CHAPTER 4

DISCUSSION

4.1 Synthesis of the chitosan polysulfate (CPS) by random substitution

Polysaccharides are increasingly used for the medical applications, either taking advantage of some intrinsic functional properties, or of some pharmacological properties elicited by the biological interactions. Biological and pharmacological properties can be induced or modified in such a polymer by chemical, especially sulfate derivatisation, (Bode and Franz, 1990). Thus, for example the therapeutic efficacy of heparin and heparinoids is extensive studied by a control of the molecular size, the degree of sulfation and the localization of specific sugar or uronic acid sequence (Bourin and Lindahl, 1993).

Since the discovery of heparin and its biological activities, a number of polysaccharide sulfates have been synthesized and their biological activities examined. Chitosan is a linear polymer of β(1→4)-linked 2-amino-2-deoxy-D-glucopyranose which can be easily prepared from chitin by N-deacetylation (Yalpani, 1992). Recently much attention has been paid to chitosan as a potential polysaccharide resource due to its specific structure and properties. Although several trials have been reported to prepare functional derivatives of chitosan by chemical modifications (Hirano, *et al.*, 1976; Moore and Goerge, 1981; Muzzarelli, *et al.*, 1983), only a few examples attained solubility in general organic solvents (Fuji, *et al.*, 1980; Kurita, *et al.*, 1982). Since chitosan is soluble in aqueous solutions of some acids, selective N-alkylidenations and N-acylations have been attempted (Muzzarelli, *et al.*, 1980; Hirano, *et al.*, 1976; Moore and Goerge, 1981). Though several

water-soluble or highly swelling derivatives were obtained, development of solubility in common organic solvents was difficult by these methods.

In general only the influence of molecular weight and/or substitution degree of sulfated polysaccharides on their biological activity is considered in the majority of investigations involving the anticoagulant or antiviral properties of these substances (Horton and Usui, 1978). The sulfation reactions of multi-functional polysaccharides are inevitably followed by the appearance of structural heterogeneity in polymer chains. When chitosan is sulfated, a structural variety of products is obtained, which may be related to the various reactivities of the three functional groups of the parent polymer, leading to different degrees of completion in the individual groups.

In this study, directed synthesis of similar heterogeneous structures and separation into small molecular weight is of great interest. In any case, the study is directed to the attentive study of the preparation conditions of these compounds and the correct identification of their structures.

Commercial chitosan derived from the shells of marine crab which a degree of deacetylation (DDA) of 0.88 was random substituted using chlorosulfonic in a mixture of N, N-dimethylformamide (DMF) as the sulfate donor called "sulfating complex". Chitosan was prepared into a gelling mixture in DMF called "solvent including chitosan" which was in a semi-heterogeneous state before sulfation. DMF in both solvent including chitosan and sulfating complex, broke the hydrogen bonding in the polymer chains and thus lead to more solubilization of the chitosan. The sulfating complex was stable at room temperature and well soluble in the solvent including chitosan. Therefore, the sulfation reaction occurred completely and high yield in product obtained. The reactions between DMF and

chlorosulfonic acid and sulfation of the chitosan were shown in Figure 52 (Mihai, *et al.*, 2001).

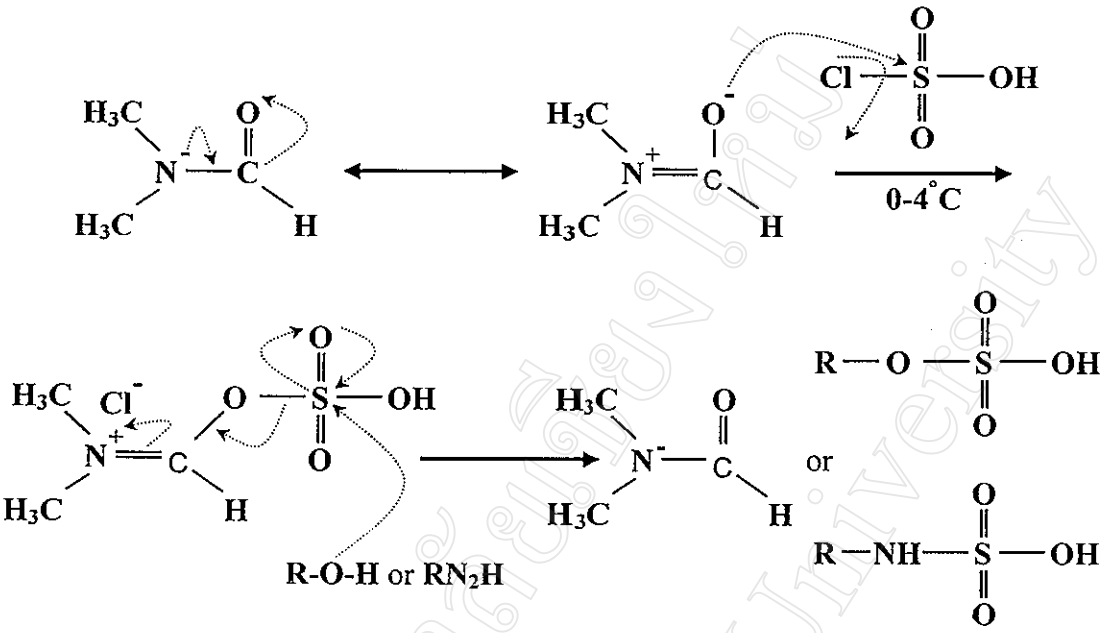
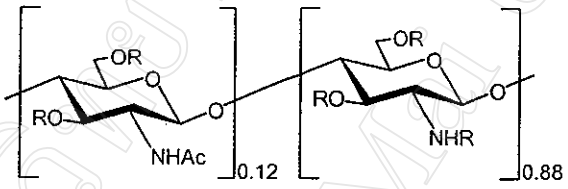


