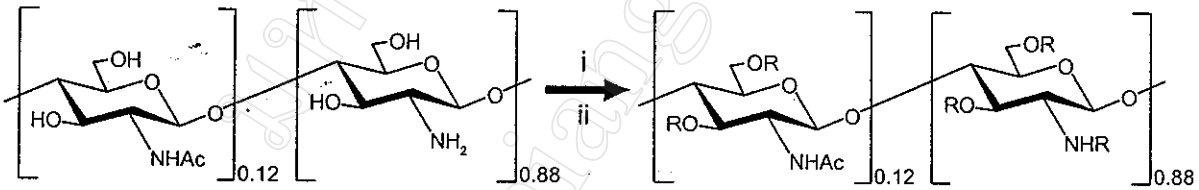
RESULTS

3.1 Preparation of chitosan polysulfate (CPS) by random substitution

3.1.1 Synthesis and purification of chitosan polysulfate (CPS)

3.1.1.1 Synthesis of chitosan polysulfate (CPS)

The starting material, chitosan was random sulfated by the conditions that was modified from the method of Gamzazade *et al*., (1997). Chitosan was solvated in DMF before adding of $HClSO_3$/DMF. The reaction was 5 hrs at room temperature. CPS product was dialysed against distilled water for 48 hrs and lyophilized, the dried CPS obtained was 2.9 gm from 3.00 gm of starting material. The yield of this step is 97%. The scheme of reaction was shown in Figure 10.



1 2

Figure 10 Scheme of sulfation reaction. 1; chitosan, 2; CPS, i; chlorosulfonic acid/DMF, ii; at room temperature for 5 hrs, R = SO_3Na or H^+

3.1.1.2 Separation and purification of CPS

A. Calibration of the Sepharose CL-6B column for the separation of various average molecular weight of CPS

The column of 1.6 x 100 cm was calibrated before gel filtration of CPS using the blue dextran (M_v 1,000 kDa) and phenol red (MW 357 Da) as the calibrators for determining the total volume (V_t) and void volume (V_o) at flow rate of 20 ml/hr. It was shown that V_t and V_o were at 58 $\pm$ 2 ml and 170 $\pm$ 2 ml, respectively as was demonstrated in Figure 11. Dextran sulfate (M_v 8, 40 and 500 kDa) were used to determine the pattern of separation of the polymers with different molecular weights. The results showed that polymers with each molecular weight provided the broad fractions of separation and there were also the overlapping between each individual molecular weight as was shown in Figure 12.

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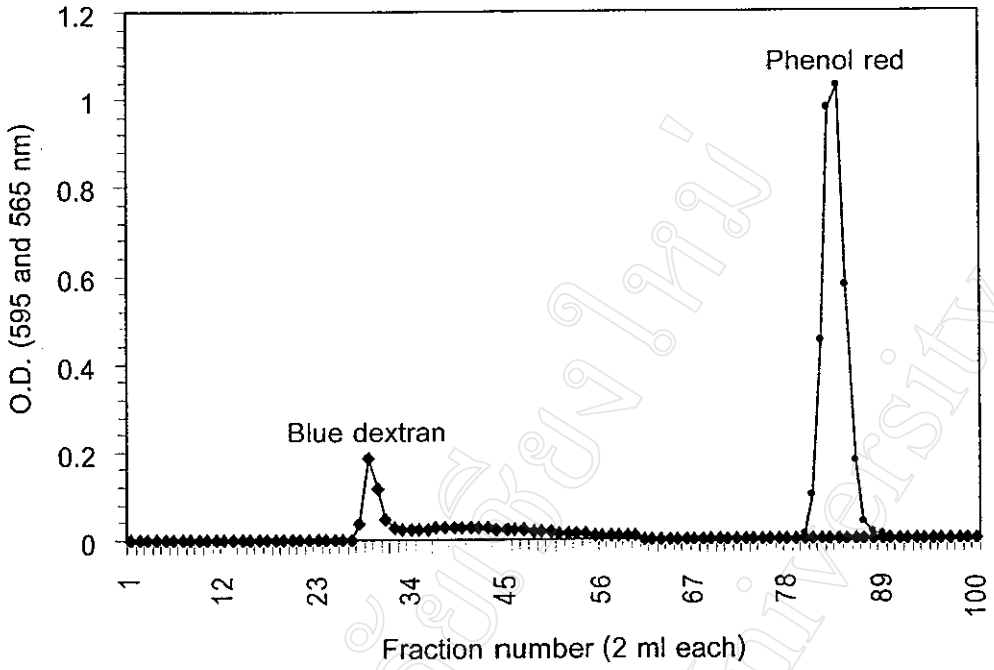

Figure 11 Standardization of the Sepharose CL-6B column (1.6 x 100 cm). Two ml of dye mixture (blue dextran and phenol red, 20 mg/ml in PBS) was loaded onto the column and eluted with PBS at flow rate of 20 ml/hr. Each fraction was detected by dimethylmethylene blue. The O.D. at 595 nm for blue dextran and 565 nm for phenol red were measured and plotted against fraction number.

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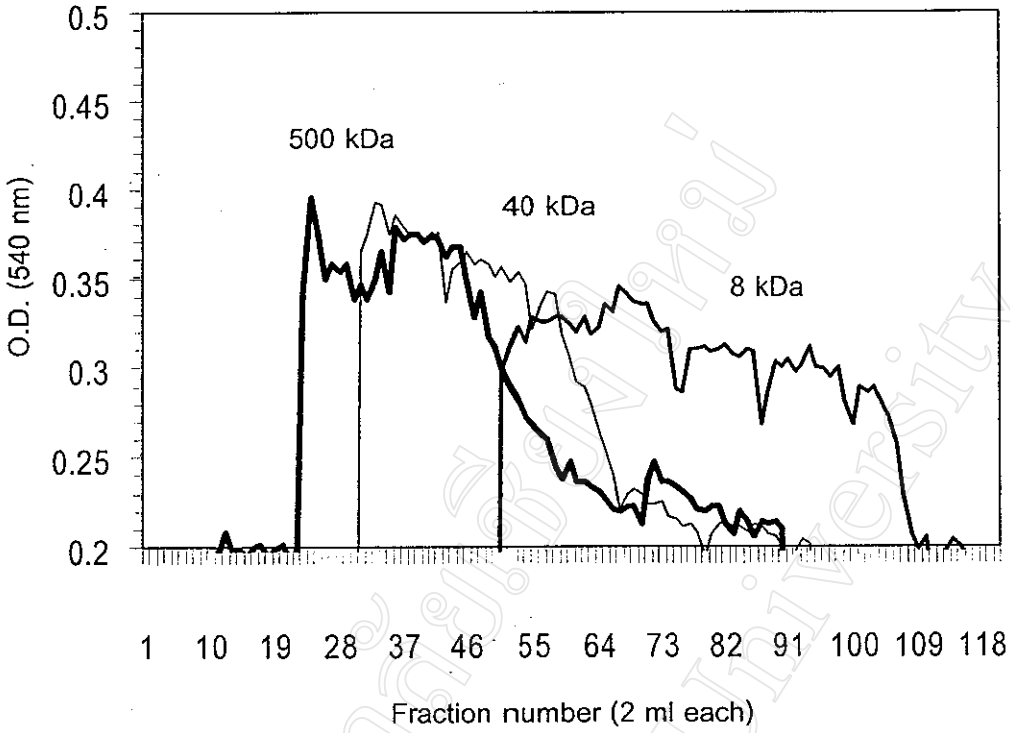


Figure 12 Patterns of gel filtration of 3 different molecular weights of dextran sulfate. Ten ml of each dextran sulfate (500, 40, or 8 kDa, 10 mg/ml) was separately loaded onto the column and eluted with PBS at flow rate of 20 ml/hr. Each fraction was detected by dimethylmethylene blue. The O.D. at 540 nm was measured and plotted against fraction number.

B. Separation of CPS with different average molecular weight (M_V)

To separate the small M_V CPS suitable for the biological activities, the prepared CPS was dissolved in the PBS pH 7.2 and applied onto the column. Each fraction was tested for the sulfate substitution by dimethylmethylene blue and the absorbance at 540 nm was measured and employed for the graph (Figure 13). CPS

showed the broad separation pattern of dimethylmethylene blue dye reaction after separation by the Sepharose CL-6B as also found in dextran sulfate. However, there were fractions with low absorbance among the whole broad pattern of separation. The objective of our study was to separate CPS into small fractions of large, medium and small size. Therefore, the separation pattern of dextran sulfate was used as a template for the decision making of 3 different size of CPS. From which, all fractions were divided into 3 peaks i.e. P1, P2 and P3. P1, P2 and P3 were collected from fraction number 21-37, 38-49 and 50-69, respectively. By the equation indicated below where V_e was the volume eluted from the start of sample application to the inflexion point (or half height of the rising part of elution peak). The K_d was used for calculation and determination of the V_e which referred to elution volume and fraction numbers of each peak in each separation. Therefore, the volume of first fraction of each peak (elution volume, V_e) together with total volume (V_t) and void volume (V_0) as calibrated was used to calculate for the dissociation constant (K_d). The result from the equation indicated below showed the 2 K_d of 0.16 and 0.58, respectively. These 2 K_d were used to calculate for the V_e which referred to the first fraction of each peak in every run of gel filtration.

$$K_d = \frac{V_e - V_0}{V_t - V_0}$$

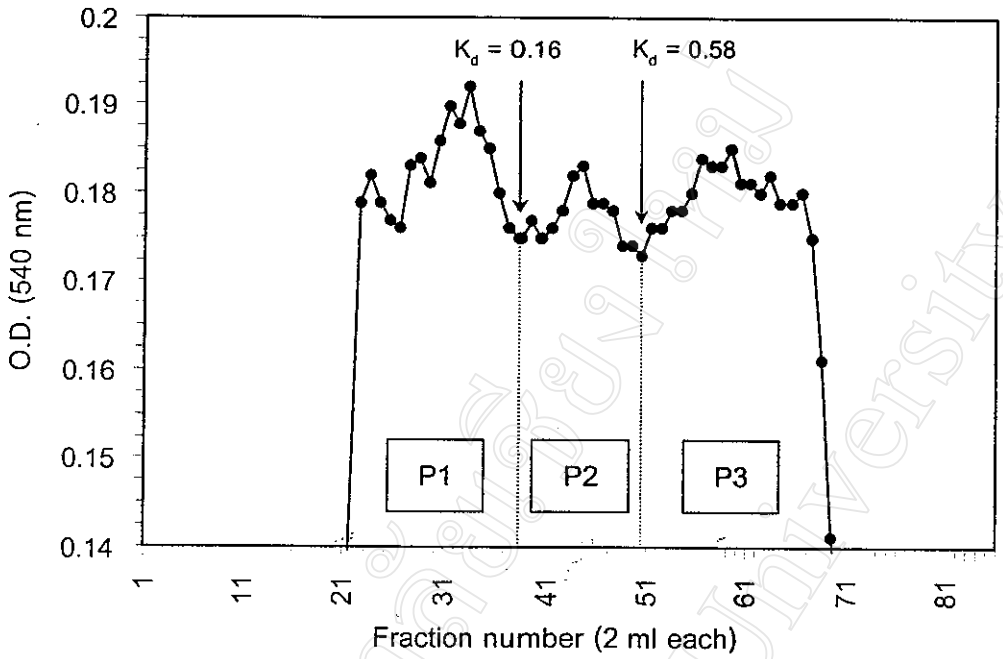


Figure 13 Separation pattern of CPS on the Sepharose CL-6B (1.6 x 100 cm). Ten ml of CPS (10 mg/ml) was loaded onto the column and eluted with PBS at flow rate of 20 ml/hr. Each fraction was detected by dimethylmethylene blue. The O.D. at 540 nm was determined and plotted against fraction number.

C. Purification of CPS

In order to eliminate the unsulfated chains, three fractions of CPS with different molecular weight obtained from B was purified by anion exchange using MonoQ column. The pattern of separation of P1-P3 which assayed by the dimethylmethylene blue were shown in Figure 14-16. The weight obtained after purification was much decreases. The yield of each fraction of purified CPS was summarized in Table 9. The overall yield of sulfation was 55%.

Table 9 Determination of the percentage purification of CPS

Sample		Start weight	Final weight	Yield (%)
Purification	P1	465.80 mg	117.60 mg	25
	P2	1211.30 mg	661.00 mg	55
	P3	587.00 mg	488.80 mg	83

Remark: The percentage yield of synthesis and purification was 55% (3.0 gm of starting chitosan yielded 1.65 gm of purified CPS)

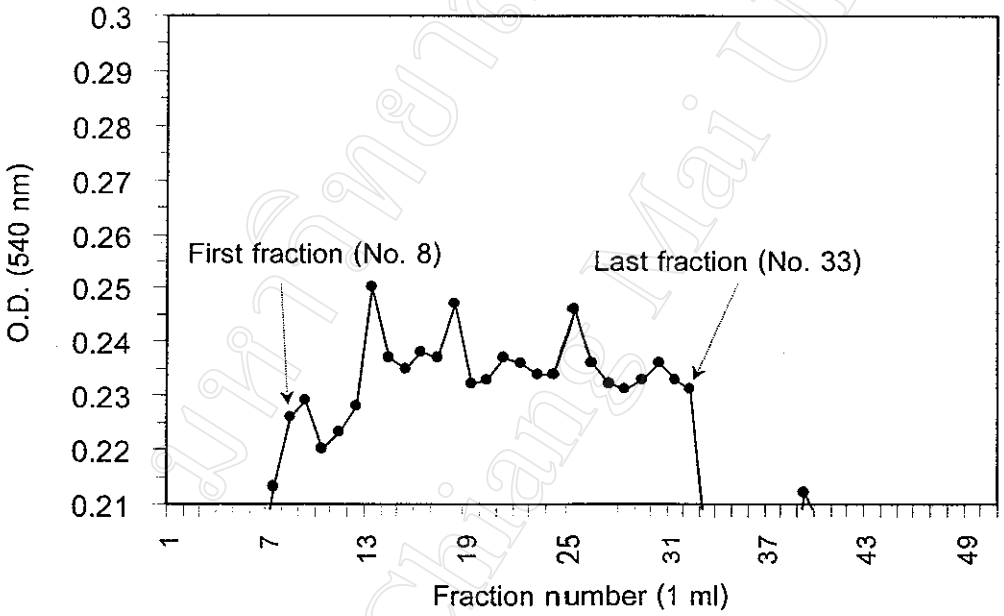


Figure 14 Pattern of purified P1 from MonoQ column as determined by dimethyl-methylene blue dye. The O.D. at 540 nm was determined and plotted against fraction number (Fraction number 8-33 were collected).

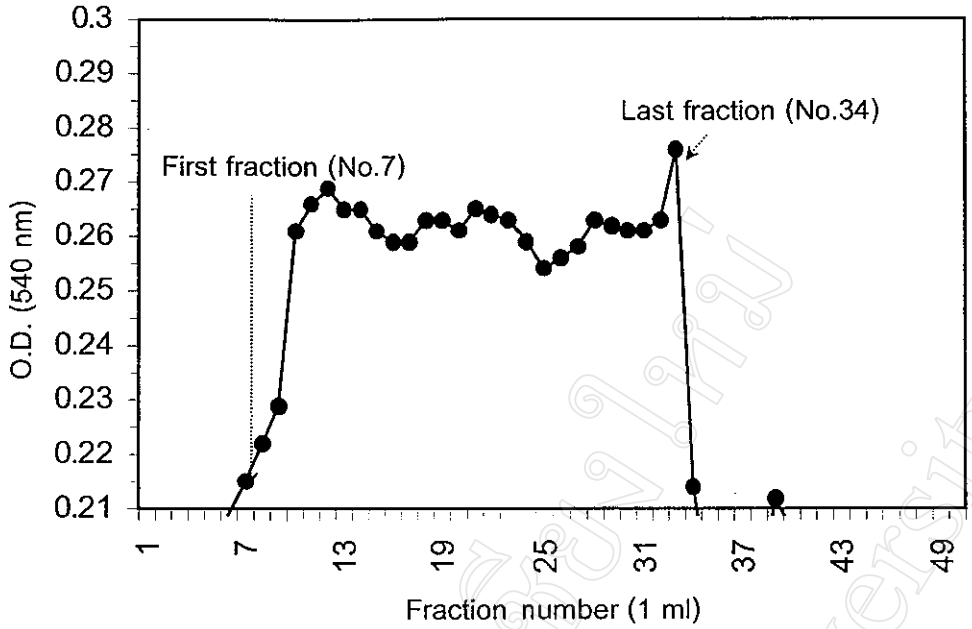


Figure 15 Pattern of purified P2 from MonoQ column as determined by dimethyl-methylene blue dye. The O.D. at 540 nm was determined and plotted against fraction number (Fraction number 7-34 were collected).

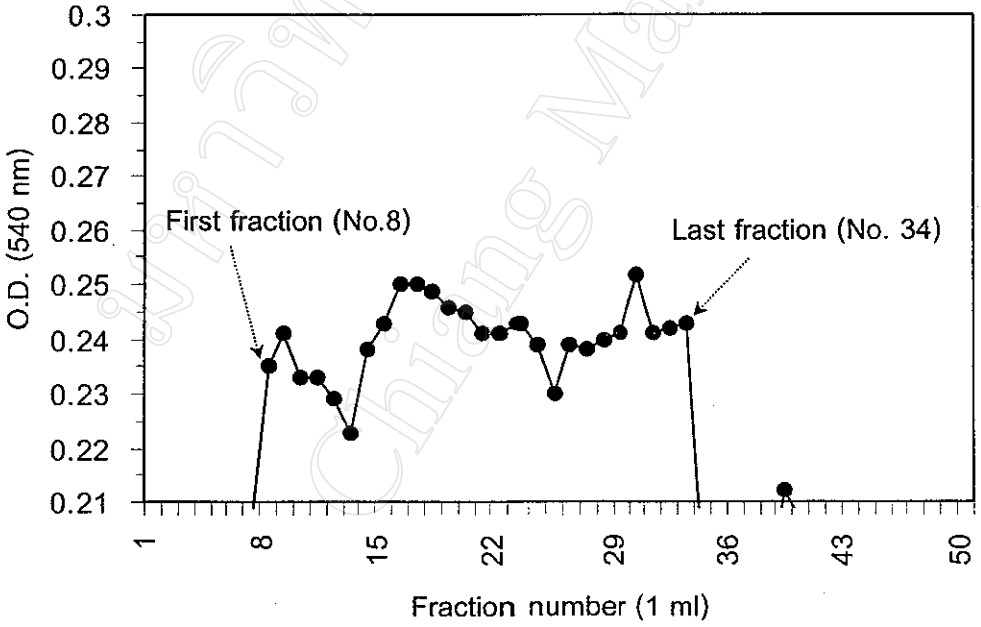


Figure 16 Pattern of purified P3 from MonoQ column as determined by dimethyl-methylene blue dye. The O.D. at 540 nm was determined and plotted against fraction number (Fraction number 8-34 were collected).

3.1.2 Chemical analysis and molecular weight determination

3.1.2.1 IR spectra

The final product, CPS was proved to be sulfate substituted by the infrared spectrometry in KBr disc. The assay was done in parallel with its starting material, chitosan and chitin from marine crab shell. The results of CPS showed the characteristic absorptions in the IR spectrum at 800 and 1240 cm^{-1} which due to sulfo assigned to C-O-S and S=O bond stretching, respectively (Figure 17). The data were summarized in Table 10.

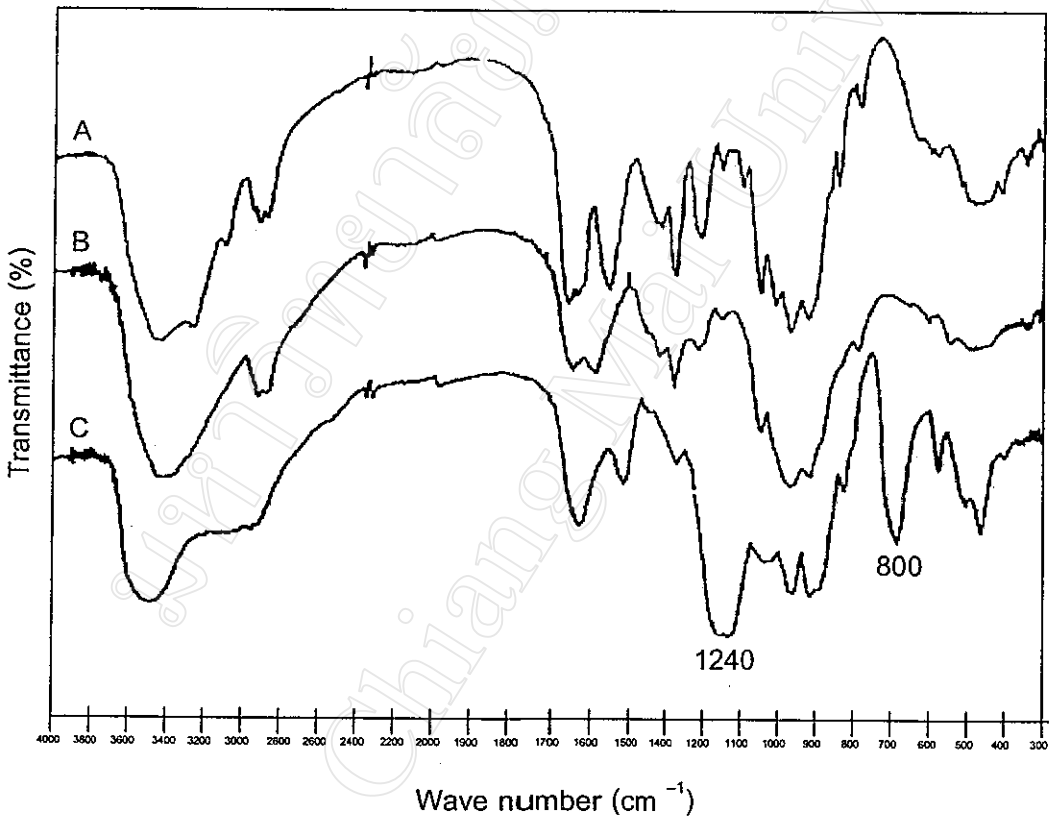


Figure 17 IR spectra of chitin (A), chitosan (B) and CPS (C) (KBr)

Table 10 Interpretation of the IR spectra of chitin, chitosan and CPS

Sample	Wave number (cm^{-1})	Functional group/vibration
Chitin	3200-3500	O-H and N-H stretching
	2890, 2910	C-H stretching
	1640, 1660	C=O stretching (amide)
	1565	N-H bending (amide)
	1425	CH_2 bending
	1381	C-H bending
Chitosan	1550	N-H bending (amine)
CPS	1240	S=O stretching (sulfate)
	800	S-O stretching (sulfate)

3.1.2.2 Specific rotation

CPS in water was determined for the optical rotation in the polarize light by polarimeter at 28 °C in order to observe its specific rotation in comparison to chitosan. The rotation (α) was recorded and calculated by the equation as described in chapter 2.

$$[\alpha]_D^{28} = \frac{(\alpha) \text{ of test}}{c \times l}$$

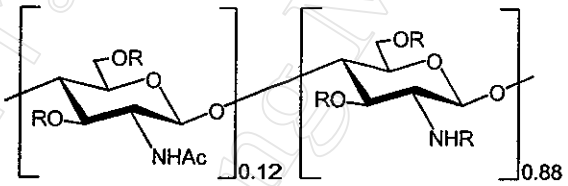
where $c = 0.005$ gm/cm^3

$l = 2.1$ dm^2

It was found that (α) of chitosan and CPS were –0.15 and –0.08, respectively. The calculated $[\alpha]_D$ were –14.3 and –7.6 for chitosan and CPS, respective. This revealed that CPS was β–D-form.

3.1.2.3 ^{1}H-NMR and ^{13}C-NMR

The structure of chitosan in solid state was determined by using CP-MAS ^{13}C-NMR (Duarte *et al.*, 2001). CPS which could be well dissolved in water was analyzed by using both ^{13}C-NMR and ^{1}H-NMR for their structures (Gamzazade *et al.*, 1997). The chemical shifts (δ) were recorded in part per million (ppm). As compare to its starting material, chitosan, it was found that there were lower chemical shifts (δ) in all three positions of carbon atom at C-2, C-3 and C-6. This revealed that there were sulfate substitutions at the three oxygen positions. The data from chemical shifts (δ) were analyzed by CP-MAS ^{13}C-NMR/ ^{13}C-NMR and ^{1}H-NMR and summarized in Table 11 and Table 12, respectively. The results indicated that sulfate molecules were substituted into 3 carbon atoms at C-2, C-3 and C-6. The structure could be predicted as shown below. The CP-MAS ^{13}C-NMR, ^{13}C-NMR and ^{1}H-NMR were shown in Figure 18-20.



R = -SO_3Na or -H

Table 11 Interpretation of the solid state CP-MAS ^{13}C-NMR spectroscopy of chitosan and ^{13}C-NMR for CPS

Sample	Chemical shift (ppm)							
	C-1	C-2	C-3	C-4	C-5	C-6	C=O	CH_3
Chitosan	105.27	57.34	75.24	82.86	75.24	60.96	175.02	23.54
CPS	100.21	58.20	80.97	79.62	75.62	69.53	177.50	25.27

Table 12 Interpretation of ^{1}H-NMR of CPS

Chemical shift (ppm)	Interpretation
4.92	1H, broad doublet, H-1
4.56	1H, broad triplet, H-3S
4.25	2H, multiplet, H-4, H-5
3.97	2H, broad doublet, H-6S
3.69	1H, broad triplet, H-6
3.45	1H, broad triplet, H-2S
3.15	1H, broad triplet, H-2
2.05	3H, singlet, CH_3

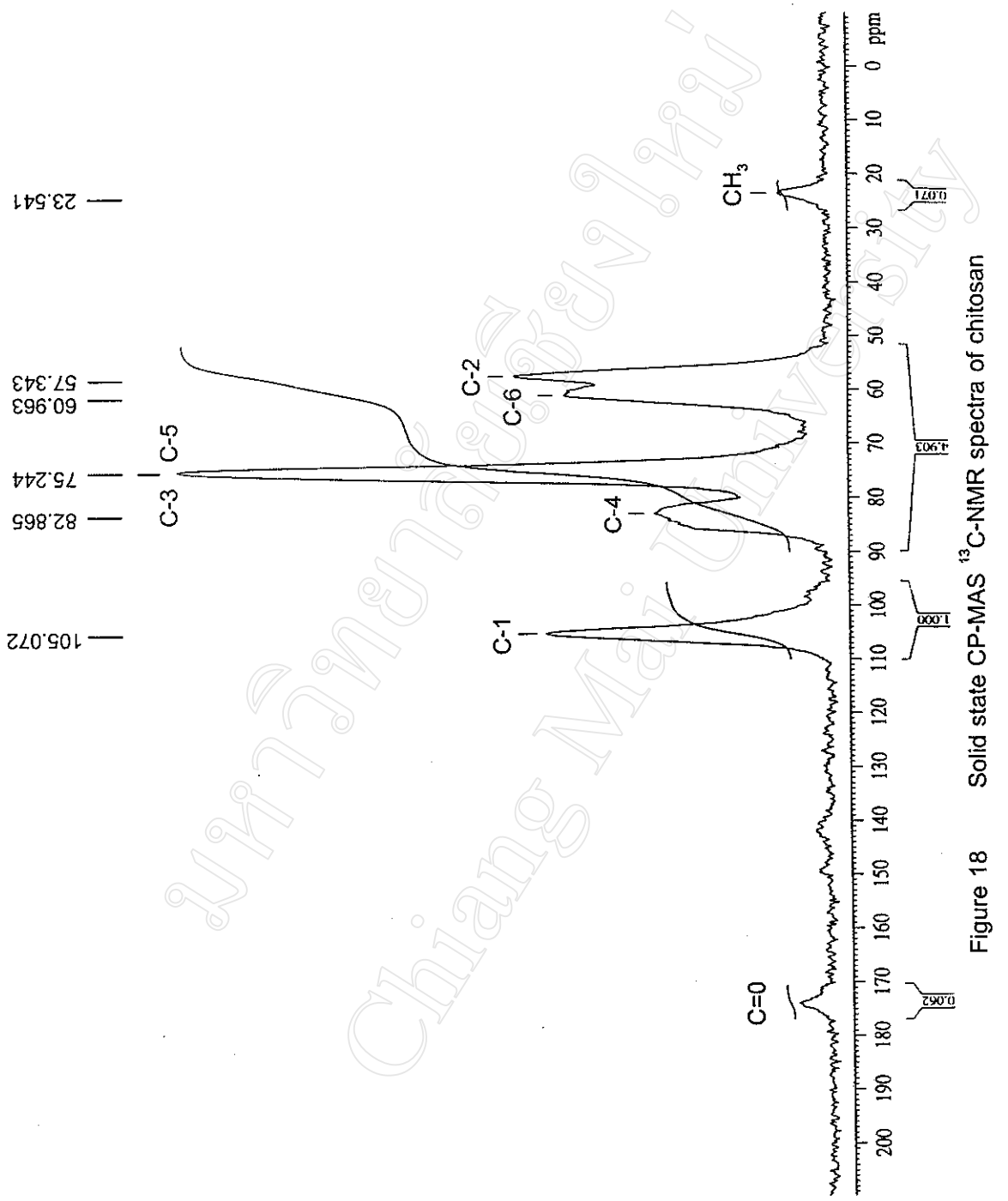


Figure 18 Solid state CP-MAS ^{13}C-NMR spectra of chitosan

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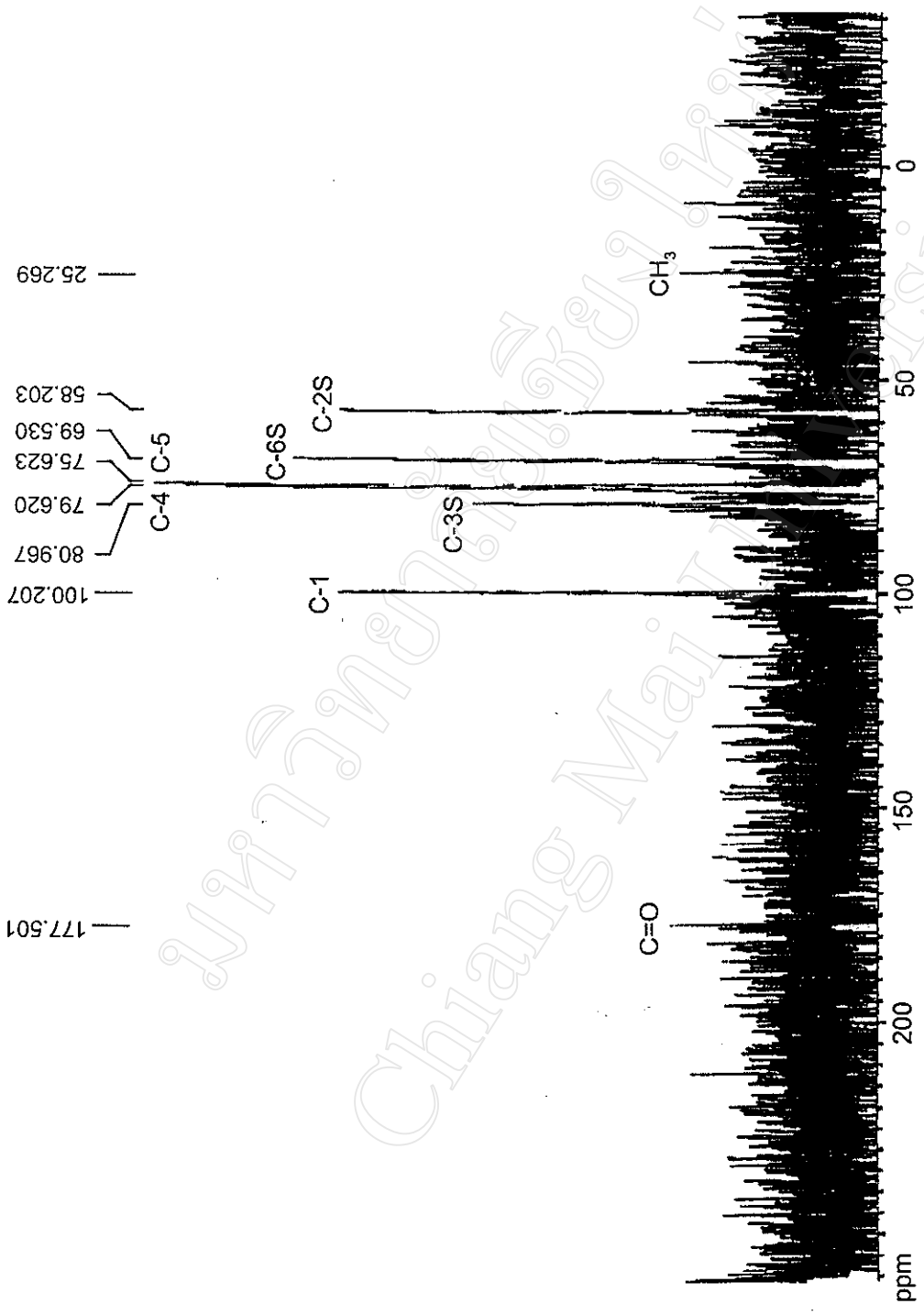

Figure 19 ^{13}C-NMR spectra of CPS (D_2O). C-3S, C-6S and C-2S; sulfate positions

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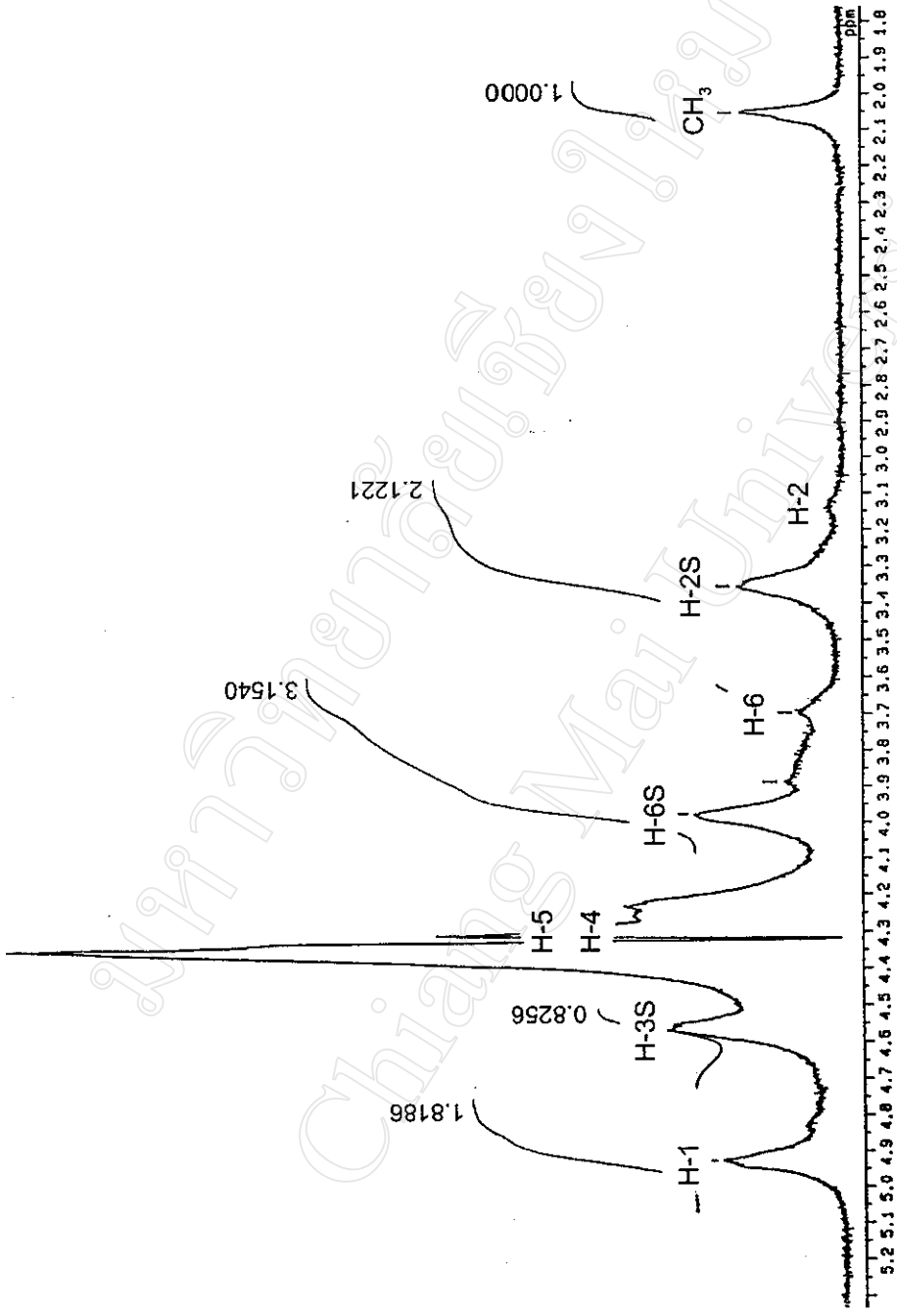

Figure 20 ^{1}H-NMR of CPS (D_2O). H-3S, H-6S and H-2S; sulfate positions

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3.1.2.4 X-ray diffraction

CPS obtained from the synthesis was well dissolved in both PBS pH 7.2 and distilled water. To verify this physical property, the analysis for its crystallinity was performed by the X-ray diffractometer. It was found that the CPS provided lower counts of the X-ray diffraction at 2θ than that of chitin and chitosan (Figure 21). The data was summarized in Table 13. The result revealed that CPS had less order in crystallinity and confirmed its physical property in water dissolvation.

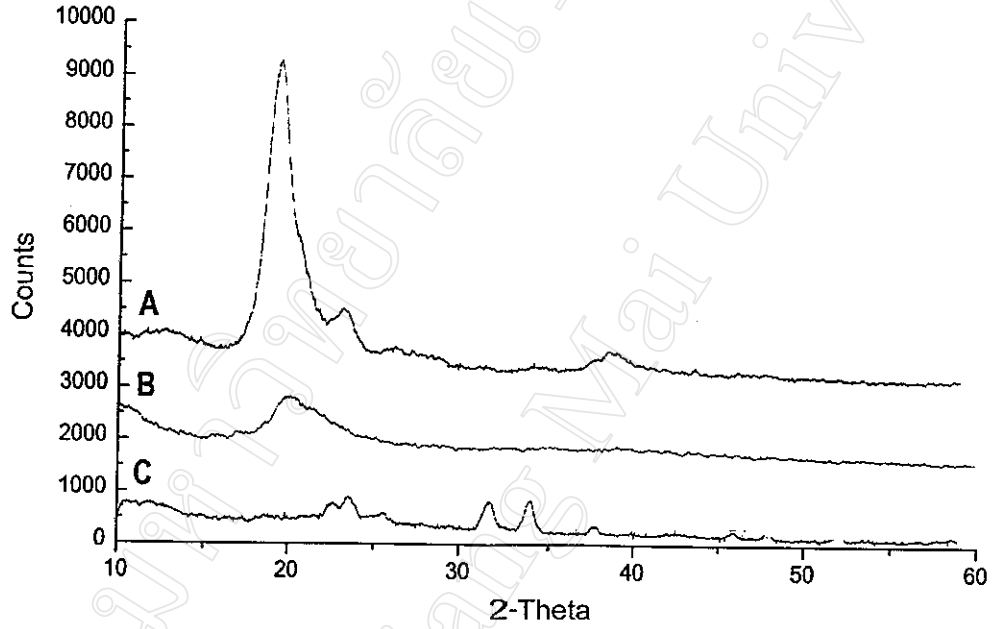

Figure 21 X-ray diffractogram at 2θ on KBr disc at 35 kV, 30 mA of chitin chitosan and its derivatives. Chitin (A), Chitosan (B), CPS (C).

Table 13 X-ray diffraction patterns at 2θ of chitin, chitosan and CPS

Sample	I/Io	d-spacing	2θ
Chitin	100	4.95	19.31
Chitosan	100	4.48	19.80
CPS	100	3.77	23.55

3.1.2.5 CHNS/O analysis

The CPS was analyzed for its composition by the CHNS/O analyzer and Atomic Absorption Spectrometer (AAS). The data was summarized in Table 14. Data from CHNS/O and Na analysis together with the degree of sulfation (Table 20) were used to calculated and predicted the simple formula of CPS as shown in Table 16. The data indicated that CPS was in the sodium salt form.

Table 14 The composition of CHNS/O and Na of chitin, chitosan and CPS

Sample [Formula]		C	H	N	S	Na
Chitin [$C_8H_{13}O_5N.\ H_2O$]	% cald.	36.55	7.61	7.11	-	-
	% found	42.09	6.45	6.20	-	-
Chitosan [$C_6H_{11}O_4N.\ H_2O$]	% cald.	40.22	7.26	7.28	-	-
	% found	40.02	7.20	7.62	-	-
CPS [$C_6H_8O_4N(C_2H_3O)_{0.12}(SO_3Na)_{2.23}(H)_{0.65}.\ 3.7H_2O$]	% cald.	15.83	3.34	3.08	16.18	11.63
	% found	16.99	3.07	3.12	15.64	11.33

Note: cald. = calculated

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

In order to determine the free amino group in the chitosan, the ninhydrin assay was performed using phenylalanine as the standard nitrogen to calibrate for the standard curve. The ratio of free amino group (%N) and total nitrogen (% total N) were calculated which referred to the degree of deacetylation (DDA) as well. Phenylalanine solution (10-80 mg/L) was prepared and performed by ninhydrin assay. The standard curve was plotted between absorbance (570 nm) and phenylanine concentration as shown in Figure 22. Two independent assays for chitosan was performed. The absorbance was converted into the equivalent of phenylalanine and calculated for nitrogen atom.