Figure 52 Scheme of the sulfating complex formation and sulfation reaction (Mihai, *et al.*, 2001)

CPS in a sodium salt was obtained as the yield of 55% after purification by FPLC. It was well dissolved in distilled water and phosphate buffered saline, pH 7.2. X-ray diffraction analysis showed that it crystallinity was less order than that of its original chitin and chitosan. This confirmed its water soluble property. The IR spectra showed characteristic absorptions at 800 and 1240 cm^{-1} which due to sulfo groups assigned to C-O-S and S=O stretching, respectively. This was revealed by ^{1}H-NMR and ^{13}C-NMR spectra. ^{1}H-NMR spectrum demonstrated the sulfate group substitution in the polymer chain according to proton shift to lower field at carbon atom that was substituted by sulfate

groups. This resulted from withdrawing of the electron by the sulfate groups. The ^{1}H-NMR spectrum of CPS obtained has been reported by Gamzazade and coworkers (1997) showing the chemical shifts at H-3S and H-6S of δ 4.6 and δ 4.0 ppm, respectively. Moreover, Holme and his coworker (1997) also reported that the chemical shifts at H-2S and H-2 were δ 3.15 and δ 2.74 ppm, respectively. Both reports were in the same range of chemical shifts in our spectrum. ^{13}C-NMR also demonstrated the sulfate substitution at C-2, C-3 and C-6 as well. The proposed formula and structure of CPS obtained was shown below.

$$C_6H_8O_4\ (C_2H_3O)_{0.12}\ (SO_3Na)_{2.23}\ (H)_{0.65}\ .\ 3.7\ H_2O$$



R = -SO_3Na or -H

The sulfation reactions proceeding in the swollen chitosan (semi-heterogeneous conditions) could promote the formation of zwitterionic structures and H-bonds in the products with participating groups at C-6 and C-2 as well as at C-3 and pyranose oxygen (Gamzazade, *et al.*, 1997). The groups at C-2 and C-3 are probably submitted to a small amount of sulfation. The specific rotation in the polarized light showed that it nature is β-D-configuration. The product obtained was analyzed for its composition and it was found

that there was 15.64% of S atom. This data was closed to 16.18% which could be calculated from its formula $(C_6H_8O_4(C_2H_3O)_{0.12}(SO_3Na)_{2.23}\ (H)_{0.65}\ 3.7H_2O)$. The sulfur content of 15.64% was enough for its anticoagulant activity as was observed by the other experiment (Bode and Franz, 1991).

The result which was observed in the IR spectra of CPS showed that there was no absorption peak at wave number of 1570-1590 which was the position of N-H bending. Moreover, the result from ninhydrin assay demonstrated the very low amount of free amino group in CPS (0.27%). This could be indicated that sulfate groups were introduced into almost all of the amine position. Taken together, total S and N content from the elemental analysis and the sulfate positions observed from the ^{1}H-NMR and ^{13}C-NMR, the DS of CPS calculated by S/N ratio was 2.23. This number referred to the number of sulfate group in each unit of amine sugar. The DS of 2.23 obtained in our product was corresponded to its anticoagulant activity as was reported by the other investigations (Ofosu, *et al.*, 1987; Akashi, *et al.*, 1996).

As the objective was to separate the CPS into small average molecular weight (M_v) fractions of high, medium and small size in order to investigate the relationship between M_v and biological activities, the gel filtration was performed using the Sepharose CL-6B (MW cutoff 10-1000 kDa). According to the separation pattern of three different M_v of dextran sulfate, as template, the elution volume of three fractions were employed to calculate for the dissociation constant (K_d). The K_d of 0.16 and 0.58 were obtained and used as a reference K_d for every batch of separation of CPS. CPS could be separated into 3 fractions (P1-P3) and the M_v as was determined by viscosity measurement were

$6.8x10^4$, $3.5x10^4$ and $2.0x10^4$ dalton, respectively where the pre-Sepharose CL-6B has M_v of $3.2x10^4$ dalton.

Purification of three fractions (P1-P3) were performed to eliminate the non-substituted polymer chains. The yield of purified products were different in each fraction where P3 gave the higher yield than P2 and P1, respectively. The results indicated that size of polymer chains could be one factor that influenced in the substitution. The sulfate molecules could not be completely substituted in the high M_v fraction since the higher M_v the lower in yield of purified product obtained. The efficiency of sulfate substitution into the smaller size of polymer chains was much more than that occurred in the larger one.