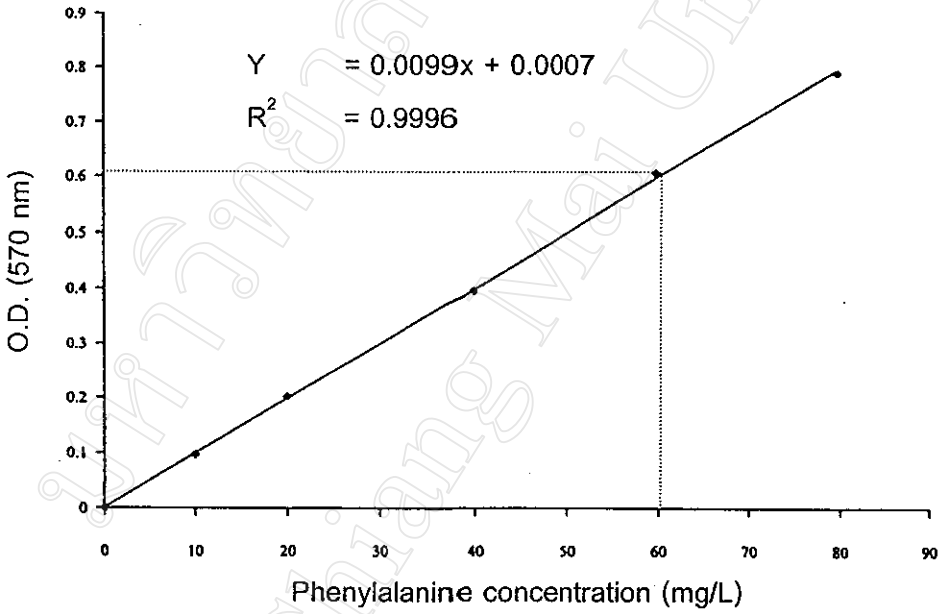

Figure 22 A typical standard curve of phenylalanine solution for the determination of free amino groups of chitosan.

Table 15 Degree of deacetylation of chitosan by ninhydrin method

Exp. No.	Weight (gm)	OD	% N ($-NH_2$)	% total N	DDA* ($-NH_2$)/N
1	0.0129	0.605	5.94	7.62	0.78
2	0.0139	0.655			

* Ratio of free NH_2 : total N was 0.78 which referred to the 78% of deacetylation or 0.78 of degree of deacetylation (DDA)

The DDA of commercial chitosan derived from marine crab was 0.85 as was indicated. This was different from the results by the ninhydrin method. The chitosan was then analyzed by the potentiometric titration method which principle was to detect the potential difference in the solid state of chitosan. Therefore the data was more valid than that of the ninhydrin method which assayed in the soluble state where all molecules were not completely dissolved. It was found that from the potentiometric titration, its DDA was 0.88.

3.1.2.7 Calculation of the degree of sulfation (DS) of CPS

(Wolfrom and Shen Han, 1953)

Chemical shifts from NMR analysis together with CHNS/O analysis were determined for the degree of sulfation (DS) by calculation of the S/N ratio. The method for calculation of DS was shown in Table 16. The result indicated that DS of CPS was 2.23.

Table 16 Calculation of the degree of sulfation (DS) of CPS

	C	H	N	S	Na
Composition	16.99	3.07	3.12	15.64	11.33
Atomic weight	12	1	14	32	23
Atomic ratio	1.42	3.07	0.22	0.49	0.49
Atom/mole	6.4	13.95	1	2.23	2.23

Remark: molecular formula was shown in Table 14

3.1.2.8 Determination of the average molecular weight (M_V) of CPS

The 3 fractions which obtained from gel filtration on a Sepharose CL-6B column (Figure 13) were purified and determined for their average molecular weights by the viscosity measurement (Nishimura *et al.*, 1986). The system of the assay was controlled at 25 ± 0.1 °C as described in the experimental section. The mean flow time was calculated for the relative viscosity (ηrel), specific viscosity (ηsp), reduce viscosity (ηred) and inherent viscosity (ηinh) by the following equations.

(1).....relative viscosity (ηrel) = t / ts

(2).....specific viscosity (ηsp) = (t-ts) / ts

(3).....reduce viscosity (ηred) = ηsp / C

(4).....inherent viscosity (ηinh) = ln ηrel / C

(when ts is the flow time of solvent and t is the flow time of solution of polymer)

The ηred and ηinh were plotted against sample concentration in gm/dl to obtain the intrinsic viscosity [η] which then afforded for the average molecular weight by the equation of Mark-Houwink-Sakurada (Muzzarelli, 1977).

$$[\eta] = kM_v^a$$

when k; constant value depending on polymer type and solvent, and temperature

$= (1.75 \times 10^{-5})$

a; constant value of interaction between polymer and solvent = 0.98

M_v; viscosity-average molecular weight

Data of the flow times of each tested sample was analyzed and calculated. The results showed that the M_v of CPS (pre-sepharose CL-6B), P1, P2 and P3 were 3.2×10^4 dalton (Table 17 and Figure 23), 6.8×10^4 dalton (Table 18 and Figure 24), 3.6×10^4 dalton (Table 19 and Figure 25) and 2.0×10^4 dalton (Table 20 and Figure 26), respectively. The average molecular weight of chitosan was determined by gel permeation chromatography since it could not be dissolved in the solvent used by this assay. The M_v of chitosan was 5.0×10^5 dalton.

Table 17 Data analysis and calculation of various viscosity of CPS

Concentration (gm/dl)	Flow time (second)	η_{rel}	η_{sp}	η_{red} (gm/dl)	η_{inh} (gm/dl)
0	38.20	-	-	-	-
0.100	40.46	1.047	0.047	0.475	0.464
0.200	42.55	1.097	0.097	0.485	0.463
0.300	44.54	1.149	0.149	0.496	0.463
0.400	46.61	1.202	0.202	0.507	0.462

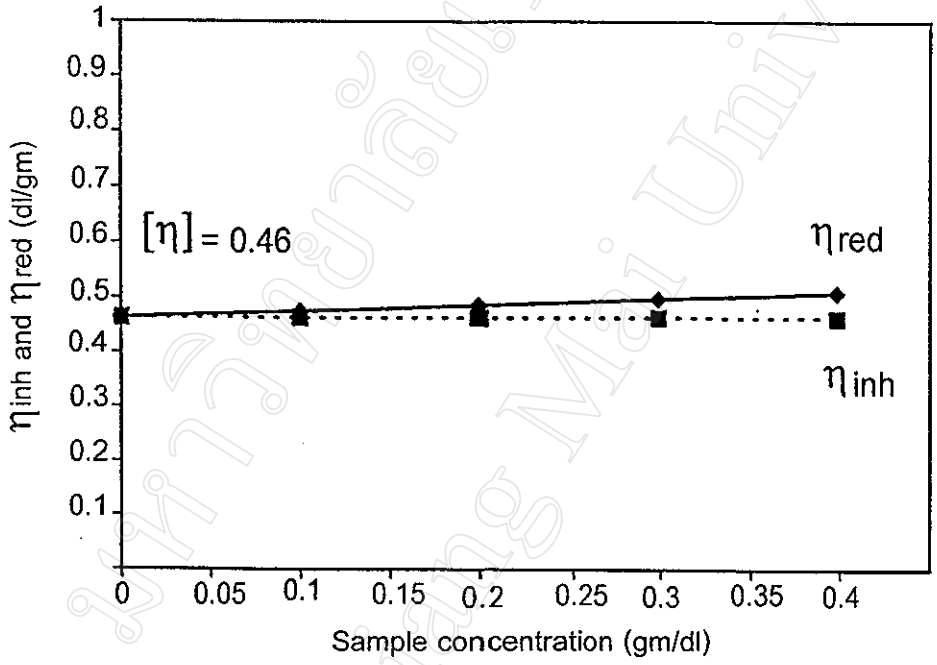

Figure 23 Extrapolation curve of η_{red} and η_{inh} of CPS (pre-Sepharose CL-6B). Various concentrations of CPS were resuspended in 0.1M NaCl and performed viscosity measurement. The flow times were calculated for η_{red} and η_{inh} to plot an extrapolation curve for the determination of $[\eta]$.

Table 18 Data analysis and calculation of various viscosity of P1

Concentration (gm/dl)	Flow time (second)	η_{rel}	η_{sp}	η_{red} (gm/dl)	η_{inh} (gm/dl)
0	38.86	-	-	-	-
0.100	42.57	1.096	0.096	0.967	0.923
0.200	46.44	1.197	0.197	0.986	0.900
0.300	50.44	1.301	0.301	1.005	0.878
0.400	54.41	1.404	0.404	1.011	0.849

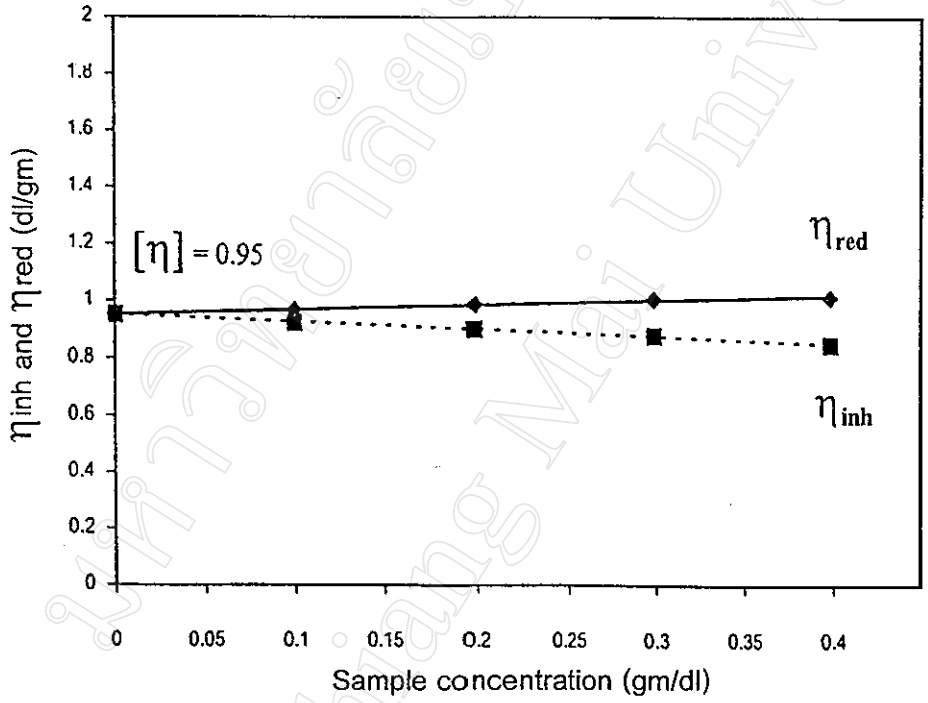

Figure 24 Extrapolation curve of ηred and ηinh of P1. Various concentrations of P1 were resuspended in 0.1M NaCl and performed viscosity measurement. The flow times were calculated for ηred and ηinh and plotted an extrapolation curve for the determination of [η].

Table 19 Data analysis and calculation of various viscosity of P2

Concentration (gm/dl)	Flow time (second)	η_{rel}	η_{sp}	η_{red} (gm/dl)	η_{inh} (gm/dl)
0	38.84	-	-	-	-
0.100	40.01	1.051	0.051	0.513	0.500
0.200	42.87	1.105	0.105	0.525	0.499
0.300	44.94	1.159	0.159	0.530	0.492
0.400	47.14	1.216	0.216	0.540	0.489

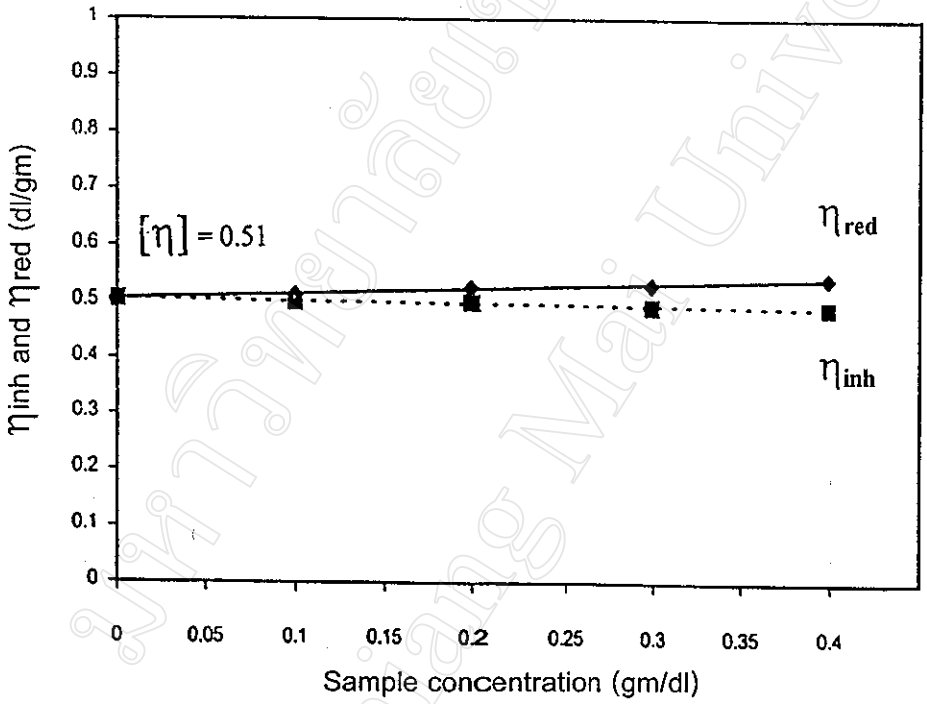

Figure 25 Extrapolation curve of ηred and ηinh of P2. Various concentrations of CPS were resuspended in 0.1M NaCl and performed viscosity measurement. The flow times were calculated for ηred and ηinh and plotted an extrapolation curve for the determination of [η].

Table 20 Data analysis and calculation of various viscosity of P3

Concentration (gm/dl)	Flow time (second)	η_{rel}	η_{sp}	η_{red} (gm/dl)	η_{inh} (gm/dl)
0	38.81	-	-	-	-
0.100	39.85	1.027	0.027	0.273	0.270
0.200	40.92	1.055	0.055	0.275	0.268
0.300	42.00	1.083	0.083	0.278	0.267
0.400	43.12	1.112	0.112	0.280	0.266

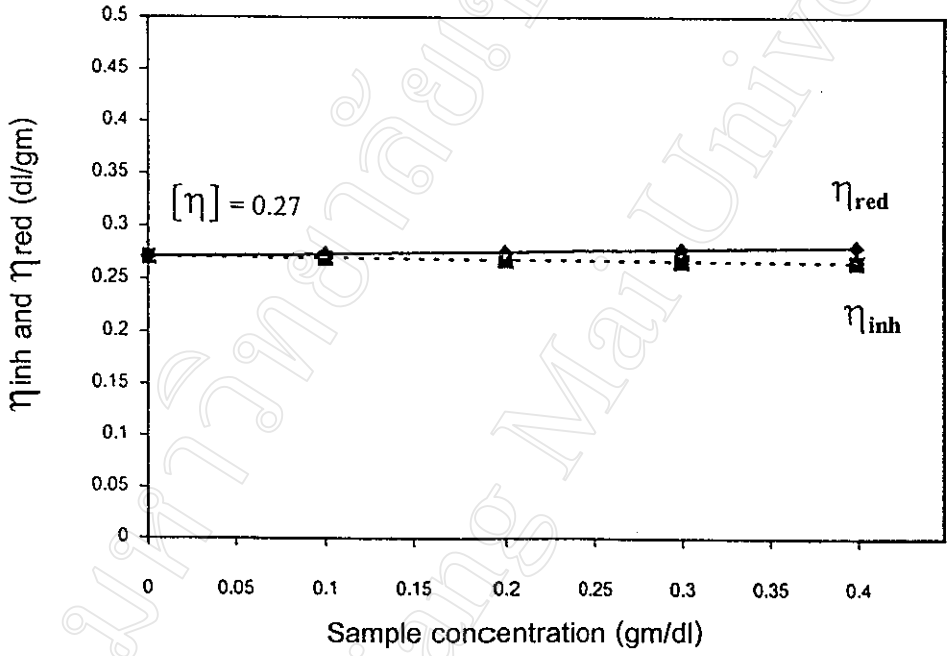

Figure 26 Extrapolation curve of ηred and ηinh and P3. Various concentrations of CPS were resuspended in 0.1M NaCl and performed viscosity measurement. The flow times were calculated for ηred and ηinh and plotted an extrapolation curve for the determination of [η].

3.2 Biological assay of CPS

3.2.1 Anticoagulant activity

3.2.1.1 Inhibition effect on Factor Xa activity

To evaluate the anticoagulant activity of CPS, the Accuclot™ Heptest was performed. Various concentrations of CPS were assayed in comparison to its starting material, chitosan, and pentosan polysulfate (PPS). Clotting times were recorded and determined the anticoagulant activity in equivalent to heparin in IU/ml by using the heparin calibration curve (Figure 27). The results showed that both CPS and PPS had the anticoagulant activity (Table 21).

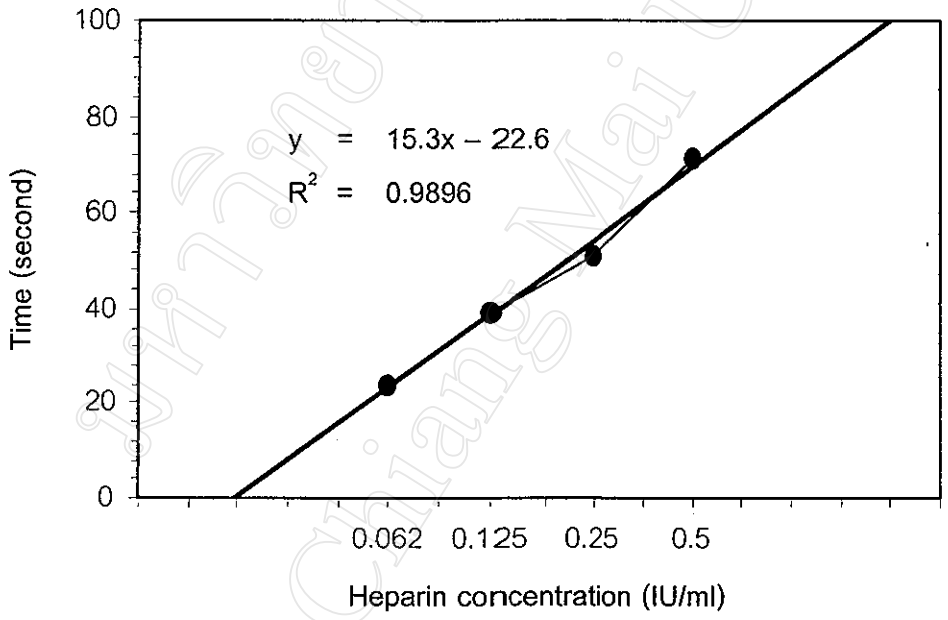

Figure 27 Heparin standard calibration curve. Various concentrations of standard therapeutic heparin were assayed by the Accuclot™ Heptest and clotting times were recorded.

Table 21 Anticoagulant activity of chitosan, CPS and PPS

Sample	Amount (µg)	Clotting time (second)	1 µg is equivalent to heparin (IU/ml)	(µg)
Chitosan	Up to 8	14	0	0
CPS	2	21	0.050	0.50
pre-Sepharose CL-6B	4	34		
	5	46		
	6	72		
	7	112		
	8	> 5 min		
CPS	2	23	0.039	0.39
P1	3	45		
	4	69		
	5	> 5 min		
CPS	2	20	0.046	0.46
P2	3	31		
	4	54		
	5	54		
	6	108		
	8	180		
CPS	2	20	0.035	0.35
P3	4	23		
	6	35		
	8	43		
	10	51		
	15	148		
	20	> 5 min		
Pentosan polysulfate	1	32	0.180	1.80
(PPS)	2	88		
	4	> 5 min		

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3.2.1.2 Effect of CPS on the inhibition of Factor Xa by antithrombin III

As CPS showed an anticoagulant activity in the presence of factor Xa, the mechanism of action was investigated. Inhibition effect through the complex formation with Antithrombin III (ATIII) was observed. In this experiment, ATIII calibration curve was performed using various concentrations of standard therapeutic heparin as shown in Figure 28. Chitosan, CPS and PPS were assayed for the inhibition of factor Xa in the presence of ATIII. The assay for measurement of the activity of the remaining factor Xa was done by adding of factor Xa substrate. The decreasing of absorbance in this colorimetric measurement could be explained that a tested sample might inhibit factor Xa activity through the complex formation with ATIII. The results showed that all except P3 inhibited factor Xa activity. In contrast, chitosan did not inhibit factor Xa and showed no effect as was demonstrated by this assay (Table 22). This demonstrated that molecular weight size of CPS might be a factor that influenced in the binding to ATIII and subsequently inhibited factor Xa activity since P3 which has the smallest M_v could not inhibit factor Xa in the presence of ATIII.

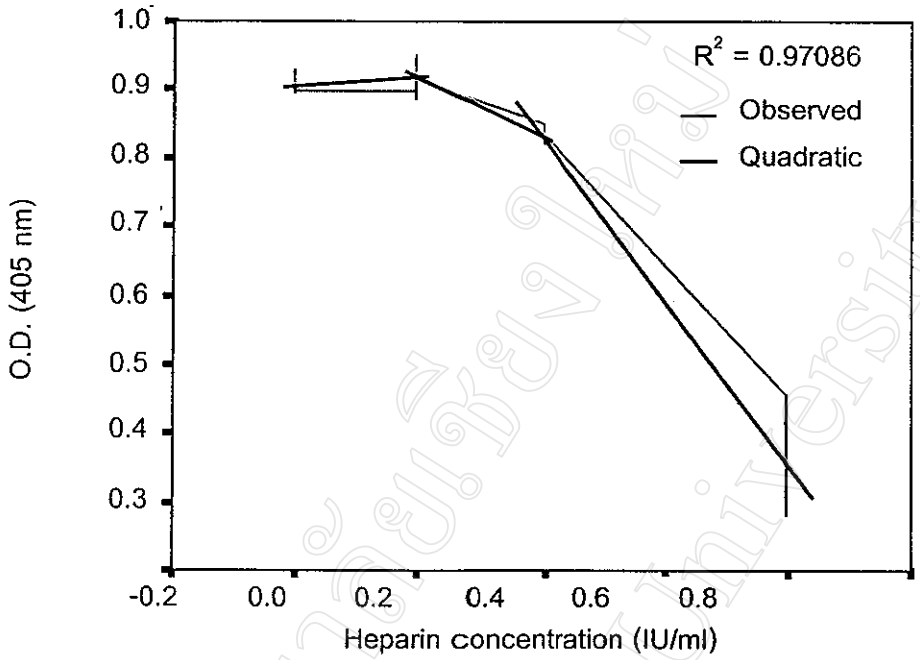

Figure 28 Antithrombin III (ATIII) calibration curve. Normal human plasma containing various concentrations of standard therapeutic heparin were incubated with ATIII for 120 seconds before adding of factor Xa substrate and O.D. at 405 nm was recorded. Gray line, curve of observed data; black line, quadratic curve from statistic analysis.

Table 22 Effect of chitosan, CPS and PPS on the inhibition of Factor Xa by antithrombin III

Sample (2 µg/ml)	O.D. (mean ± S.D.)	Equivalent to heparin (IU/ml)
Chitosan	0.986 ± 0.035	0
CPS (pre-Sepharose CL-6B)	0.769 ± 0.014	0.35
CPS (P1)	0.579 ± 0.035	0.52
CPS (P2)	0.760 ± 0.071	0.36
CPS (P3)	0.931 ± 0.028	0
Pentosan polysulfate (PPS)	0.421 ± 0.037	0.64

3.2.1.3 Determination of antithrombin activity

When antithrombin III (ATIII) forms complex with heparin or heparinomimetic drugs, it interferes the coagulation process by inhibition both factor Xa and thrombin. However, the first action is much more efficient than the latter. To evaluate that CPS promoted the activity of ATIII in inhibition of thrombin, the determination of antithrombin activity was assayed by measurement of the remaining thrombin in the presence of heparin or a tested sample and adding of the thrombin substrate. The colorimetric measurement showed no change in absorbance if the tested ATIII had no effect on thrombin. In this assay, the standard antithrombin was plotted for the antithrombin activity calibration curve (Figure 29). The results showed that all tested CPS and PPS had no effect on

antithrombin activity as shown in Table 23. The data indicated that complex formation of CPS and ATIII had effected only on the activity of factor Xa but not on thrombin.

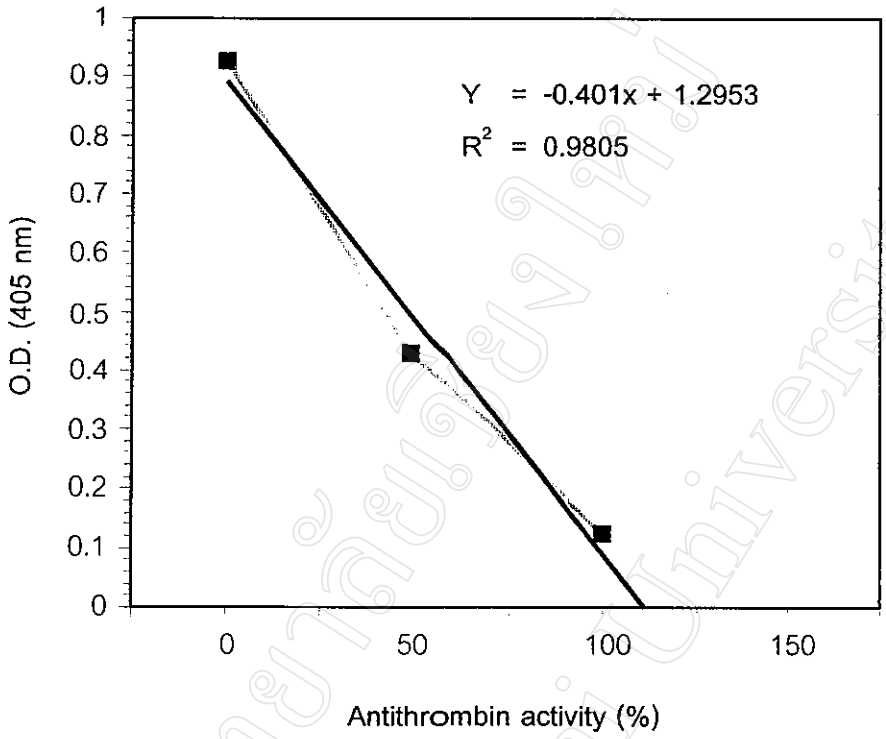

Figure 29 Antithrombin activity calibration curve. Various concentrations of prewarmed antithrombin standard were incubated with Heparin/thrombin reagent for 120 seconds before adding of thrombin substrate and O.D. at 405 nm was recorded.

Table 23 Effect of chitosan, CPS and PPS on the inhibition of thrombin by antithrombin III

Sample (2 µg/ml)	O.D. (405 nm)	Antithrombin activity (%)
Normal human plasma (NHP)	0.242	98
Chitosan	0.162	105
CPS (pre-Sepharose CL-6B)	0.209	102
CPS (P1)	0.264	97
CPS (P2)	0.279	95
CPS (P3)	0.284	95
Pentosan polysulfate (PPS)	0.296	94
Heparin (0.1 IU/ml)	0.283	95

Normal antithrombin activity = 79-125%

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3.2.1.4 Determination of thrombin time (TT)

To investigate that CPS could directly inhibit the activity of thrombin, the thrombin time assay was performed. Various concentrations of CPS, PPS and standard therapeutic heparin were assayed in parallel with the normal control. The data in Table 24 showed that all preparations of CPS could prolong the thrombin time as well as the standard therapeutic heparin and PPS. It was indicated that CPS could directly inhibit thrombin activity and this mechanism was the same as heparin action.

Table 24 Inhibition of thrombin activity of CPS, PPS and heparin

Sample		Thrombin time (TT) second (mean ± SD)			
		Control	Test		
			Sample concentration (µg)		
			1	5	8
Normal human plasma		13.1± 1.3			
Control I (A4589)		10.4± 0.3			
Heparin (IU/ml)	0.1	22.8± 0.8			
	0.2	47.1± 2.1			
	0.4	139.7 ± 3.4			
Chitosan			14.1 ± 1.1	12.9 ± 0.3	12.9
CPS (pre-Sepharose CL-6B)			29.7 ± 0.4	70.4 ± 4.9	69.3 ± 4.8
CPS (P1)			27.8 ± 0.9	91.6 ± 0.2	ND
CPS (P2)			22.2 ± 0.4	159.7 ± 27.6	123.6 ± 14.8
CPS (P3)			22.4 ± 1.3	47.1 ± 7.9	50.0 ± 0.9
Pentosan polysulfate (PPS)			20.8 ± 0.5	35.9 ± 2.9	ND

ND = not determine

3.2.1.5 Determination of prothrombin time (PT)

Heparin had an anticoagulant activity by inhibition of factor Xa and thrombin but it had no effect on the factor in extrinsic pathway. The major coagulation factor of extrinsic pathway was factor VII. To investigate that CPS could not inhibit the activity of coagulation factors in extrinsic pathway as well as the heparin, it was investigated for the prothrombin time (PT) in comparison to the standard therapeutic heparin and PPS. The mean of clotting times was calculated and reported in the International Normalized Ratio (INR) according to the ISI value as indicated by the instruction manual (Hirst *et al.*, 1992). INR was calculated by the equation described in the experimental section. The result shown in Table 25 indicated that all tested sample could not prolong the PT. This demonstrated that the mechanism of action of CPS and PPS was closed to heparin and had no effect on the coagulation factors in the extrinsic pathway.

Table 25 Prothrombin time (PT) of CPS, PPS and heparin

Sample (2 µg)	Clotting time (mean)	R (test/normal)	INR (R^{ISI})
Normal human plasma	15.0	1.00	1.00
Heparin (0.05 IU/ml)	14.0	0.93	0.91
Heparin (0.1 IU/ml)	14.5	0.97	0.96
Chitosan	15.0	1.00	1.00
CPS (pre-Sepharose CL-6B)	19.5	1.30	1.39
CPS (P1)	19.0	1.27	1.35
CPS (P2)	19.0	1.27	1.35
CPS (P3)	18.5	1.30	1.39
Pentosan polysulfate (PPS)	17.0	1.13	1.17

ISI = 1.25 (Thromboplastin–HS, Lot no. 031K6145)

3.2.1.6 Determination of the effect of CPS on fibrin polymerization

The results from all assays mentioned previously indicated that CPS was able to inhibit the coagulation process. The mechanism of action was both inhibiting factor Xa in the presence of ATIII and directly inhibit thrombin activity. The blood coagulation might be effected from interfering of the fibrin polymerization. To investigate that the inhibition effect of these tested materials was not involve in the fibrin polymerization, the atroxin time was performed. CPS and heparin were assayed in comparison to the commercial normal control. It was found that all tested materials could not prolong the atroxin time as shown in Table 26. This indicated that CPS as well as heparin had no effect on fibrin polymerization.

Table 26 Effect of CPS and heparin on atroxin time

Sample (10 µg/ml)	Clotting time (second)
Normal human plasma	17
Control I (A4589)	18
Heparin (0.5 IU/ml)	20
Chitosan	18
CPS (P1)	17
CPS (P2)	17
CPS (P3)	17

3.2.2 Involvement in the immune response *in vitro*

3.2.2.1 Effect on cell proliferation

A. Determination of the optimal conditions for the detection of cell proliferation by the colorimetric method

To evaluate the effect of CPS in the proliferation of peripheral blood mononuclear cell (PBMCs), the optimal conditions for the detection assay was performed. PPD and PHA of various concentrations (0-10 µg/ml) were used to optimize the experimental assay. The results showed that the optimal BrdU labeling time was 21 hrs (Figure 30-31). The incubation time and temperature for anti-BrdU reaction was 2 hrs at 37 °C and the reaction time for TMB was 15 minutes at room temperature as recommended by the manufacturer.

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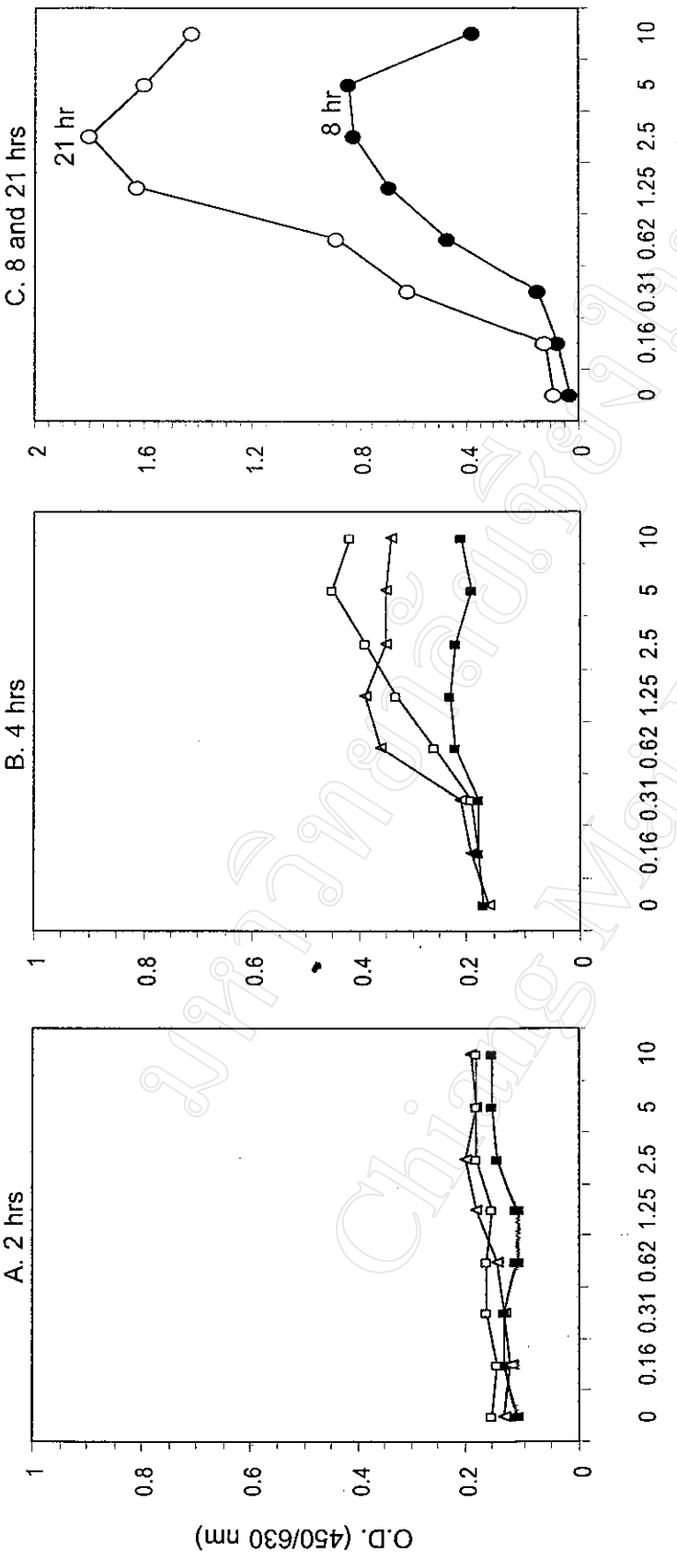

Figure 30 Optimization of the BrdU-labeling time in PHA stimulated PBMC proliferation assay. PBMCs isolated from 3 healthy donors were cultured in the presence of various concentration of PHA for 2 days before labeling with BrdU for 2 hrs (A), 4 hrs (B), 8 and 21 hrs (C). Cell culture supernatant was collected and assayed for the cell proliferation by the colorimetric method.

■: donor-1, □: donor-2, △: donor-3, ●: 8 hr-labeling, ○: 21 hr-labeling.

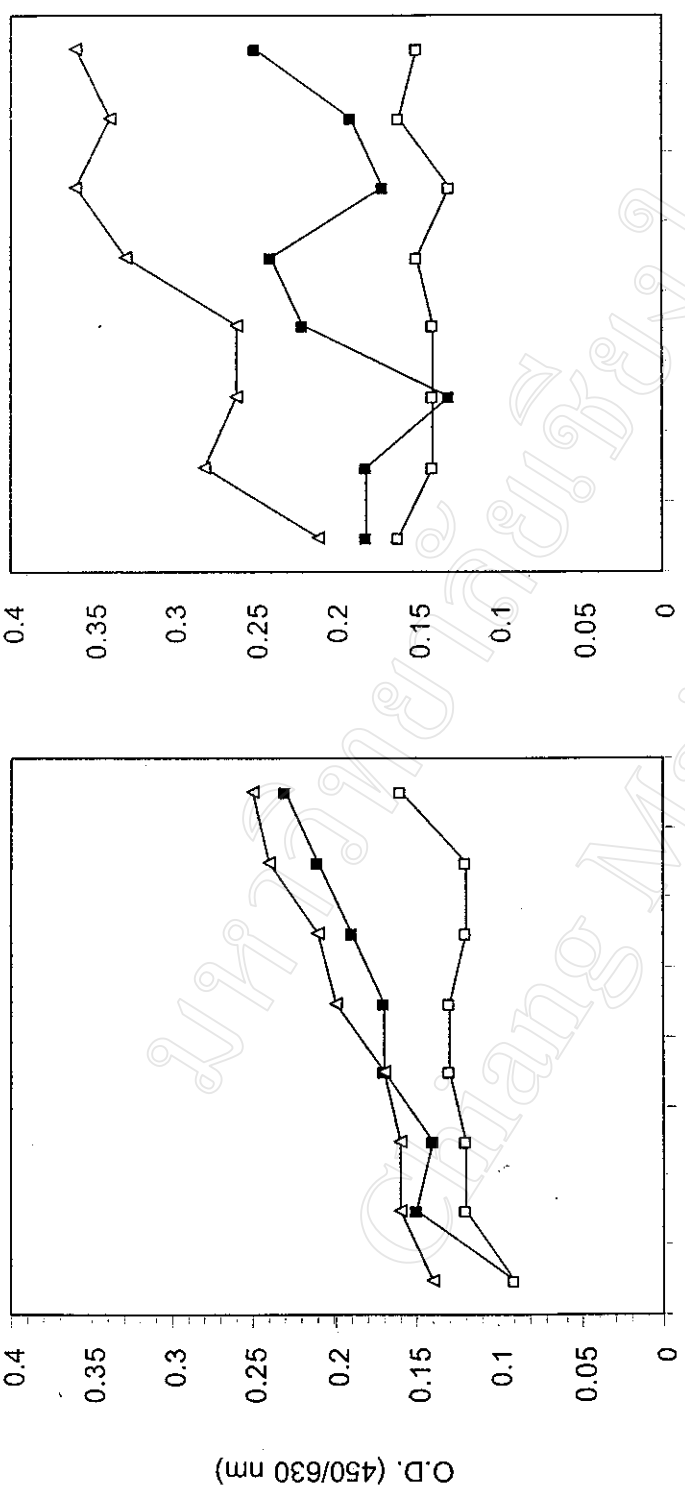

Figure 31 Optimization of the BrdU-labeling time in PPD stimulated PBMC proliferation assay. PBMCs isolated from 3 healthy donors were cultured in the presence of various concentration in the presence of various concentration of PPD for 5 days before labeling with BrdU for 8 hrs (A) and 21 hrs (B). Cell culture supernatant was collected and assayed for the cell proliferation by the colorimetric method. ■ : donor-1, □ : donor-2, △: donor-3.

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B. Determination of the sub-optimal concentration of PPD

In order to study the effect of CPS on the PPD stimulated cell proliferation, sub-optimal concentration of PPD was optimized. Peripheral blood mononuclear cells (PBMCs) of 3 healthy donors were studied. Triplicate aliquots of PBMCs (5 x 10^4 cells/ml) were cultured in the presence or absence of various concentrations of PPD (0-20 µg/ml) for 5 days before an assay of cell proliferation by colorimetric method. The results showed that O.D. observed at 2 PPD concentrations of 0.31 µg/ml and 0.62 µg/ml were not clearly different although they were demonstrated to be the suitable sub-optimal concentrations (Figure 32). Therefore, both concentrations were selected for further on assay.

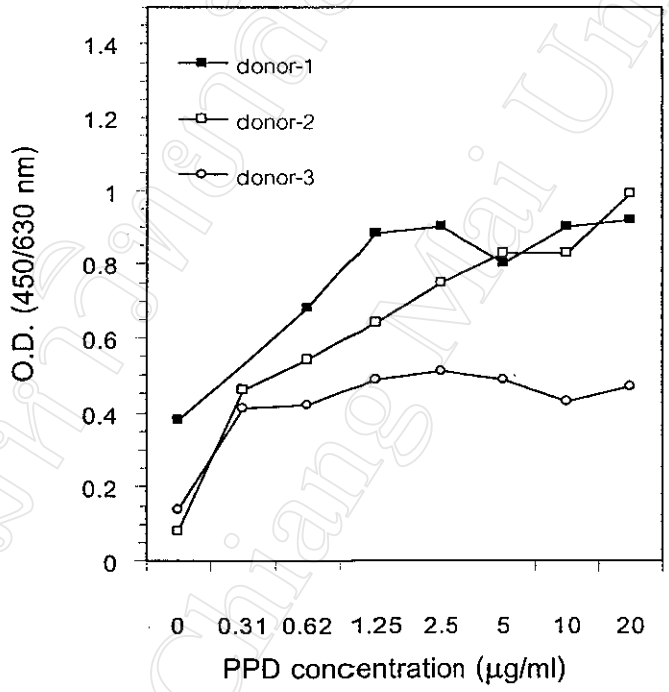

Figure 32 Determination of the sub-optimal concentration of PPD. PBMCs isolated from 3 healthy donors were cultured in the presence of various concentrations of PPD for 5 days before 21 hr-BrdU labeling following with the detection for the cell proliferation by the colorimetric method.