The first objective of this study was to modify the chitosan by random sulfation. The method of Gamzazade and his coworker (1997) was adapted. The first point was to precipitate the solvent including chitosan with 20% NaOH instead of 0.01M NaOH. The reason was that by 20% NaOH, the higher amount of solvent including chitosan was obtained. Moreover, by this adapted technique, the time needed for the whole process was about 8-9 hrs which much less than that was indicated in the method of Gamzazade. It was because the step in preparation of solvent including chitosan was reduced from about 15 hrs to 4 hrs since the chitosan was absolutely dissolved in aqueous acetic within 3 hrs. In addition, there was no need to incubate the chitosan in DMF for 12 hr as was indicated. However, the other change was to extend the sulfation reaction time from 1 hr to 5 hrs since the longer of the reaction time, the more degree of substitution success (Akashi, *et al.*, 1996). In addition to this reason, it was reported that increased sulfation could improve the anticoagulant activity of the glycosaminoglycans (Ofosu, *et al.*, 1987). The reaction was performed at room temperature to prevent degradation of the polymer

chain (Yalpani, *et al.*, 1992) although there was a report that increasing the reaction temperature to 80 °C could increase the sulfur content to 15.81% (Nagasawa, *et al.*, 1972). The objective of this study was to randomly substitute the sulfate molecules into the whole polymeric molecules. The technique should be feasible and easily perfomed in the laboratory. Therefore, the reaction was done without any requirement of the temperature-controlled device. However, by this mild condition, the obtained CPS yielded 15.64% of sulfur content and was demonstrated to have the anticoagulant activities as was expected while the products obtained by Gamzazade have not been investigated for its biological activities.

4.2 Biological assay

4.2.1 Anticoagulant activity

Since the propose of this study was to synthesize the chitosan polysulfate that has the anticoagulant activity. Therefore, the second main objective of the present study is to test the blood anticoagulant activities of the synthetic CPS with various M_v. To address this, the product was investigated for the inhibition effect on blood coagulation. The principle of Heptest® is to monitor heparin or heparin-like compound in the plasma in the presence of factor Xa and tissue factor which derived from rabbit brain. Heparin itself can exhibit little anticoagulant activity. It requires a plasma cofactor, antithrombin III (ATIII), for its anticoagulant activity. Specifically, heparin is a catalyst that accelerates the neutralization of activated clotting factors such as factor Xa and thrombin. The inhibition of factor Xa by ATIII is greatly accelerated by trace amount of heparin (Yin and Wessler,

1970). By this mechanism, under optimal conditions the amount of factor Xa activity neutralized during a predetermined time period is directly proportional to the concentration of heparin or heparin-like compounds in the reaction mixture. Various concentrations of chitosan, and CPS were assayed in comparison to pentosan polysulfate (PPS), the synthetic sulfated polysaccharide which was reported to have high anticoagulant activity. Various concentrations of the standard therapeutic heparin were used to calibrate for the heparin standard curve. The ability in the inhibition of coagulation was reported in equivalent to heparin. The results showed that chitosan has no inhibition activity while our products provided the significant anticoagulant activity as compared to heparin but less than PPS. The activity of each fraction could be graded from high to low as followed: pre-Sepharose CL-6B preparation, P2, P1 and P3, respectively. From this study, the ability of CPS in inhibition of factor Xa was shown to be related to the appropriate M_v since M_v of pre-Sepharose CL-6B preparation and P2 was closed to each other (3.2 and $3.5x10^4$ dalton, respectively). The result obtained was as mentioned by Hirano and his coworker (1985). However, the other factors such as degree of substitution and sequence of sulfate patterns was of interest regarding to the high activity of PPS although it has a small M_v of only $4.5x10^3$ dalton.

The principal inhibitor of thrombin, factor Xa and other coagulation serine proteases in plasma was ATIII. The rate of inhibition under normal conditions was slow, but could be increased several thousand-fold by heparin (Hirst, 1991). This mechanism accounts for the anticoagulant effect of heparin. Low molecular weight therapeutic heparin preparations appear to catalyze the reaction between factor Xa and ATIII more readily than the reaction between thrombin and ATIII (Teien, *et al.*, 1976). Thus the factor Xa inhibition test was the