C. Determination of the sub-optimal concentration of PHA

The effect of CPS on the mitogen stimulated cell proliferation was also evaluated. The sub-optimal concentration of PHA was determined. The assay was done in the triplicate aliquots of PBMCs (5 x 10^4 cells/m). The PBMCs from 3 healthy donors were cultured in the presence or absence of various concentrations of PHA (0-5 µg/ml) for 2 days before an assay of cell proliferation and using colorimetric method. The results from all donors were quite clear that the sub-optimal concentration of PHA was 0.5 µg/ml as shown in Figure 33.

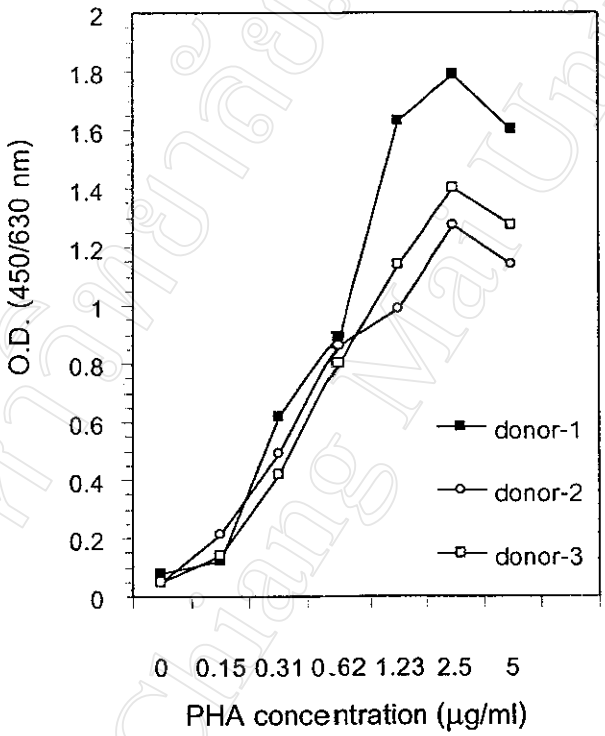

Figure 33 Determination of the sub-optimal concentration of PHA. PBMCs isolated from 3 healthy donors were cultured in the presence of various concentrations of PHA for 2 days before 21 hr-BrdU labeling following with the detection for the cell proliferation by the colorimetric method.

D. Effect of P1-P3, PPS and heparin on PPD stimulated cell proliferation

To evaluate the effect of CPS on the PPD stimulated cell proliferation, PBMCs from 3 healthy donors were used. P1-P3 was studied in parallel to the other synthetic polysaccharide, PPS and standard therapeutic heparin. The PBMCs ($5x10^4$ cells/ml) were stimulated with PPD (0.31 or 0.62 μg/ml) in the presence of various concentrations of P1-P3, PPS (0-8 μg/ml) or heparin (0-0.8 IU/ml) for 5 days. All assay were done in triplicate. Controls were performed in parallel by culture PBMCs alone, or in the presence or absence of antigen or tested materials. The cell proliferation was assayed by colorimetric method. It was found that all tested materials significantly inhibited the PPD stimulated cell proliferation in dose dependent manner ($p<0.01$, MANOVA test). The inhibition effect in dose dependent manner was clearly remarked when cells were cultured in the presence of the tested materials with small molecular weight such as P3, PPS and heparin. Result from one of all 3 donors was shown in Figure 34 and all data were shown in Table 27.

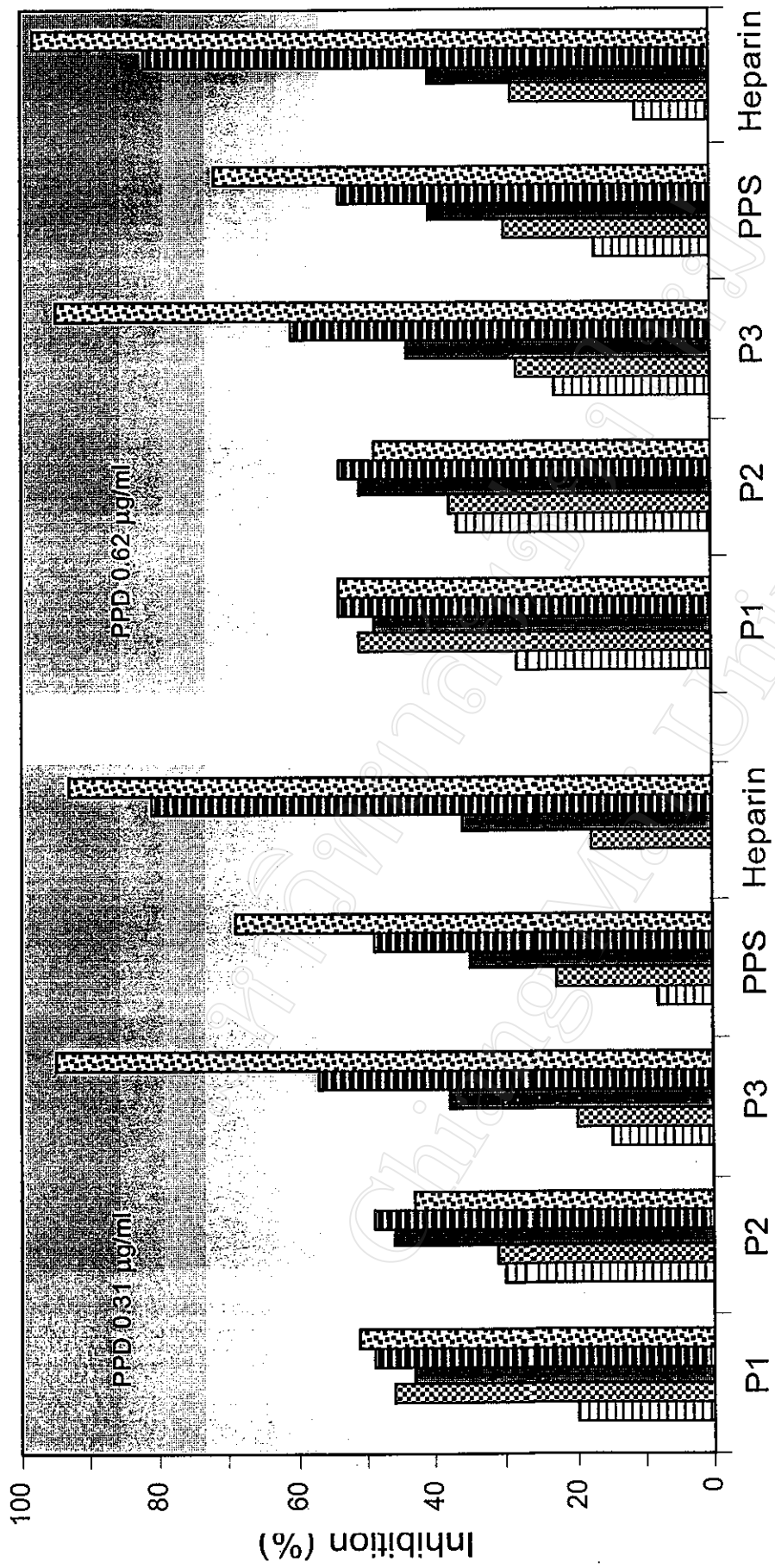

Figure 34 Effect of P1-P3, PPS and heparin on PPD stimulated cell proliferation. PBMCs ($5x10^4$ cells/ml) were cultured in the presence or absence of various concentrations of tested materials and PPD (0.31 and 0.62 µg/ml) for 5 days. All assays were done in triplicate and means of O.D. were used to calculate for the inhibition of cell proliferation (%).

Table 27 Effect of P1-P3, PPS and heparin on PPD stimulated cell proliferation of 3 healthy donors

Tested Materials	Concentration	Inhibition (%)					
		Donor-1		Donor-2		Donor-3	
		PPD (µg/ml)		PPD (µg/ml)		PPD (µg/ml)	
		0.31	0.62	0.31	0.62	0.31	0.62
P1 (µg/ml)	0	0	0	0	0	0	0
	0.5	7	12	20	28	28	54
	1.0	34	23	46	51	52	59
	2.0	44	28	43	49	59	58
	4.0	34	30	49	54	53	65
	8.0	39	41	51	54	42	54
P2 (µg/ml)	0	0	0	0	0	0	0
	0.5	24	5	30	37	36	39
	1.0	38	22	31	38	54	55
	2.0	32	38	46	51	49	65
	4.0	29	37	49	54	62	63
	8.0	41	24	43	49	58	52
P3 (µg/ml)	0	0	0	0	0	0	0
	0.5	22	21	15	23	25	52
	1.0	27	33	20	28	46	45
	2.0	28	29	38	44	73	61
	4.0	32	26	57	61	78	76
	8.0	34	34	95	95	76	71
PPS (µg/ml)	0	0	0	0	0	0	0
	0.5	24	5	8	17	11	39
	1.0	17	28	23	30	26	61
	2.0	32	31	35	41	46	61
	4.0	46	40	49	54	62	61
	8.0	62	57	69	72	66	66
Heparin (IU/ml)	0	0	0	0	0	0	0
	0.05	13	4	0	11	26	64
	0.10	12	13	18	29	48	66
	0.20	24	13	36	41	66	63
	0.40	26	36	81	82	76	86
	0.80	58	63	93	98	92	94

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E. Effect of P1-P3, PPS and heparin on PHA stimulated cell proliferation

The effect of tested materials on PHA stimulated was also evaluated. P1-P3 was studied in parallel to the other synthetic polysaccharide, PPS and standard therapeutic heparin. The PBMCs ($5x10^4$ cells/ml) were stimulated with PHA (0.5 μg/ml) in the presence of various concentrations of P1-P3, PPS (0-8 μg/ml) or heparin (0-0.8 IU/ml) for 2 days. All assay were done in triplicate. Controls were performed in parallel by culture PBMCs alone, or in the presence or absence of mitogen or tested materials. In the presence of PHA at 0.5 μg/ml and various concentrations of P1-P3 or PPS, PBMC of all 3 healthy donors showed no significantly change in proliferation ($p>0.05$, MANOVA test). Only heparin that significantly inhibited cell proliferation in dose response curve ($p<0.05$, MANOVA test). It was demonstrated that CPS has no significant effect on mitogen stimulated cell proliferation. Results from one of all 3 donors was shown in Figure 35 and all data were shown in Table 28.

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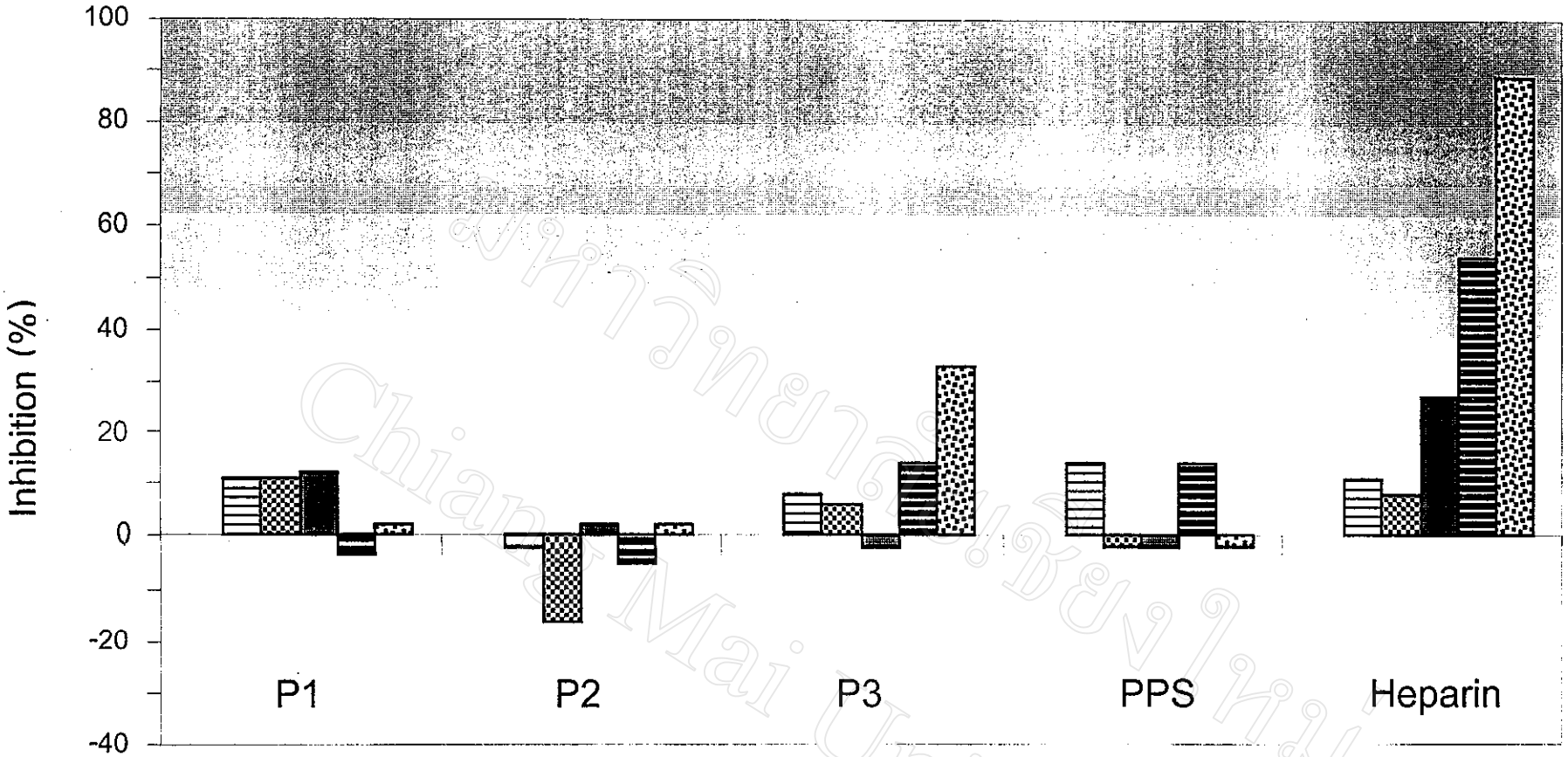

Figure 35 Effect of P1-P3, PPS and heparin on PHA stimulated cell proliferation. PBMCs ($5x10^4$ cells/ml) were cultured in the presence or absence of various concentrations of tested materials and PHA (0.5 μg/ml) for 2 days. All assays were done in triplicate and means of O.D. were used to calculate for the inhibition of cell proliferation (%).

□ 0 μg/ml ⊟ 0.5 μg/ml ▩ 1.0 μg/ml ■ 2.0 μg/ml ▤ 4.0 μg/ml ⊡ 8.0 μg/ml

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Table 28 Effect of P1-P3, PPS and heparin on PHA (0.5 µg/ml) stimulated cell proliferation of 3 healthy donors

Tested Materials	Concentration	Inhibition (%)		
		Donor-1	Donor-2	Donor-3
P1 (µg/ml)	0	0	0	0
	0.5	-7	11	25
	1.0	2	11	33
	2.0	-9	12	22
	4.0	-9	-3	16
	8.0	-18	2	17
P2 (µg/ml)	0	0	0	0
	0.5	7	-2	12
	1.0	10	-16	12
	2.0	5	2	12
	4.0	9	-5	16
	8.0	-7	2	9
P3 (µg/ml)	0	0	0	0
	0.5	1	8	17
	1.0	6	6	14
	2.0	9	-2	28
	4.0	24	14	14
	8.0	26	33	17
PPS (µg/ml)	0	0	0	0
	0.5	21	14	3
	1.0	-9	-2	16
	2.0	18	-2	16
	4.0	20	14	25
	8.0	8	-2	12
Heparin (IU/ml)	0	0	0	0
	0.05	13	11	19
	0.10	16	8	25
	0.20	24	27	50
	0.40	40	54	75
	0.80	71	89	92

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3.2.2.2 Effect on cell mediated cytotoxicity

A. Determination of the optimal target cell concentration

To evaluate the effect of CPS on cell mediated cytotoxicity, K562 cells were optimized and used as the target cells. Various concentrations of K562 ($1x10^3$-$200x10^3$ cells/well) were assayed for the spontaneous LDH release and maximum LDH release which indicated the baseline and maximal rate of cell death. The assay was done in triplicate. The results showed that O.D. observed at the cell concentration of $2x10^4$ cells/well of both low and high control were clearly different (Figure 36). This difference was suitable to evaluate the effect of tested material on the cell mediated cytotoxicity. Therefore, the concentration of K562 at was selected for further investigation.

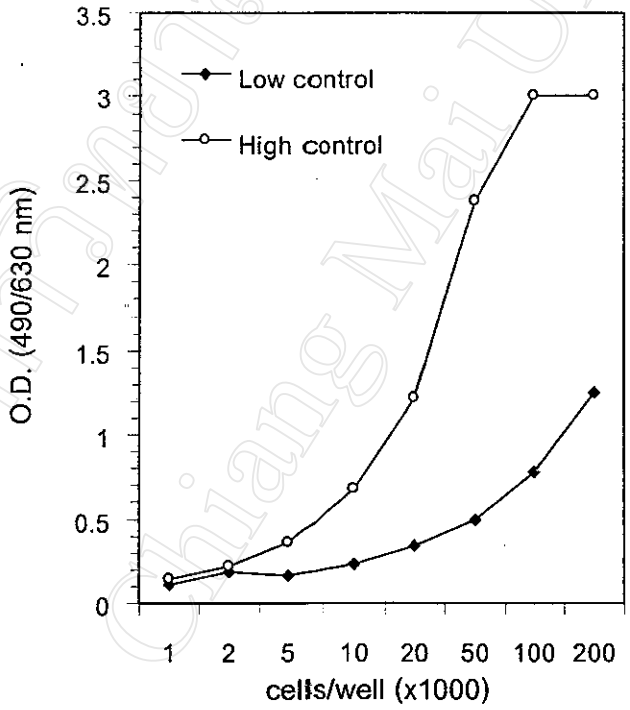

Figure 36 Optimal K562 concentration for the determination of cell mediated cytotoxicity assay. Various concentrations of K562 were cultured in the presence of triton X-100 (final concentration of 1%, high control) or absence (low control) for 4 hrs before an assay of LDH release.

B. Determination of Effector : Target cell ratio (E:T)

To determine the E:T ratio for the cell mediated cytotoxicity assay, PBMCs from 5 healthy donors were used. The PBMCs were serial diluted into various cell concentrations and cultured in the presence of fixed amount of K562 ($2x10^4$ cells/well). Various E:T ratio (6.3 : 1–200 : 1) were assayed to obtained the optimal E:T ratio. All assay were performed in triplicate. The cytotoxicity (%) was calculated from the equation indicated in chapter 2 and plotted against E:T ratio. The results from 5 donors were shown in Figure 37. Each donor expressed his/her own response in cell mediated cytotoxicity. In the lower E:T ratio, some donors showed higher O.D. of effector control which resulted in the below-zero in cytotoxicity. The cytotoxicity was increased when increasing the E:T ratio. Since there was some controversy in the results from 5 donors, the 2 E:T ratio of 100 : 1 and 50 : 1 were selected for further investigated in order to evaluate the effect of tested materials at both points

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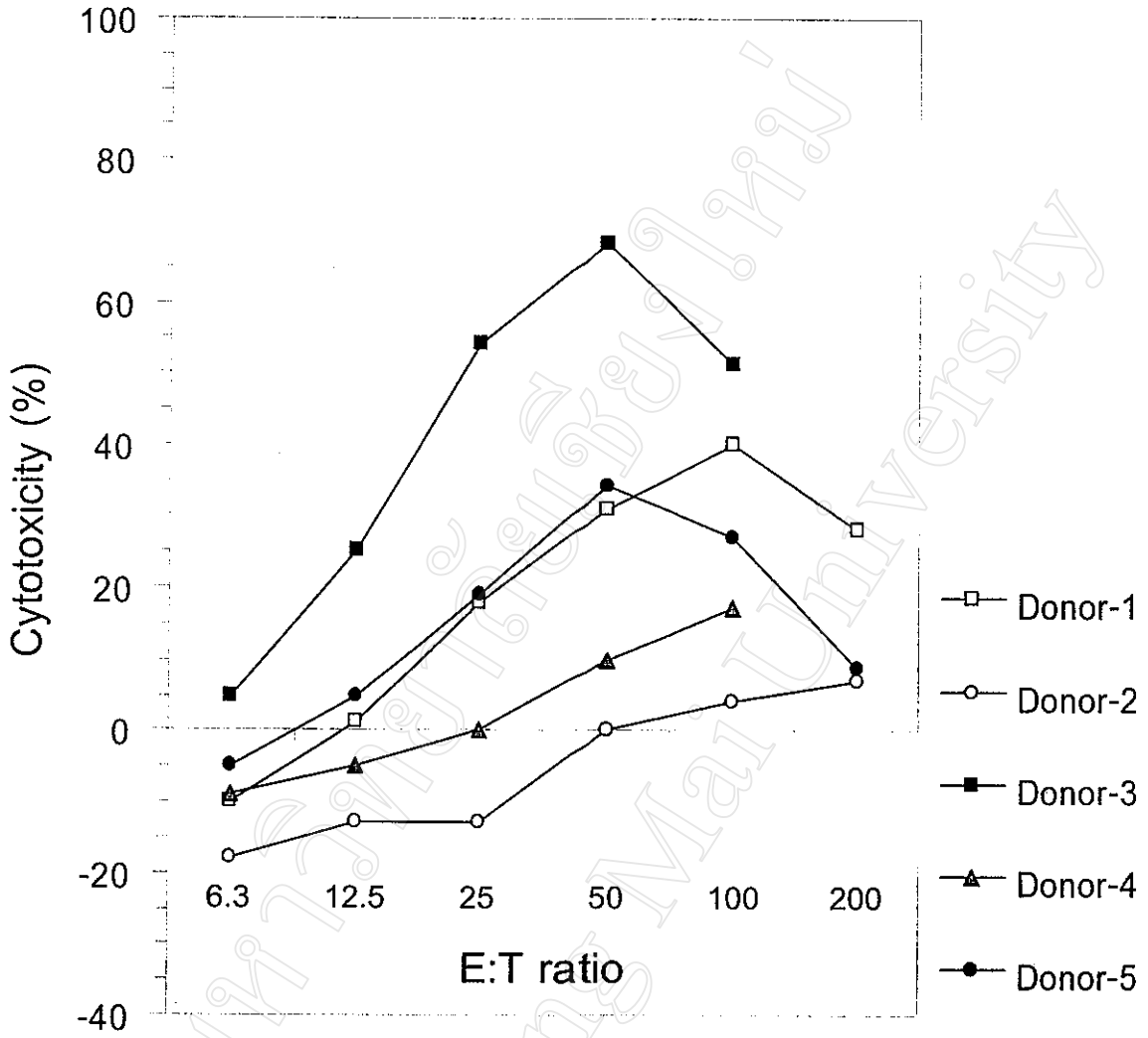

Figure 37 Determination of the optimal E:T ratio for cell mediated cytotoxicity assay. PBMCs from 5 healthy volunteers were serial diluted and cultured with K562 ($2x10^4$ cells/ml) for 4 hrs before an assay of LDH release. The cytotoxicity (%) was calculated and plotted against the E:T ratio.

C. Effect of P1-P3, PPS and heparin on cell mediated cytotoxicity

To evaluate the effect of tested samples on cell mediated cytotoxicity, PBMCs from 5 healthy donors were used. The PBMCs were cultured in the presence of various concentrations of P1-P3, PPS and heparin. The K562 ($2x10^4$ cells/ml) was added before 4-hr cultivation. The results demonstrated that all tested materials could not affected the cell mediated cytotoxicity in all 5 donors. The cytotoxic potential of tested materials to PBMCs were also performed and it was found that all has no toxic to PBMCs (data not shown). The changes in cell mediated cytotoxicity were not significant ($p>0.05$, Pearson's test) on both conditions of E:T ratio. Results from one of all 5 donors was shown in Figure 38-42 and all data were shown in Table 29.

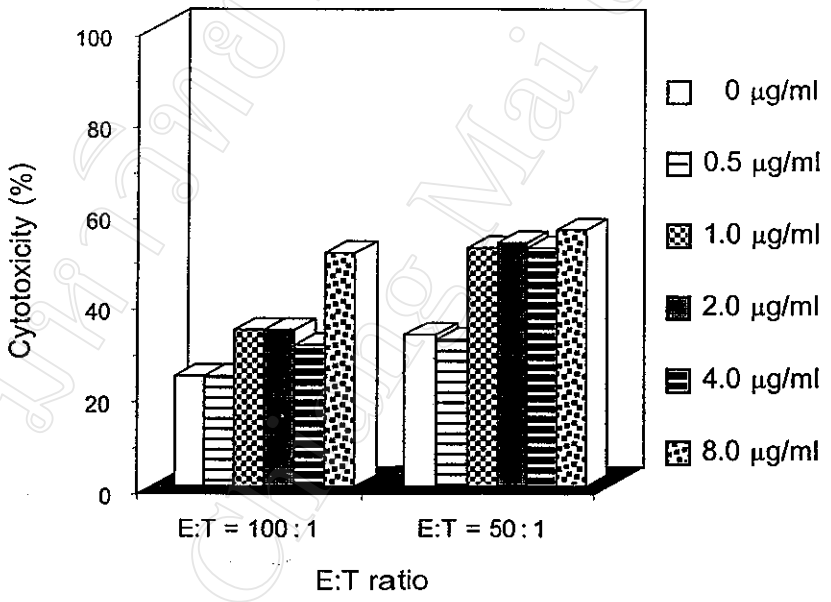

Figure 38 Effect of P1 on cell mediated cytotoxicity. PBMCs were cultured with K562 in the E:T ratio of 100 : 1 and 50 : 1 in the presence or absence of P1 for 4 hrs before an assay of LDH release. All assays were done in triplicate and means of O.D. were used to calculate for the cytotoxicity (%).

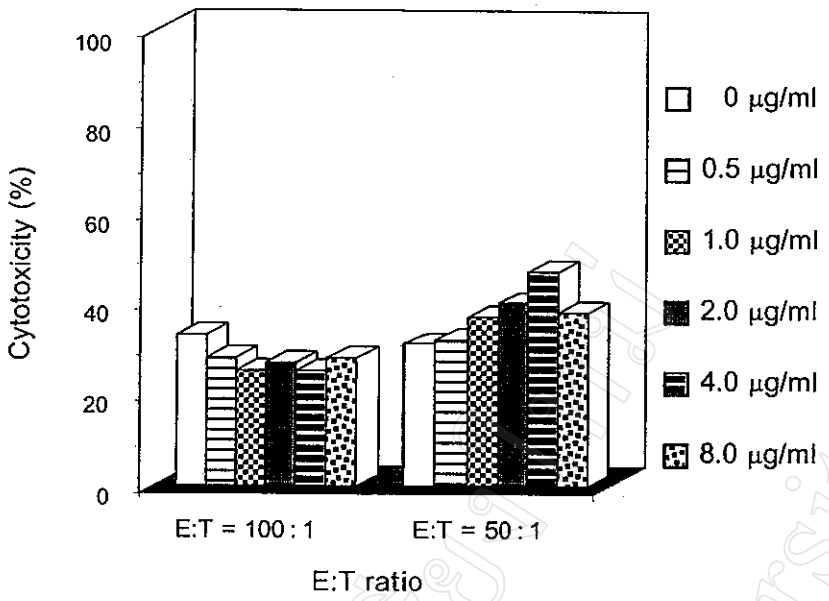

Figure 39 Effect of P2 on cell mediated cytotoxicity. PBMCs were cultured with K562 in the E:T ratio of 100 : 1 and 50 : 1 in the presence or absence of P2 for 4 hrs before an assay of LDH release. All assays were done in triplicate and means of O.D. were used to calculate for the cytotoxicity (%).

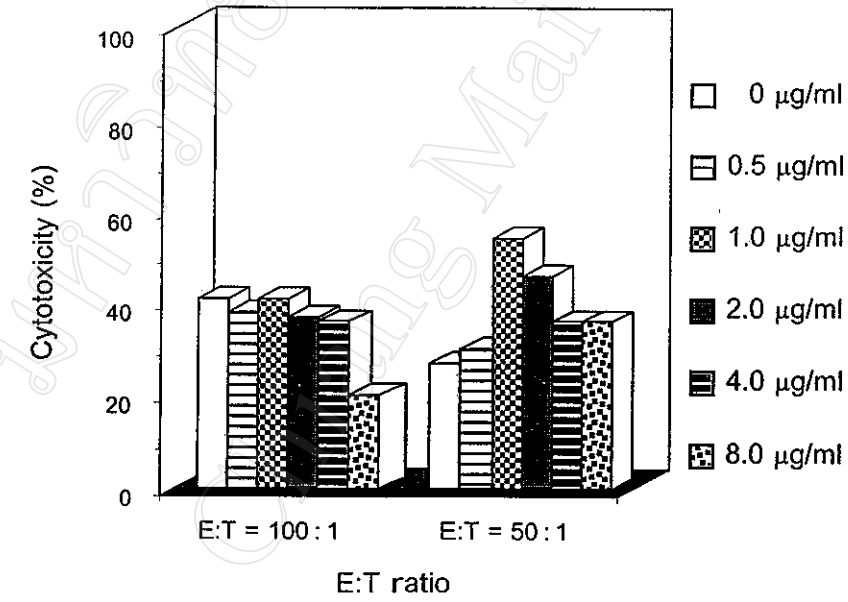

Figure 40 Effect of P3 on cell mediated cytotoxicity. PBMCs were cultured with K562 in the E:T ratio of 100 : 1 and 50 : 1 in the presence or absence of P3 for 4 hrs before an assay of LDH release. All assays were done in triplicate and means of O.D. were used to calculate for the cytotoxicity (%).

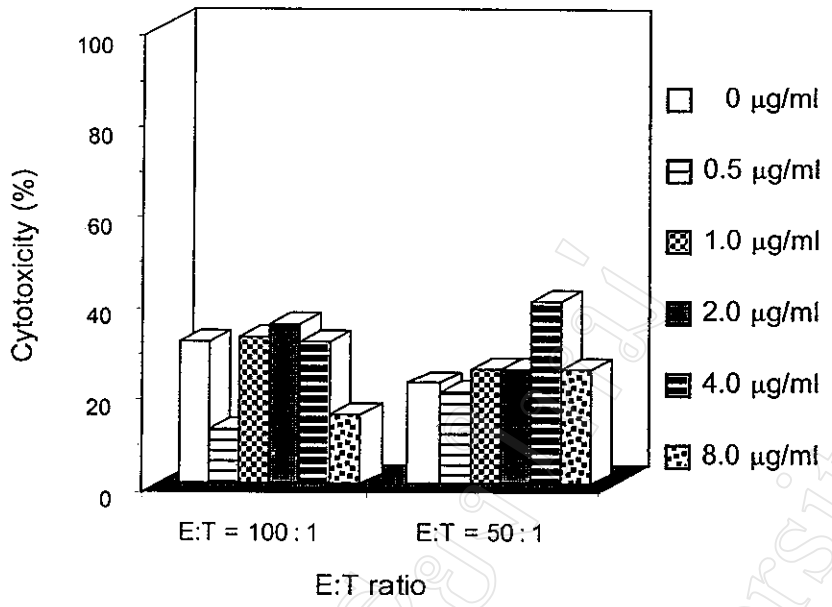

Figure 41 Effect of PPS on cell mediated cytotoxicity. PBMCs were cultured with K562 in the E:T ratio of 100 : 1 and 50 : 1 in the presence or absence of PPS for 4 hrs before an assay of LDH release. All assays were done in triplicate and means of O.D. were used to calculate for the cytotoxicity (%).

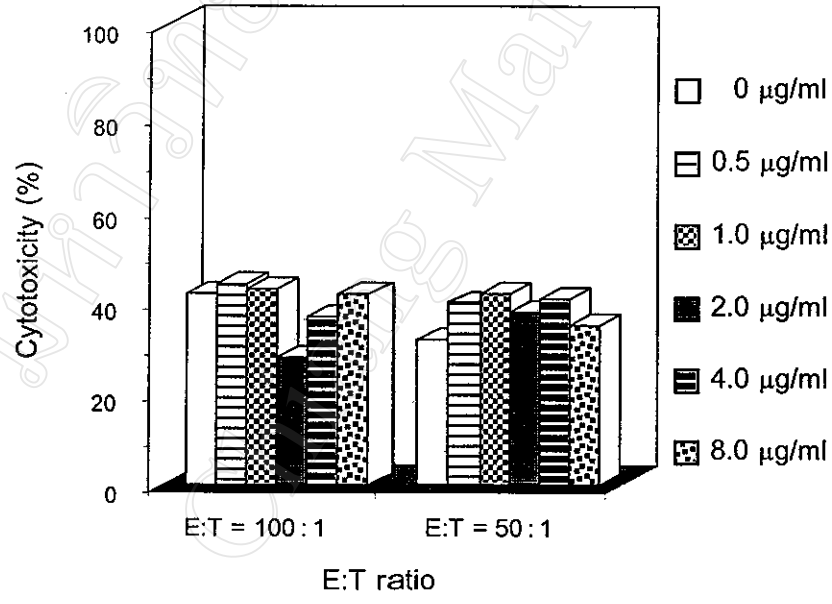

Figure 42 Effect of heparin on cell mediated cytotoxicity. PBMCs were cultured with K562 in the E:T ratio of 100 : 1 and 50 : 1 in the presence or absence of heparin for 4 hrs before an assay of LDH release. All assays were done in triplicate and means of O.D. were used to calculate for the cytotoxicity (%).

Table 29 Effect of P1-P3, PPS and heparin on cell mediated cytotoxicity assay

Tested Matenals	Concentration	Cytotoxicity (%)									
		Donor-1		Donor-2		Donor-3		Donor-4		Donor-5	
		E:T ratio		E:T ratio		E:T ratio		E:T ratio		E:T ratio	
		100:1	50:1	100:1	50:1	100:1	50:1	100:1	50:1	100:1	50:1
P1 (µg/ml)	0	12	6	24	33	13	9	30	24	56	62
	0.5	15	14	24	32	37	4	24	19	53	44
	1.0	15	11	34	52	28	17	46	34	65	64
	2.0	19	11	34	53	21	22	33	29	66	62
	4.0	15	11	31	52	29	29	37	34	71	65
	8.0	7	18	51	56	39	43	33	42	75	68
P2 (µg/ml)	0	17	4	33	31	34	17	33	43	62	61
	0.5	16	12	28	32	41	22	34	28	64	52
	1.0	6	20	25	37	36	32	35	30	64	66
	2.0	5	4	27	40	38	39	39	26	65	61
	4.0	5	15	25	47	32	46	30	16	69	65
	8.0	6	0	28	38	26	38	39	25	81	69
P3 (µg/ml)	0	20	9	41	27	21	36	30	18	48	52
	0.5	17	15	38	30	20	25	37	23	53	48
	1.0	22	24	41	54	28	33	35	30	51	53
	2.0	19	18	37	46	30	29	35	23	64	48
	4.0	18	18	36	36	26	38	29	30	53	48
	8.0	15	14	20	36	11	25	38	29	60	57
PPS (µg/ml)	0	15	14	31	22	32	30	29	25	45	48
	0.5	13	21	12	20	22	21	23	16	42	44
	1.0	21	19	32	25	24	18	24	28	49	44
	2.0	17	18	35	25	22	22	24	16	49	45
	4.0	9	18	31	40	24	22	23	10	47	40
	8.0	7	11	15	25	11	22	28	18	51	35
Heparin (IU/ml)	0	26	25	42	32	25	37	38	34	57	60
	0.05	22	22	44	40	48	33	37	26	58	48
	0.10	26	24	43	42	48	34	43	35	58	26
	0.20	17	23	28	38	37	33	34	28	48	42
	0.40	16	19	37	41	28	18	33	29	61	47
	0.80	13	13	42	35	16	22	32	23	64	53

D. Determination of the cytotoxic potential of P1-P3, PPS and heparin on K562

Parallel to the evaluation of cell mediated cytotoxicity, the cytotoxic potential of each tested material to target cells (K562) was studied. In order to investigate that the tested materials had no natural cytotoxic potential to target cells, various concentrations of P1-P3 and PPS (0-8 µg/ml) and heparin (0-0.8 IU/ml) were cultured with K562 (2×10^4 cells/ml). The assay was done in triplicate and the mean O.D. was measured. The mean of O.D. was used to calculate for cytotoxicity (%) by the equation mentioned in chapter 2. The results demonstrated that all tested materials had no cytotoxic potential to K562 (Table 30).

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Table 30 Cytotoxic potential of P1-P3, PPS and heparin on K562

Tested materials	Concentration	Cytotoxicity (%)
P1 (μg/ml)	0	0
	0.5	0
	1.0	0
	2.0	0
	4.0	0
	8.0	0
P2 (μg/ml)	0	0
	0.5	0
	1.0	0
	2.0	0
	4.0	0
	8.0	4
P3 (μg/ml)	0	0
	0.5	0
	1.0	0
	2.0	0
	4.0	0
	8.0	4
PPS (μg/ml)	0	0
	0.5	0
	1.0	0
	2.0	0
	4.0	0
	8.0	2
Heparin (IU/ml)	0	0
	0.05	4
	0.1	0
	0.2	0
	0.4	0
	0.8	3

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3.2.2.3 Effect on the immunoglobulin production

A. Determination of the optimal conditions for the assay of immunoglobulin production

To assay the effect of tested materials on the immunoglobulin production, the optimal conditions were investigated. In this assay, the optimal dilution of first antibody for coating plate and peroxidase conjugated secondary antibody was determined. Various dilutions of goat anti-human immunoglobulin polyvalent (Igs) were coated, standard human immunoglobulins (G, A, M) was used as a source of antigen and peroxidase conjugated goat anti-human immunoglobulins (1:2500) was used as the secondary antibody. The optimal dilution of first antibody was demonstrated to be 1:1000 as it showed the high absorbance obtained even in the high titer of standard human immunoglobulin tested (Figure 43A). The optimal dilution of peroxidase conjugated goat anti-human Igs was also studied. The results showed that the optimal dilution of peroxidase conjugated goat anti-human Igs was 1:2500 (Figure 43B). While the other classes of anti-human immunoglobulins used in this study are derived from goat, anti-human IgG class was derived from rabbit and it should be optimized as well. However, the first trial showed that optimal dilution of peroxidase conjugated rabbit anti-human IgG was 1:20000. Therefore, it was not optimized since this dilution provided high absorbance even at high titer of standard human immunoglobulin tested. The incubation times for antigen-antibody reaction and the conjugate reaction was 2 hrs at 37 °C. The substrate incubation time was 30 minutes at room temperature (Coligan *et al.*, 1994)

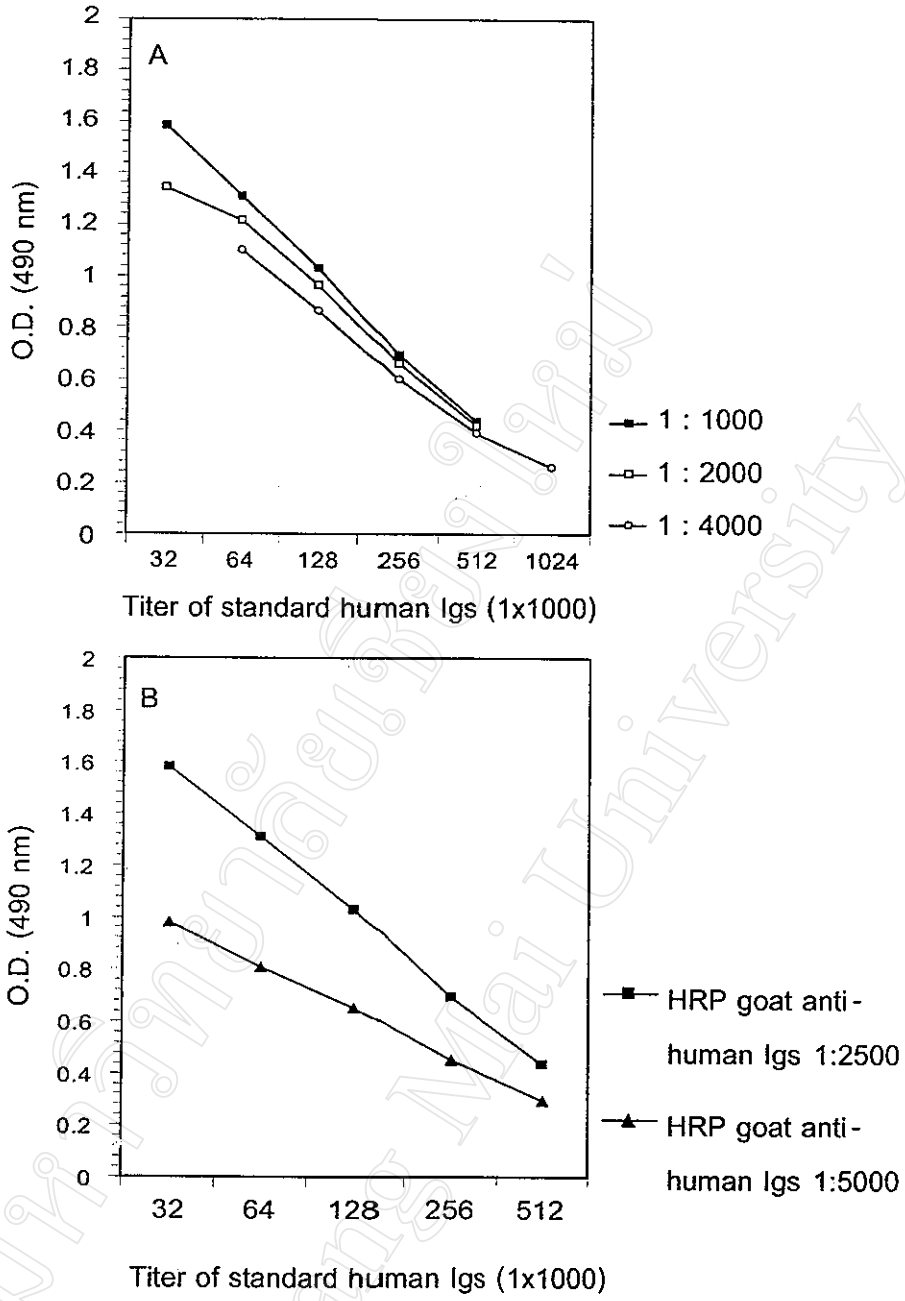

Figure 43 Optimal conditions for the detection of the immunoglobulin. (A) optimal dilution of goat anti-human Igs for coating plate. Various dilutions of goat anti-human Igs were coated on plates and peroxidase conjugated goat anti-human Igs (1;2500) was used as the secondary antibody. (B) Optimal dilution of peroxidase conjugated goat anti-human Igs. Goat anti-human Igs (1:1000) was coated on plates and standard human immunoglobulins (G, A, M) was used as a source of antigen.