most useful test for assaying the widest variety of therapeutic heparin or heparin-like compound preparations. The mechanism of action was further investigated by the Accucolor™ Heparin. In this method, when both factor Xa and ATIII were present in excess, the rate of factor Xa inhibition was directly proportional to the heparin concentration. Thus the residual factor Xa activity, measured with a factor Xa-specific chromogenic substrate, was inversely proportional to the heparin concentration (Teien and Lie, 1977). The results were reported in equivalent to heparin as well. The ATIII calibration was in a quadratic curve not linear as was indicated by the manufacturer. The reason might be due to the small size of sample tested. However, the R^2 was 0.97 which could be statistical accepted. It was shown that our products significantly decreased the activity of factor Xa. The inhibition effect could be resulted from its reaction and complex formation with ATIII and subsequently inhibit factor Xa activity.. Therefore, the mechanism of action could be reasonably proposed to mimic the heparin. The activity of pre-Sepharose CL-6B preparation is closed to P2, however less than P1. This was controversy to the first experiment. In addition, P3 showed no detectable activity by this experiment while PPS has high ability to form complex with ATIII and inhibit factor Xa activity. From these experiments, it was demonstrated that the ability of CPS to form complex with ATIII depended on its M_v since the higher M_v could promote the higher binding to ATIII while P3 showed no detectable activity. However, not only M_v that had the influence in the ability, configuration according to pattern and sequence of sulfation might also influenced since PPS which M_v of 4500 dalton could bind to ATIII and subsequently inhibit factor Xa by this experiment and its ability was much higher than that of CPS. P3 which could inhibit coagulation but had no detectable antithrombin activity might have its

own specific configuration that did not fit to the ATIII molecules. The inhibition effect of P3 on anticoagulation, therefore due to another mechanism that differ from the other fractions or PPS. The results from this experiment indicated that only some fractions of CPS with appropriate M_v and sulfation pattern could inhibit factor Xa activity *via* the complex formation with ATIII and subsequently, inhibit the coagulation pathway.

The CPS was also investigated whether it could directly inhibit factor Xa. The assay performed in the absence of ATIII. It was found that residual factor Xa concentration was not decreased. The result revealed that CPS itself did not directly inhibit factor Xa.

ATIII is the major inhibitor of plasma thrombin and factor Xa as described. The inhibitory activity of ATIII towards thrombin is greatly increased (2-3 orders of magnitude) in the presence of heparin (Hirst, 1991). To address the antithrombin activity of ATIII in the presence of CPS, the antithrombin activity assay was performed. In the two-stage method, thrombin was added to a plasma dilution containing AT in the presence of heparin or CPS. After an initial incubation period (stage 1) residual thrombin was determined with the thrombin-specific chromogenic substrate (stage 2). The residual thrombin activity is inversely proportional to the antithrombin concentration. The results showed that all fractions of CPS, PPS and heparin (0.1 IU/ml) provided the normal antithrombin activity. It could be indicated that when CPS formed complex with ATIII, the main effect is on only factor Xa not thrombin. By this experiment, heparin showed the normal antithrombin activity although heparin itself when form complex with ATIII, could promote the antithrombin activity of ATIII (Hirst, 1991). In this study, CPS obtained was not analyzed for its sequence, however, its structure could be predicted to have the specific sequence resemble to that of PPS and the specific pentasaccharide sequence in heparin. Heparin is

not a homogeneous substance; it consists of a family of glycosaminglycans of various molecular weight ranging from 4-30 kDa (M_v of 12-15 kDa)(Hirst, 1991). There were reports indicated that a specific pentasaccharide (Figure 6) is the structural element that is crucial for the high-affinity binding of heparin to ATIII and thus for heparin's anticoagulant activity (Rosenberg and Lam, 1979; Lindahl, *et al.*, 1979; Choay, *et al.*, 1983). Only about one-third of the molecules in a solution of pharmaceutical-grade heparin contain this pentasaccharide, and these account for at least 80% of heparin's thrombin-neutralizing biological activity (Lam, *et al.*,1976; Andersson, *et al.*, 1976). The balance of specific pentasaccharide in the backbone has very low affinity for antithrombin and contribute little to heparin's ability to inhibit the action of thrombin or other activated clotting factors. Although such "inert" members of a population of heparin molecules may contribute to the hemorrhagic side effects of heparin by interfering with platelet function (Salzman, *et al.*, 1980).

CPS was also evaluated for its direct inhibition effect on thrombin activity by thrombin clotting time assay (TT). The TT is an important screening procedure for disorders of thrombosis and hemostasis as well as the presence of heparin or heparin-like compounds (Coleman, *et al.*, 1987). TT testing is a rapid assay procedure that measures the polymerization of fibrinogen to fibrin. Any interference with this conversion will be reflected in a prolongation in the clotting time of the test procedure. The system provides thrombin for fibrinogen hydrolysis. Abnormal TT occurs in cases of low or deficiency of fibrinogen. It is also prolonged in the presence of inhibitors such as heparin, myeloma proteins and fibrin/fibrinogen degradation product (FDPs) which block either thrombin cleavage of fibrinopeptides or fibrin monomer polymerization (Miale, 1977; Williams, *et al.*,

1977; Wintrobe, *et al.*, 1981). In order to investigate for the direct inhibition effect of CPS on thrombin, the TT was assayed with normal human plasma containing various concentrations of tested materials. The results showed that all fractions prolonged the TT clotting time with dose dependent when compared to the effect of heparin and PPS. The ability of pre-Sepharose CL-6B was over P1 and P2, respectively. It could be indicated that the anticoagulant activity of the 3 fractions including pre-Sepharose CL-6B, P1 and P2 based on 2 mechanisms, firstly, via ATIII complex formation thereafter inhibit factor Xa, and secondly directly inhibition of thrombin activity. P3, whereas provided a mechanism of anticoagulant activity only by directly inhibited thrombin activity.

As TT is also prolonged in the presence of fibrin/fibrinogen degradation products (FDPs) as well as heparin or myeloma proteins. To observe that CPS may have a negative feed back effect on fibrin polymerization, the atroxin time assay was performed. Atroxin time or plasma clotting time is an important screening procedure as well as TT for disorders of thrombosis and hemostasis. They are rapid tests that measure the polymerization of fibrinogen to fibrin. Atroxin is a purified enzyme fro *Bothrops atrox* venom which is based on the preclinical studies, would appear useful for evaluating disturbances associated with fibrin formation such as arterial and venous thrombosis, disseminated intravascular coagulation (DIC) and congenital dysfibrinogenemia (Anaclerio, *et al.*, 1980; Markwardt and Nowak, 1980; Yoshikawa, *et al.*, 1983). Atroxin time is not sensitive to the presence of plasma heparin or heparin-like compounds (Bonilla, 1975). The results showed that none of tested materials prolonged the atroxin time. It was indicated that CPS as well as PPS or heparin did not interfere with the fibrin polymerization.

To demonstrate whether CPS provided the inhibition effect on the other coagulation factors especially in the extrinsic pathway, prothrombin time (PT) was assayed. PT is a widely accepted as the means to monitor patients on oral anticoagulant therapy (Errichetti, *et al.*, 1984; Hirst, *et al.*, 1992)) and for the assay of the specific coagulation factors in the extrinsic and common pathways of coagulation. The test was performed in parallel to heparin and PPS. The PT was reported in term of INR (international normalized ratio). All tested materials showed the normal range of PT. This could be indicated that CPS as well as heparin and PPS had no inhibition effect on other coagulation factors in the extrinsic and common pathway including factor II, VII, IX, X, protein C and protein S.

Taken together, it could be indicated that CPS with DS of 2.23 which synthesized in the mild conditions provided significant different anticoagulant activity based on its M_v and configuration according to its sulfation patterns. The pre-Sepharose CL-6B, P1 and P2 with M_v of $3.2x10^4$, $6.8x10^4$ and $3.5x10^4$ dalton, respectively, have two different mechanisms in the inhibition of anticoagulation; inhibited factor Xa via ATIII complex formation and directly inhibited thrombin activity. A fraction with M_v of $2.0x10^4$ dalton (P3) was demonstrated to inhibit only the thrombin activity. The activity of CPS was much less than PPS. CPS was proved to have no effect on fibrin polymerization or inhibition of other coagulation factors in extrinsic and common pathway and did not directly inhibit factor Xa as well. The mechanism of anticoagulant activity was similar to heparin. The results showed that the smallest size of CPS was much larger than that of PPS but it still provided the anticoagulant activity. This revealed that difference in mechanism of action of CPS was influenced by both size and sequence of sulfate molecules which referred to its difference in configuration.

4.2.2 Effect on the immune response *in vitro*

To synthesize the heparin-like compound and intend to use as a biomedical material, it is necessary to study whether it has an adverse effects especially on the immune response. Study of the immune response consists of 1) humoral mediated immune response (HMI) which can be assayed by the detection of the mitogen-induced immunoglobulin production, 2) cell mediated immune response (CMI) which can be assayed by the detection of both antigen and mitogen-induced cell proliferation, 3) cell mediated cytotoxicity which can demonstrate the activity of CTLs, and 4) the analysis of cytokine production. Various *in vitro* assays were performed in order to evaluate for the involvement of CPS in the human immune response.

4.2.2.1 Effect on the antigen and mitogen stimulated cell proliferation

To investigate the effect of CPS on cell proliferation both stimulated by mitogen and antigen, the optimal conditions of the system and sub-optimal concentration of mitogen and antigen were studied. The optimal conditions were first studied by varying the time of BrdU labeling from 2-21 hrs. The BrdU labeling time was 2-4 hrs as manufacturer's recommendation. However, it was shown that from PHA induced cell proliferation, the absorbance (O.D.) at 405/630 nm which indicated the amount of BrdU incorporation was very low and could not be used as the reference condition for further investigation. We decided to extend the BrdU labeling time to 8 and 21 hrs reasoning on our facility provided in laboratory works. The results showed that the higher O.D. was obtained. In the ELISA detection step, the instruction also recommended to incubate the HRP-anti-BrdU for 1 hr at room temperature. However, by the recommended conditions, the O.D. obtained was very low (data not shown). We, therefore, extended the incubation

time for HRP-anti-BrdU reaction to 2 hrs at 37°C which was normally used in ELISA technique. By these conditions, the resulting in O.D. of each concentrations of antigen and mitogen could be interpreted. The sub-optimal concentration of PHA optimized from 3 healthy donors in this study was 0.5 μg/ml. This concentration provided an O.D. which was quite distinguished from the O.D. of both concentrations at 0.31 and 0.62 μg/ml. Therefore, the concentration of PHA at 0.5 μg/ml was selected to investigate since we could differentiate the inhibitory effect from cell proliferation. The study of the sub-optimal dose of PPD was different from that of PHA. All three donors showed different responsive patterns and there was no clear difference in the O.D. when each concentration of PPD was used to optimize. The results showed that the concentrations of PPD at 0.31 and 0.62 μg/ml were the most appropriate. However, as the O.D. at the two positions were not significant distinct and there was no data at 0.31 μg/ml from one donor, both concentrations were selected for the investigation of the effect of CPS on antigen stimulated proliferation. The reason was that the effect on the proliferation at both positions should be parallel to each other.