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

As in the study of immunoglobulin production, PWM was used to stimulate the PBMCs for 10 days before an assay of the immunoglobulins in the cell culture supernatant. Optimal concentration of PWM was investigated. PBMCs (5×10^5 cells/ml) isolated from 3 healthy donors were cultured in the presence or absence of PWM (0-10 μg/ml) for 10 days. The assay of the immunoglobulin production in all 3 donors showed that the optimal concentration of PWM was 2.5 μg/ml (Figure 44). Since it provided the high absorbance that was suitable for further investigation for the effect of tested material on the PWM stimulated immunoglobulin production.

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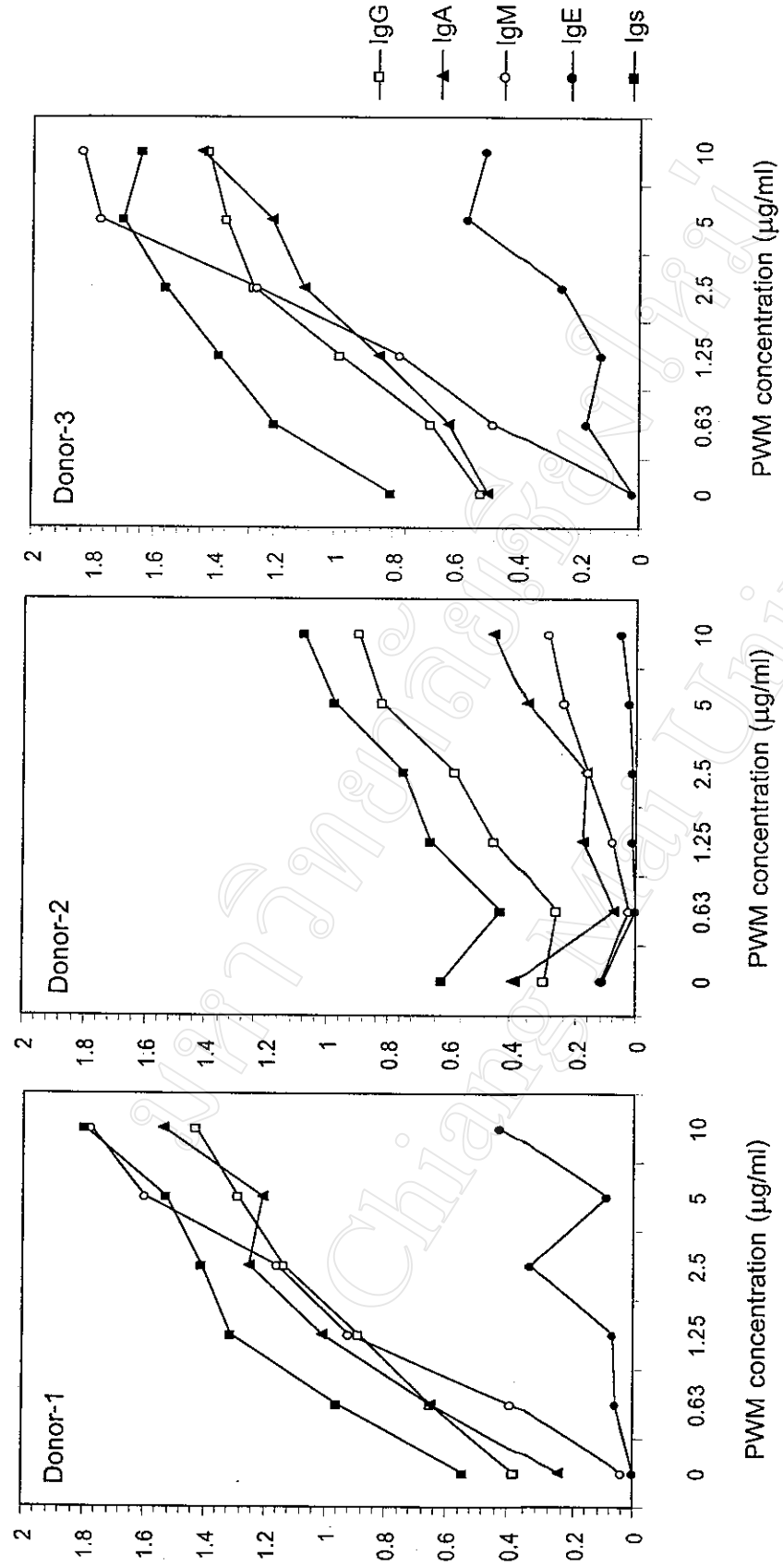

Figure 44 Determination of the sub-optimal concentration of PWM. PBMCs ($5x10^5$ cells/ml) isolated from 3 healthy donors were cultured with various concentrations of PWM for 10 days. Culture supernation were studied for the production of various classes of human immunoglobulins.

C. Effect of P1-P3, PPS and heparin on the immunoglobulin production

To evaluate the effect of CPS on the immunoglobulin production, the assay was done in parallel to PPS and heparin. PBMCs isolated from 3 healthy donors were cultured with various concentrations of tested materials in the presence or absence of PWM (2.5 μg/ml) for 10 days. The cell-free culture supernatants were assayed for the changes in the existent of each class of human immunoglobulin. The results from the cultivation of PBMCs in the various concentrations of each tested materials with no PWM showed that all tested materials did not significantly effect the immunoglobulin production ($p<0.01$, pair T-test). Moreover, each donor expressed his/her individual status of amount and classes of the immunoglobulin secretion into the cell culture supernatants after 10 days of cultivation. In contrast, in the presence of PWM, the immunoglobulin level was significantly decreased and in dose dependent manner ($p<0.01$, pair T test). In addition, the inhibition effect on IgM production was clearly remarked when compared to the other classes. However, there was no different between P1, P2 and P3 which were different in M_v. The result also demonstrated that heparin provided the different pattern from other tested materials where the inhibitory effect was not quite clear and was not in dose dependent manner. The results of one from 3 donors tested were shown in Table 31-35 and Figure 45-49.

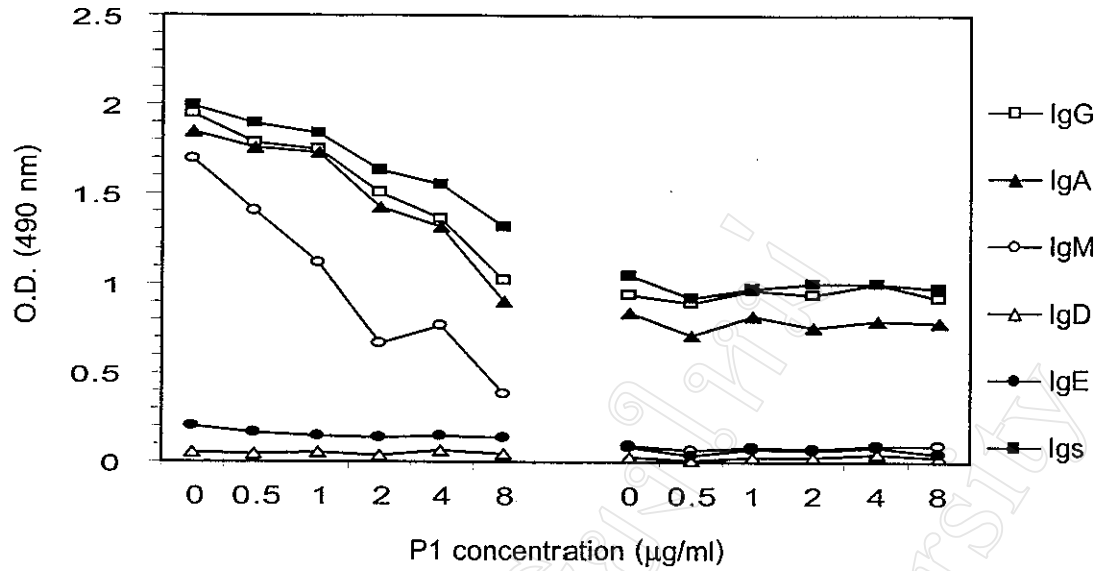

Figure 45 Inhibition effect of P1 on the immunoglobulin production. PBMCs ($5x10^5$ cells/ml) were cultured with various concentrations of P1 in the presence of PWM (2.5 µg/ml) for 10 days. Culture supernatants were assayed for the production of various classes of human immunoglobulins.

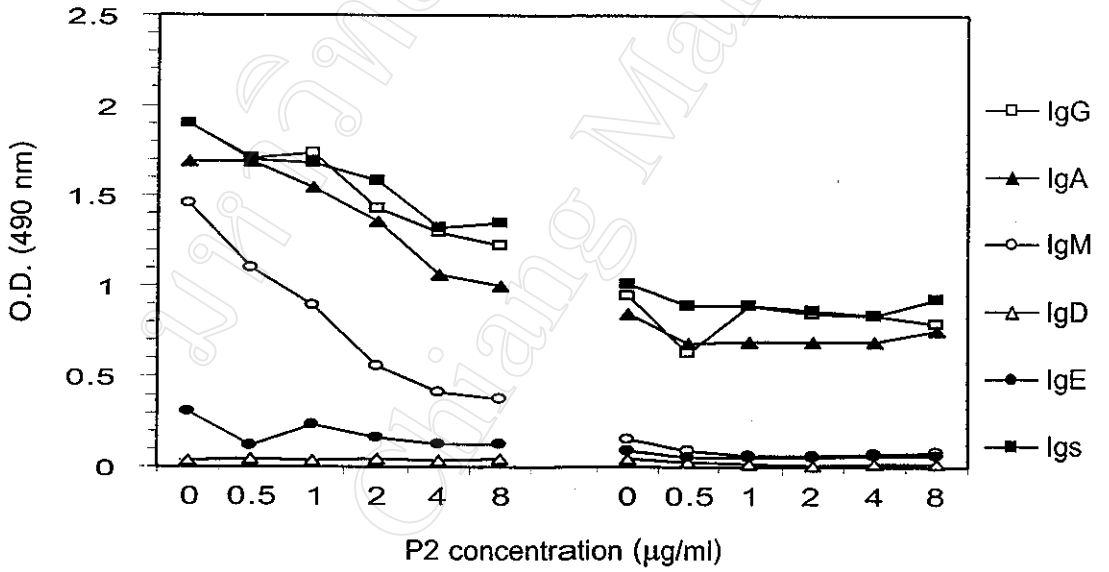

Figure 46 Inhibition effect of P2 on the immunoglobulin production. PBMCs ($5x10^5$ cells/ml) were cultured with various concentrations of P2 in the presence of PWM (2.5 µg/ml) for 10 days. Culture supernatants were assayed for the production of various classes of human immunoglobulins.

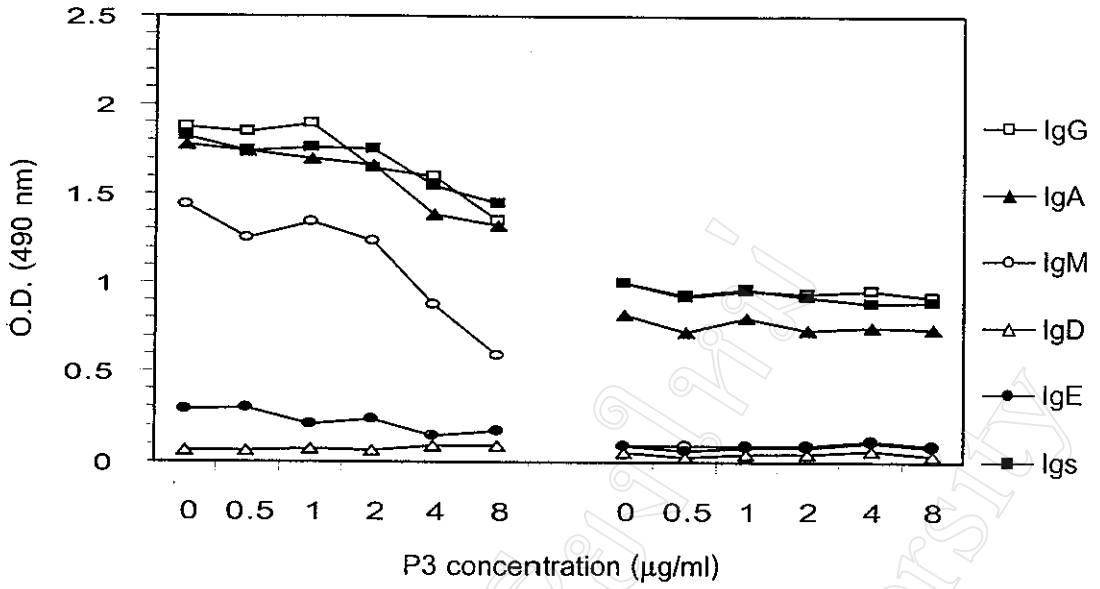

Figure 47 Inhibition effect of P3 on the immunoglobulin production. PBMCs ($5x10^5$ cells/ml) were cultured with various concentrations of P3 in the presence of PWM (2.5 µg/ml) for 10 days. Culture supernatants were assayed for the production of various classes of human immunoglobulins.

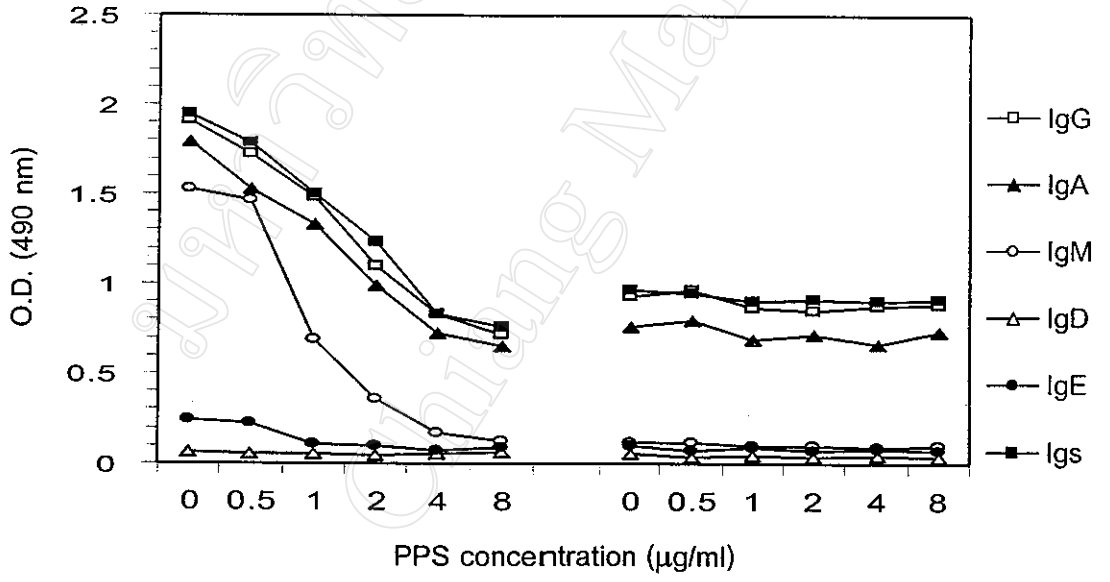

Figure 48 Inhibition effect of PPS on the immunoglobulin production. PBMCs ($5x10^5$ cells/ml) were cultured with various concentrations of PPS in the presence of PWM (2.5 µg/ml) for 10 days. Culture supernatants were assayed for the production of various classes of human immunoglobulins.

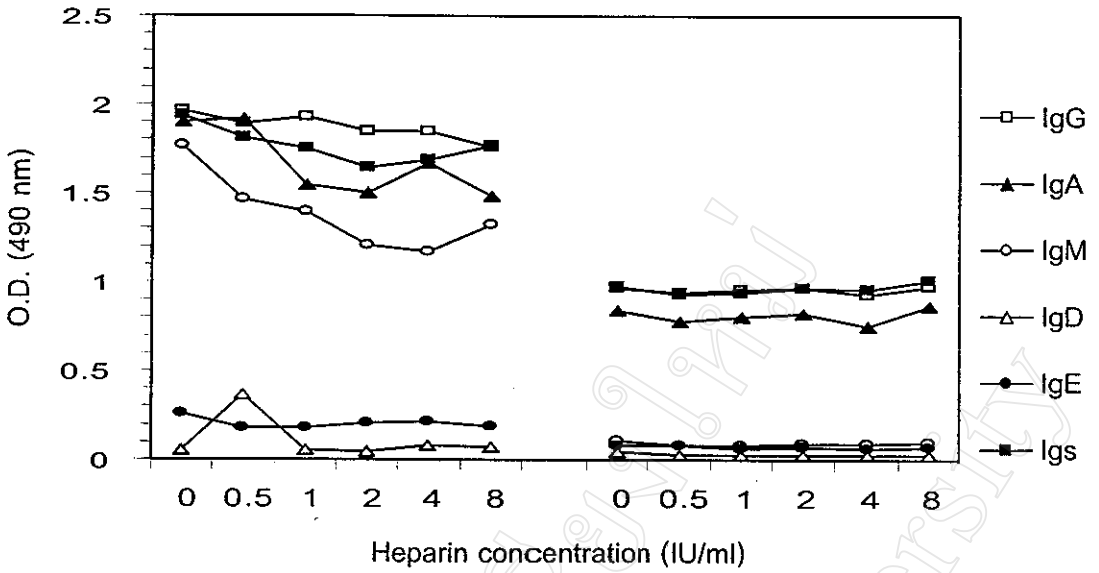

Figure 49 Inhibition effect of heparin on the immunoglobulin production. PBMCs ($5x10^5$ cells/ml) were cultured with various concentrations of heparin in the presence of PWM (2.5 μg/ml) for 10 days. Culture supernatants were assayed for the production of various classes of human immunoglobulins.