Cultivation of PBMCs ($5x10^4$ cells/well) in the presence of PPD (0.31 or 0.62 μg/ml) and various concentrations of CPS in comparison to PPS and heparin, showed that all of tested materials have the significant effect on the inhibition of cell proliferation ($P<0.01$). This effect was in dose dependent manner. More interesting, the dose dependent effect was more distinct in product of smaller M_v such as P3, PPS and heparin than those of P1 and P2. This suggested that the interference in PPD stimulated cell proliferation might be influenced by the size and/or sulfation patterns in the polymer chains. Previously, there was a report that size and sulfating sequence of polysaccharide sulfates were the factors

that influenced their biological activities especially in the cell interaction (Roden, 1989). To investigate that the inhibition in cell proliferation did not cause by the toxicity of products, the viability test was also performed by trypan blue dye exclusion assay. Viability of cells cultured in different concentrations of CPS and PPS (0-8 μg/mL) or heparin (0-0.8 IU/mL) were up to 98% until day 5 of cultivation (data not shown).

It was shown that, in contrast to the result of PPD stimulation, stimulatory effect of P1-P3 and PPS on PHA stimulated cell proliferation was not at the significant level ($p > 0.05$). Only heparin inhibited PHA stimulated cell proliferation in a dose dependent manner and this effect was not from the product toxicity to the cells as well ($p<0.01$).

Since there was no report in the study of polysaccharide sulfate and the PBMCs proliferation, we herein proposed the mechanism of inhibition of CPS, PPS and heparin on the PBMCs proliferation. With their polyanionic nature, CPS and PPS provided highly negative ionic charges that could prevent cell-cell interaction. More specifically, it may interfere with the antigen processing and presentation process of antigen presenting cells which expressed the processed PPD via CD1 molecules. Secondly, it might interfere with the processing of PPD antigen on the membrane of presenting cells such as monocytes since chitosan itself can bind protein (Yalpani, 1992). There was a report that CD14 is a key mediator in LPS-induced cytokine production and activation of endothelial cells (von Esmuth, *et al.*, 1993). Moreover, LPS and neutral-soluble chitosan shared a binding site on monocytes that involves CD14 (Otterlei, *et al.*, 1994). According to our observations, processing of PPD by the antigen presenting cells such as monocytes might be interfered by the cross-linking of sulfated polysaccharides (CPS, PPS and heparin) and receptors such as CD14, mannose receptor, or CR3 and/or CPS-receptor complex formation on the

cell membrane which caused stearic hindrance. Thirdly, some of synthetic CPS with smaller sizes might get into cells. Phagocytosis of particulates, probably by a postmembrane event such as interiorization, appears to be important to disrupt the antigen processing within antigen presenting cells. This was revealed in our experiment that the smaller M_v such as P3 (20 kDa), PPS (4,500 Da) and heparin (4-30 kDa) inhibited cell proliferation in dose dependent manner. The results were much more distinguished than those of P1 and P2. This was unclear but there was a report that chitosan particles, the bioadhesive polymer which can bind to protein, could adhere onto the gastrointestinal cell membrane and induced endocytosis into such cells (Roy, *et al.*, 1999). Lastly, the inhibition might due to the inactivation of secretory cytokine involved in the proliferation by tested materials. However, the data from PHA stimulated cell proliferation showed that only heparin, hat had an inhibitory effect. This was elucidated that the proposed mechanism of binding and inactivation of IL-2 secreted from activated T lymphocytes could not be accepted. Two explanations for the mechanism of the inhibition effect of CPS, PPS and heparin on PPD stimulated cell proliferation might be consisted of the following; 1) the interfering in the interaction of antigen presenting cells and T lymphocytes resulted from their highly negative charges was the most interesting subject and 2) interfering in the antigen processing and/or uptaking resulted from cross-linking to various receptors and/or their specific receptors on the cell membrane. It should be required, therefore, to clarify the exact mechanism of inhibitory effect of CPS on PPD stimulated cell proliferation.

In contrast, CPS did not inhibit the PHA stimulated cell proliferation. PHA has separate pathway of activation. It was plant lectin that could bind to oligosaccharide motif on glycoprotein existing on the T cell receptor molecules by type and configuration specific.

This mimic the activation process of T cells and resulted in T cell proliferation (Coligan, *et al.*, 1997). PHA stimulated cell proliferation was not affected from the inhibition of PHA by CPS as well since CPS has no specific configuration which served for PHA binding. Taken together, the results of this study showed that CPS synthesized from the shell of marine crabs with anticoagulant activity has an inhibitory effect only on recall antigen not mitogen stimulated cell proliferation. This effect supported its involvement in the human immune response whenever it would be applied as an anticoagulant biomedical material. The proposed hypothesis raised up to explain its mechanism was shown in Figure 53.

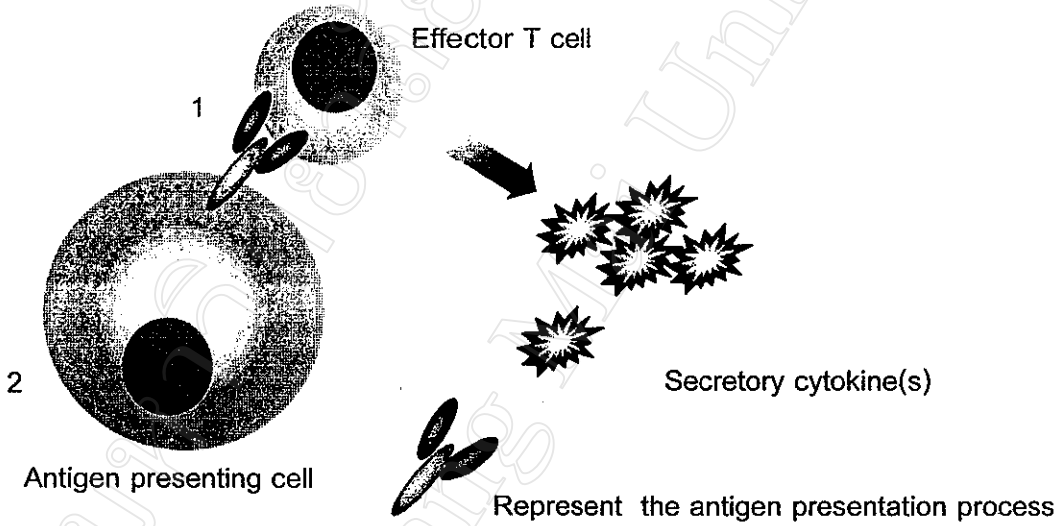


Figure 53 Proposed mechanism of action of CPS in the inhibition of PPD stimulated cell proliferation. 1; highly negative charges of CPS prevent cell-cell interaction and/or antigen presentation, 2; cross-linking of various receptors and specific receptor of CPS interfere with the processing of antigen bound onto membrane.

4.2.2.2 Effect on the cell mediated cytotoxicity

The activity of cytotoxic T lymphocytes (CTLs) function was investigated in order to evaluate the effect of CPS on non-specific killing activity of CTLs. Moreover, its cytotoxic potential was also studied whether it would be used as an anticoagulant material in human body without any defective effect. To optimize for the optimal E:T ratio for an assay of the cell mediated cytotoxicity, PBMCs from five healthy donors were studied. The results showed variations in individual response. Some donors have high activity in cell mediated cytotoxicity while some showed low activity. By these results, E:T ratio of 50:1 and 100:1 were used for further evaluation to clarify the effect of tested materials at both E:T ratio in parallel. In the absence of tested materials, degree of cytotoxicity (%) in the condition of E:T ratio 100:1 was higher than that in the condition of E:T ratio 50:1. However, there was no change in the cytotoxicity (%) in the presence of tested materials. It was demonstrated that various concentrations of all tested materials showed no significant effect on the cell mediated cytotoxicity ($p>0.05$, Pearson correlation test). Moreover, tested materials itself has no cytotoxic potential as well. The results were as was reported by Mori and his coworker (Mori, *et al.*, 1997). However, this was different from our results since his work was done on the fibroblast cell lines and human endothelial cell lines not human PBMCs. There was no report on the effect of polyanionic compounds employing human CTLs assay. This could be the first report which demonstrated that sulfate derivatives of chitosan in comparison to PPS and heparin has no cytotoxic effect on the human PBMCs, thus it could be applied as an anticoagulant material without interfering with the CTLs function.

4.2.2.3 Effect on the immunoglobulin production

Chitosan was reported to increase IgM production in human-human hybridoma cell lines and PBMCs (Maeda, *et al.*, 1992). It induced only IgM and has no effect on other subtype. There was no report of CPS and its effect on the immunoglobulin production on PBMCs. To address this effect, an assay was performed using PWM as a stimulant. The effect of tested materials on the immunoglobulin production of PBMCs in the absence of PWM was also performed in parallel. Each donor demonstrated the individual characteristics in the immunoglobulin production which depended on individual basic status. The results demonstrated that PBMCs from different donors had distinct ability to produce immunoglobulin both in terms of level and class. In the absence of PWM, tested materials did not affect the immunoglobulin production even at the high concentrations of 8 μg/ml of CPS and PPS or at the concentration of 0.8 IU/ml of heparin. The inhibitory effects on immunoglobulin production stimulated by sub-optimal dose of PWM were observed in all tested CPS (P1-P3). In addition, there was no correlation between size and bioactivity. More interesting, the CPS could significantly inhibited IgM production compared to other immunoglobulin classes. In contrast, PPS showed the most effective inhibitory effect among all tested materials while heparin demonstrated the specific different inhibitory patterns. The inhibitory effect of heparin was not as strong as CPS and PPS and was not in dose dependent manner. PWM is a plant mitogen that has a specific binding property to N-acetylglucosamine and it can stimulate both T and B cell. Stimulation of B cell is independent of T cell help (Coligan *et al.*, 1994). First proposed mechanism of inhibitory effect was that it could be blocked by binding to its specific sites on tested materials. This effect, therefore, reduced the concentration of PWM in the culture.