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Table 31 Effect of P1 on PWM stimulated immunoglobulin production of 3 healthy donors

Donor number	Condition	Ig Class	O.D. (490 nm) P1 concentration (µg/ml) 0	0.5	1.0	2.0	4.0	8.0
1	with PWM	IgG	1.943	1.786	1.746	1.505	1.356	1.010
		IgA	1.846	1.759	1.726	1.425	1.310	0.900
		IgM	1.693	1.402	1.110	0.657	0.758	0.376
		IgD	0.055	0.041	0.050	0.036	0.063	0.043
		IgE	0.197	0.160	0.145	0.134	0.148	0.138
		Igs	1.994	1.897	1.838	1.627	1.550	1.316
	without PWM	IgG	0.931	0.885	0.956	0.937	0.996	0.917
		IgA	0.833	0.702	0.811	0.753	0.786	0.779
		IgM	0.089	0.065	0.079	0.069	0.093	0.089
		IgD	0.024	0.012	0.029	0.026	0.047	0.023
		IgE	0.082	0.038	0.068	0.067	0.083	0.042
		Igs	1.043	0.914	0.965	0.994	0.992	0.969
2	with PWM	IgG	1.716	1.652	1.447	1.320	1.323	1.154
		IgA	0.507	0.280	0.348	0.275	0.263	0.293
		IgM	0.960	1.034	0.828	0.603	0.402	0.203
		IgD	0.098	0.085	0.108	0.089	0.113	0.123
		IgE	0.131	0.121	0.166	0.088	0.091	0.077
		Igs	1.464	1.493	1.391	1.173	1.234	1.023
	without PWM	IgG	0.774	0.715	0.784	0.809	0.842	0.831
		IgA	0.234	0.192	0.207	0.219	0.256	0.265
		IgM	0.077	0.030	0.065	0.075	0.095	0.074
		IgD	0.092	0.063	0.087	0.096	0.110	0.098
		IgE	0.042	0.018	0.044	0.051	0.095	0.047
		Igs	0.669	0.588	0.625	0.661	0.673	0.707
3	with PWM	IgG	1.422	1.231	1.273	1.185	1.120	1.131
		IgA	0.907	0.967	0.892	0.974	1.078	0.999
		IgM	0.867	0.828	0.763	0.839	0.457	0.471
		IgD	0.047	0.041	0.059	0.041	0.068	0.062
		IgE	0.100	0.089	0.104	0.142	0.099	0.118
		Igs	1.327	1.300	1.298	1.197	1.164	1.177
	without PWM	IgG	1.109	0.949	1.028	1.017	1.007	1.053
		IgA	0.919	0.766	0.848	0.816	0.810	0.862
		IgM	0.089	0.061	0.114	0.111	0.130	0.111
		IgD	0.017	0.035	0.044	0.040	0.063	0.047
		IgE	0.048	0.031	0.079	0.069	0.095	0.080
		Igs	1.075	0.886	0.920	0.958	0.871	0.984

Table 32 Effect of P2 on PWM stimulated immunoglobulin production of 3 healthy donors

Donor number	Condition	Ig Class	O.D. (490 nm) P2 concentration (µg/ml) 0	0.5	1.0	2.0	4.0	8.0
1	with PWM	IgG	1.901	1.712	1.732	1.428	1.294	1.219
		IgA	1.690	1.690	1.550	1.355	1.060	0.999
		IgM	1.458	1.099	0.890	0.557	0.415	0.374
		IgD	0.039	0.043	0.037	0.041	0.037	0.047
		IgE	0.309	0.114	0.234	0.166	0.129	0.122
		Igs	1.895	1.697	1.686	1.584	1.321	1.349
	without PWM	IgG	0.946	0.632	0.887	0.848	0.835	0.79
		IgA	0.842	0.687	0.693	0.696	0.691	0.757
		IgM	0.149	0.091	0.064	0.067	0.070	0.077
		IgD	0.045	0.025	0.018	0.012	0.017	0.020
		IgE	0.088	0.050	0.054	0.054	0.062	0.059
		Igs	1.006	0.891	0.894	0.866	0.833	0.926
2	with PWM	IgG	1.582	1.559	1.438	1.347	1.240	1.243
		IgA	0.341	0.295	0.350	0.334	0.253	0.232
		IgM	0.948	0.736	0.658	0.557	0.384	0.264
		IgD	0.106	0.106	0.108	0.109	0.124	0.125
		IgE	0.105	0.092	0.069	0.072	0.071	0.128
		Igs	1.30	1.340	1.259	1.142	1.122	1.055
	without PWM	IgG	0.787	0.700	0.699	0.761	0.765	0.711
		IgA	0.279	0.235	0.229	0.192	0.200	0.245
		IgM	0.098	0.065	0.080	0.071	0.072	0.062
		IgD	0.119	0.096	0.097	0.098	0.104	0.098
		IgE	0.065	0.050	0.052	0.045	0.055	0.052
		Igs	0.627	0.562	0.521	0.603	0.529	0.529
3	with PWM	IgG	1.156	1.252	1.077	1.082	1.162	1.096
		IgA	0.961	0.865	0.733	0.749	0.824	0.853
		IgM	1.054	0.713	0.542	0.548	0.511	0.523
		IgD	0.053	0.046	0.047	0.044	0.055	0.071
		IgE	0.102	0.103	0.073	0.084	0.097	0.148
		Igs	1.280	1.217	1.042	0.952	1.118	1.107
	without PWM	IgG	0.994	0.964	0.946	0.923	0.982	0.991
		IgA	0.830	0.781	0.720	0.686	0.735	0.812
		IgM	0.120	0.119	0.104	0.089	0.108	0.120
		IgD	0.044	0.037	0.034	0.033	0.035	0.038
		IgE	0.075	0.067	0.071	0.029	0.060	0.069
		Igs	0.926	0.886	0.820	0.818	0.785	0.891

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Table 33 Effect of P3 on PWM stimulated immunoglobulin production of 3 healthy donors

Donor number	Condition	Ig Class	O.D. (490 nm) P3 concentration (µg/ml) 0	0.5	1.0	2.0	4.0	8.0
1	with PWM	IgG	1.878	1.848	1.891	1.644	1.599	1.352
		IgA	1.778	1.739	1.692	1.660	1.380	1.321
		IgM	1.440	1.248	1.342	1.231	0.871	0.593
		IgD	0.065	0.062	0.070	0.061	0.085	0.086
		IgE	0.282	0.293	0.209	0.230	0.146	0.174
		Igs	1.824	1.745	1.760	1.754	1.544	1.442
	without PWM	IgG	0.997	0.917	0.959	0.937	0.954	0.922
		IgA	0.817	0.727	0.805	0.733	0.750	0.739
		IgM	0.091	0.086	0.093	0.089	0.112	0.091
		IgD	0.054	0.030	0.042	0.045	0.062	0.037
		IgE	0.093	0.063	0.083	0.079	0.107	0.078
		Igs	1.000	0.931	0.968	0.924	0.882	0.896
2	with PWM	IgG	1.163	1.358	1.346	1.213	1.084	1.191
		IgA	0.659	0.789	0.734	0.736	0.661	0.938
		IgM	1.055	0.756	0.832	0.822	0.595	0.721
		IgD	0.032	0.032	0.036	0.037	0.034	0.054
		IgE	0.062	0.077	0.083	0.100	0.073	0.091
		Igs	1.027	1.114	1.115	0.977	0.918	1.118
	without PWM	IgG	0.739	0.689	0.728	0.734	0.748	0.761
		IgA	0.217	0.194	0.186	0.192	0.197	0.215
		IgM	0.053	0.023	0.056	0.055	0.072	0.047
		IgD	0.079	0.057	0.079	0.078	0.096	0.082
		IgE	0.035	0.013	0.034	0.03	0.05	0.041
		Igs	0.551	0.526	0.508	0.542	0.495	0.544
3	with PWM	IgG	1.262	1.196	1.220	1.131	1.100	1.191
		IgA	0.917	0.834	0.845	0.840	0.786	0.783
		IgM	1.104	0.915	0.503	0.660	0.580	0.473
		IgD	0.044	0.046	0.049	0.046	0.055	0.061
		IgE	0.115	0.073	0.094	0.114	0.071	0.107
		Igs	1.257	1.219	1.061	1.003	1.013	1.096
	without PWM	IgG	1.125	0.981	0.967	0.947	0.988	0.976
		IgA	0.797	0.704	0.668	0.679	0.630	0.697
		IgM	0.128	0.094	0.096	0.085	0.097	0.116
		IgD	0.046	0.032	0.035	0.039	0.035	0.038
		IgE	0.065	0.075	0.064	0.062	0.059	0.062
		Igs	0.973	0.819	0.775	0.792	0.746	0.826

Table 34 Effect of PPS on PWM stimulated immunoglobulin production of 3 healthy donors

Donor number	Condition	Ig Class	O.D. (490 nm) PPS concentration (µg/ml) 0	0.5	1.0	2.0	4.0	8.0
1	with PWM	IgG	1.911	1.719	1.479	1.097	0.830	0.710
		IgA	1.795	1.531	1.327	0.989	0.727	0.652
		IgM	1.524	1.468	0.685	0.359	0.166	0.126
		IgD	0.063	0.053	0.056	0.047	0.054	0.064
		IgE	0.240	0.223	0.103	0.094	0.073	0.088
		Igs	1.942	1.790	1.502	1.232	0.829	0.758
	without PWM	IgG	0.933	0.967	0.868	0.852	0.878	0.880
		IgA	0.760	0.798	0.687	0.715	0.657	0.736
		IgM	0.112	0.113	0.099	0.095	0.091	0.094
		IgD	0.051	0.040	0.041	0.034	0.041	0.034
		IgE	0.100	0.072	0.087	0.074	0.081	0.070
		Igs	0.964	0.950	0.901	0.912	0.900	0.908
2	with PWM	IgG	1.685	1.584	1.283	1.046	0.737	0.732
		IgA	0.341	0.336	0.225	0.169	0.167	0.174
		IgM	0.803	0.617	0.380	0.235	0.078	0.087
		IgD	0.095	0.091	0.094	0.089	0.094	0.102
		IgE	0.060	0.106	0.056	0.052	0.041	0.064
		Igs	1.330	1.333	1.035	0.867	0.540	0.538
	without PWM	IgG	0.860	0.709	0.752	0.713	0.749	0.761
		IgA	0.213	0.226	0.199	0.200	0.205	0.205
		IgM	0.081	0.059	0.054	0.058	0.058	0.059
		IgD	0.096	0.083	0.086	0.085	0.088	0.087
		IgE	0.052	0.037	0.044	0.036	0.039	0.037
		Igs	0.663	0.526	0.510	0.519	0.536	0.560
3	with PWM	IgG	1.292	1.216	1.145	1.001	0.860	0.842
		IgA	0.903	0.839	0.874	0.686	0.704	0.655
		IgM	0.762	0.729	0.785	0.482	0.313	0.145
		IgD	0.047	0.037	0.052	0.037	0.068	0.074
		IgE	0.108	0.077	0.083	0.089	0.080	0.078
		Igs	1.098	1.113	1.091	0.804	0.794	0.707
	without PWM	IgG	0.957	0.871	0.953	0.930	0.956	0.973
		IgA	0.769	0.676	0.643	0.635	0.635	0.675
		IgM	0.118	0.081	0.102	0.096	0.115	0.094
		IgD	0.047	0.024	0.044	0.036	0.065	0.043
		IgE	0.074	0.044	0.070	0.080	0.089	0.055
		Igs	0.823	0.752	0.723	0.512	0.717	0.745

Table 35 Effect of heparin on PWM stimulated immunoglobulin production of 3 healthy donors

Donor number	Condition	Ig Class	O.D. (490 nm) heparin concentration (IU/ml) 0	0.5	1.0	2.0	4.0	8.0
1	with PWM	IgG	1.963	1.896	1.933	1.849	1.844	1.762
		IgA	1.898	1.923	1.547	1.502	1.673	1.481
		IgM	1.766	1.460	1.392	1.207	1.171	1.324
		IgD	0.057	0.368	0.051	0.049	0.083	0.072
		IgE	0.258	0.182	0.176	0.203	0.210	0.185
		Igs	1.939	1.812	1.750	1.639	1.690	1.770
	without PWM	IgG	0.960	0.940	0.956	0.963	0.933	0.976
		IgA	0.838	0.781	0.802	0.817	0.754	0.863
		IgM	0.105	0.083	0.077	0.085	0.086	0.099
		IgD	0.046	0.030	0.030	0.029	0.029	0.030
		IgE	0.082	0.078	0.065	0.068	0.061	0.070
		Igs	0.975	0.925	0.935	0.960	0.956	1.005
2	with PWM	IgG	1.895	1.662	1.746	1.708	1.457	1.567
		IgA	0.387	0.250	0.362	0.387	0.293	0.284
		IgM	1.213	0.821	0.841	0.906	0.562	0.711
		IgD	0.100	0.098	0.101	0.098	0.102	0.114
		IgE	0.192	0.107	0.098	0.069	0.082	0.074
		Igs	1.473	1.366	1.388	1.349	1.220	1.358
	without PWM	IgG	0.795	0.716	0.781	0.760	0.810	0.799
		IgA	0.234	0.213	0.201	0.223	0.201	0.185
		IgM	0.097	0.052	0.057	0.064	0.051	0.047
		IgD	0.097	0.083	0.101	0.088	0.086	0.088
		IgE	0.049	0.033	0.035	0.035	0.035	0.033
		Igs	0.631	0.525	0.537	0.571	0.550	0.585
3	with PWM	IgG	1.163	1.358	1.346	1.213	1.084	1.191
		IgA	0.659	0.789	0.734	0.736	0.661	0.938
		IgM	1.055	0.756	0.832	0.822	0.595	0.721
		IgD	0.032	0.032	0.036	0.037	0.034	0.054
		IgE	0.062	0.077	0.083	0.100	0.073	0.091
		Igs	1.027	1.114	1.115	0.977	0.918	1.118
	without PWM	IgG	0.995	0.980	0.958	0.967	1.059	1.051
		IgA	0.698	0.601	0.603	0.595	0.559	0.673
		IgM	0.100	0.088	0.087	0.076	0.067	0.092
		IgD	0.033	0.026	0.026	0.024	0.025	0.023
		IgE	0.066	0.045	0.052	0.071	0.043	0.051
		Igs	0.804	0.742	0.678	0.735	0.719	0.797

3.2.2.4 Effect on the stimulation of cytokine production

The CPS was assayed for its effect on the stimulation of IL-2 and IFN-γ production in parallel with PPS and heparin. PBMCs ($1x10^6$cells/ml) from 3 healthy donors were cultured for 24 hrs in the presence or absence of various concentrations of tested materials. PMA/Ionomycin was used to stimulate cells to perform the positive control. The standard calibration curve of IL-2 and IFN-γ was performed to determine the concentration of cytokine in the cell culture supernatant (Figure 50-51). The results showed that the O.D. at 450/550 nm from each of experiments were very low. The concentration of both cytokines could not be reported since the O.D. was lower than that of the lowest concentration of standard cytokine used in the calibration of the standard curve. It was indicated that all tested materials had no effect on the stimulation of IL-2 and IFN-γ production at 24 hrs of cultivation. The results were shown in Table 36.

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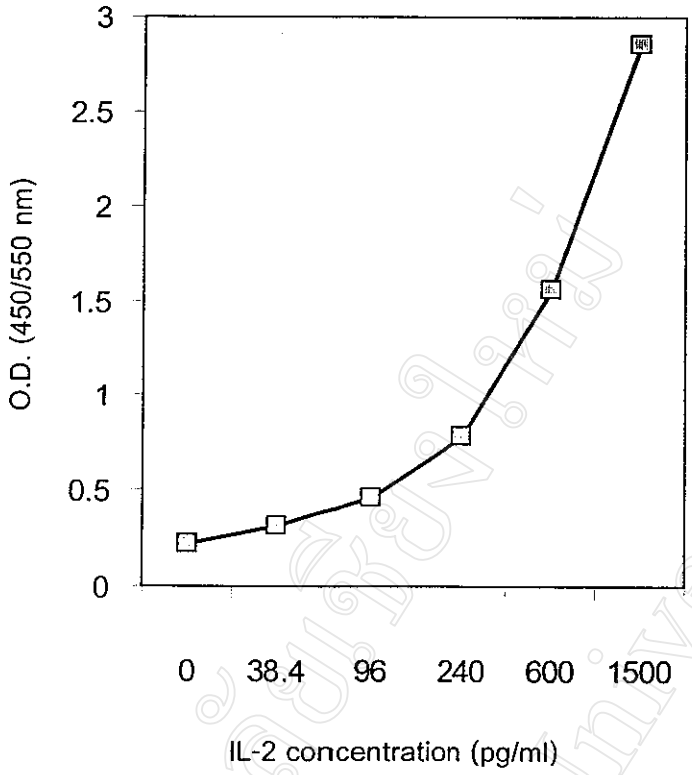

Figure 50 Standard calibration curve of IL-2. Various concentrations of IL-2 and biotinylated antibody reagent were incubated with anti-human IL-2 precoated on well plates for 2 hrs at room temperature before washing. Streptavidin-HRP was added and incubated for 30 min at room temperature. The reaction was washed prior to the TMB reaction for 30 min following with the stop solution. The O.D. was read at 450/550 nm.

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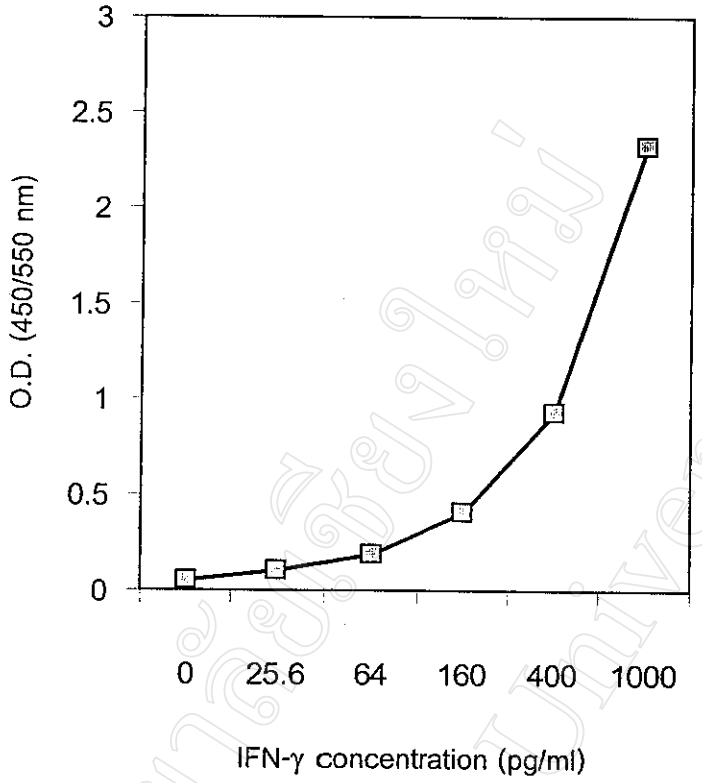

Figure 51 Standard calibration curve of IFN-γ. Various concentrations of IFN-γ and biotinylated antibody reagent were incubated with anti-human IFN-γ. precoated on well plates for 3 hrs at room temperature before washing. Streptavidin-HRP was added and incubated for 30 min at room temperature. The reaction was washed prior to the TMB reaction for 30 min following with the stop solution. The O.D. was read at 450/550 nm.

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Table 36 The stimulation effect of P1-P3, PPS and heparin on the cytokine production of 3 healthy donors

Tested Materials	Concentration	O.D. (450/550 nm)					
		IL-2			IFN-γ		
		Donor-1	Donor-2	Donor-3	Donor-1	Donor-2	Donor-3
P1 (μg/ml)	0	0.117	0.118	0.126	0.067	0.086	0.077
	1	0.115	0.112	0.120	0.093	0.084	0.074
	2	0.114	0.091	0.107	0.099	0.083	0.068
	4	0.099	0.084	0.116	0.088	0.076	0.068
	8	0.111	0.092	0.114	0.099	0.073	0.062
P2 (μg/ml)	0	0.117	0.118	0.126	0.067	0.086	0.077
	1	0.107	0.080	0.084	0.112	0.080	0.063
	2	0.101	0.069	0.094	0.079	0.100	0.069
	4	0.106	0.072	0.098	0.083	0.079	0.060
	8	0.110	0.078	0.090	0.092	0.071	0.062
P3 (μg/ml)	0	0.117	0.118	0.126	0.067	0.086	0.077
	1	0.093	0.062	0.078	0.091	0.075	0.061
	2	0.083	0.059	0.079	0.085	0.060	0.065
	4	0.094	0.064	0.074	0.081	0.062	0.060
	8	0.077	0.076	0.074	0.087	0.074	0.049
PPS (μg/ml)	0	0.117	0.118	0.126	0.067	0.086	0.077
	1	0.117	0.077	0.109	0.131	0.097	0.074
	2	0.117	0.072	0.101	0.100	0.082	0.067
	4	0.101	0.065	0.221	0.103	0.085	0.069
	8	0.117	0.070	0.095	0.118	0.072	0.069
Heparin (IU/ml)	0	0.117	0.118	0.126	0.067	0.086	0.077
	0.1	0.093	0.083	0.098	0.085	0.080	0.070
	0.2	0.081	0.066	0.097	0.070	0.071	0.062
	0.4	0.081	0.070	0.081	0.077	0.074	0.062
	0.8	0.085	0.068	0.108	0.074	0.070	0.068
Medium Control		0.231	0.151	0.231	0.056	0.084	0.079
PMA/I (Dilution)	Undiluted	> 3.000	> 3.000	> 3.000	> 3.000	> 3.000	> 3.000
	1:2.5	2.042	ND	ND	> 3.000	ND	ND
	1:5	1.199	1.390	1.776	> 3.000	> 3.000	> 3.000
	1:10	0.739	0.822	0.854	1.875	1.808	2.088

PMA/I; phorbol myristate acetate/ Ionomycin, ND = not determine.