However, this proposed mechanism was not conclusion. The reason were that, firstly, the degree of acetylation in CPS is only 0.12 and moreover it was demonstrated that degree of sulfation was 2.23 which revealed that almost all of N-acetylglucosamine units in the backbone were substituted. Therefore, the binding sites of PWM in the CPS is very rigid. Secondly, the sub-optimal concentration of PWM was used in the assay. The sub-optimal concentration was optimized in order to differentiate the effect of tested materials on the assay. Therefore, the inhibitory effect could not be caused by the low concentration of mitogen used. Thirdly, PPS which is the synthetic polysaccharided sulfate has its own natural backbone of xylan (Soria, *et al.*, 1980) and which has no specific binding sites for PWM could strongly inhibit the effect of PWM. The second proposed mechanism was that the binding of PWM and its specific oligosaccharides on the membrane glycoprotein of T lymphocytes may be interfered with highly anionic charge of tested materials. In addition, the interaction between polysaccharide sulfate and may be its specific receptor on the cell membrane (Otterei, *et al.*, 1994). The mechanism of CPS and PPS as well as heparin in inhibiting the PWM stimulated immunoglobulin production was not quite clear. In addition, each tested material is different in backbone and configuration patterns, therefore, the configuration concerning to its sulfate pattern, degree of sulfation and together with the structure of the backbone should be discussed.

4.2.2.4 Effect on cytokine production

Chitin and its derivatives have been used in a variety of biomedical applications. Chitin and chitosan apparently permit regeneration of normal tissue elements in skin wounds (Biagini, *et al.*, 1991; Chandy and Sharma, 1990, Okamoto, *et al.*, 1993).

Chitin, chitosan and its sulfate derivatives were demonstrated to induce the production of cytokine involved in the angiogenesis and wound healing process. It was demonstrated that chitosan induced IL-8 production of the L929, fibroblast cell line. In the *in vivo* assay, it also induced angiogenesis and migration of neutrophils (Mori, *et al.*, 1997). Sulfated 70% deacetylated chitosan (S-DAC70) was demonstrated to affect the function by inducing the production of IL-1β, IL-6, IL-8 and TNF-α but not proliferation of human endothelial cell lines (HUVECs). However, there was no report of CPS in the production of IL-2 and IFN-γ in human PBMCs. It was demonstrated that chitin and chitosan could bind to Toll-like receptor (TLR) which acted the same as PRR, leads to the cytokine production and subsequently cell proliferation. In our assay, PBMCs which included monocyte were used. The monocytes also expressed TLR which could bind CPS and resulted in stimulation of TNF-α production and subsequently IL-2 production. In order to evaluate the effect of CPS on the cytokine production, IL-2 and IFN-γ were of interest in this study. IL-2 is a T cell-derived cytokine which was first described as a T cell growth factor (TCGF) (Callard and Gearing, 1994). It is now known to stimulate growth and differentiation of T cells, B cells, NK cells, LAK cells, monocytes, macrophages and oligodendrocytes (Smith, 1984; Smith, 1988; Kuziel and Greene, 1991)). On the other hand, IFN-γ is pleiotropic involved in the regulation of nearly all phases of the immune and inflammatory responses, including the activation, growth and differentiation of T cells, B cells, macrophages, NK cells and other cell types such as endothelial cells and fibroblasts (Callard and Gearing, 1994). Investigation of the involvement of CPS in comparison to PPS and heparin in both cytokine production showed that they did not induce the IL-2 and IFN-γ production. The inhibition of mitogen stimulated cytokine production was not performed since the objective of the study

aims to demonstrate whether the CPS which has a strong anticoagulant activity may have the stimulatory effect on the cytokine production. The study in the molecular level was not assay since the demonstration in the cellular level such as cellular secretion or functional assay is superior indicator for the cell activation.

From all assays on the involvement in the human immune response *in vitro*, the results showed that CPS with the strong anticoagulant activity significantly inhibited PPD stimulated cell proliferation in a dose dependent manner. In contrast, no effect was observed when cells were stimulated with PHA. CPS did not affect the immunoglobulin production but it significantly inhibited PWM stimulated immunoglobulin production. Additionally, it has no effect on IL-2 and IFN-γ production as well as cell mediated cytotoxicity.

It is important to note that CPS could be synthesized in a mild conditions with some adaptations in the process. The adapted process reduced the time consumed and could be performed easily in the general laboratory. The product obtained was demonstrated to have a high anticoagulant activity and could inhibit antigen induced cell proliferation but together with mitogen-induced immunoglobulin production. These effects on human immune response *in vitro* has not yet been reported.