HOKKAIDO UNIVERSITY

Title	GENERATION OF CARBOXIMETHYL-CHITIN-METAL ION COMPLEX AND ITS PROPERTY
Author(s)	Uraki, Yasumitsu
Degree Grantor	北海道大学
Degree Name	博士(理学)
Dissertation Number	乙第3640号
Issue Date	1989-12-25
Doc URL	https://hdl.handle.net/2115/32568
Type	doctoral thesis
File Information	3640.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

GENERATION OF CARBOXYMETHYL-CHITIN - METAL ION COMPLEX AND ITS PROPERTY

YASUMITSU URAKI

A DOCTRAL DISSERTATION

FACULTY OF SCIENCE,
HOKKAIDO UNIVERSITY,
SAPPORO, JAPAN

CONTENT

	Page
ABSTRACT	
INTRODUCTION	1
I. Preparation of various CM-chitins and its identification	19
EXPERIMENTAL	20
RESULTS AND DISCUSSION	25
CONCLUSION	42
II. Adsorption of metal ions to CM-chitin and surface property of the complex.	43
EXPERIMENTAL	44
RESULTS AND DISCUSSION	50
CONCLUSION	103
III. Preparation of peptide and its adsorption to CM-chitin	104
EXPERIMENTAL	105
RESULTS AND DISCUSSION	108
CONCLUSION	115
CONCLUDING REMARKS	116

ACKNOWLEDGEMENT 119

REFERENCES 120

ABSTRACT

Variously substituted 6-O-carboxymethyl-chitins(O-CM-chitin) and 3,6-O-CM-chitin were prepared from α- and β-chitins, and their characterization were performed with ^{13}C-NMR, FT-IR and others. At first, metal ion-binding properties of these materials were investigated exhaustively. Either 6-O-CM-chitins, prepared from α- or β-chitin adsorbed calcium ion specifically and tightly among various divalent metal ion. The adsorption was found to be contributed not only by the electrostatic interaction of carboxyl group but also by acetamide group, as well as by the primary and secondary hydroxyl groups, (suggesting the existence of specific adsorption site, constructed with multiple way of binding). The specific adsorption of 6-O-CM-chitin toward calcium ion disappeared by the stretching process in the spinning of the CM-chitin fiber owing to the appreciable enhancement of the adsorption capacities for other metal ions. This phenomena suggested the importance of geometrical arrangement of those functional groups for the conformation of the metal-binding sites.

Nevertheless 6-O-CM-chitin from β-chitin forms gel by the addition of transition metals owing to the wide distributed carboxyl group, the specific adsorption for calcium ion still remained, so suggested the geometrical arrangement of binding site could not be destroyed. On the other hand, Non-specific binding for calcium ion was found in 3,6-O-CM-chitin ,neither from α- or β-, even though it formed gel by

addition of some metal ions owing to high negative charge density.

The second, the adsorption of amino acids to the Ca^{2+}-CM-chitin complex was examined in order to identify the configuration aspects of specific binding sites of calcium ion. Adsorption of amino acids to the complex was found to be proportional to the hydrophobicity of amino acid. Enormously high quantities of phenylalanine was adsorbed and their chiral specific property was observed. The peptide containing Phe was also found to be adsorbed to Ca-6-O-CM-chitin from α-chitin complex tightly and the adsorbed peptide in the complex could not be hydrolyzed by peptidase. It was noted that the peptide fragment splitted out from complex when it was treated sequentially by lysozyme and peptidase remaining only the Phe residue in the complex.

These results suggested the existence of specific adsorption site for aromatic ring of Phe and in calcium specific adsorption site, that is to say, the side chain of phenyl group is incorporated to generate tight complex constructed with CM-chitin, Ca^{2+} ion and Phe residue.

Since 6-O-CM-chitin was found to protect the peptide from peptidase and stabilize it in animal body, 6-O-CM-chitin was suggested to be a useful material as a polymeric drug, especially, as a drug carrier of sustained release system.

The present study will also proposed a model to explain the mechanism of the interaction of CM-chitin with blood protein.

INTRODUCTION

Chitin is a polysaccharide consisting of N-acetyl-glucosamine(2-acetamido-2-deoxy-D-glucopyranose) residueslinked by the β-1,4-glycoside bonds. The deacetylated derivative of chitin is called as "chitosan". Chitin is known to exist in the large number of living organism, including bacteria, plants and animals. In bacteria and plants, chitin exists in a large amount in the cell wall of bacterium, mainly in mold, yeast and mushroom, and is also found in some algae groups. Polysaccharide as components of cell wall is not chitin but peptideglycan in the more primitive living organisms, while is cellulose in higher order plants. In the attaching bacteria, on the other hand, the cell wall contains chitosan also in addition to chitin(1).

In animal, chitin distributes in the various animals from *Protozoa* to *Pogonophora*. Ability to synthesize chitin, which was established in the beginning of the evolution, has been kept in a number of *Diproblastica* and most *Triproblastica*, but the ability disappeared on the process of evolution to *Deuteromia* except a part of living organisms(2,3). Namely chitin exists in *Crustacea* such as crab or shrimp, in *Arthropoda* such as spider or tick, and in *Nematoda*. However, it does not exist in the animals of higher order. These seems to be seriously concerned with physiological "ecdysis". As chitin has very rigid poly-saccharide chain as described below, it is used as the

protection cloth in *Arthropoda*, and also used as the supporting material for stigma, sensitive organ, vessel of secret glandura and both end of digestive tube in addition to the cuticle. The cuticle is consisted of, in addition to chitin, proteins, lipids, inorganic salts and catechols to protect effectively from severe natural environment. Thus, the cuticle prevents the evapolation of water from surface and invasion of foreign enemies(4,5). *Arthropoda* occupies 80% of total animal species and has been prospered for a long period by this way. Since the rigid and less expensive cuticle dose not match well for the growth of animals, these animals need the ecdysis. In the case of the higher order animals, chitin seems to be converted to proteins such as collagen to obtain the ability of more rapid growth.

Considering the evolution of animal cuticle from the aspects of chemical structure, peptideglycan in the very primitive bacteria seems to be converted to cellulose simply to form the plant cuticle. On the other hand, chitin which has a skeleton such as cellulose and amide bonds similar to protein is an intermediate material on the process of evolution to proteins in higher animals.

Chitin belongs to so-called "mucopolysaccharides(proteo-glycan)" together with other mucopolysaccharides such as heparin, heparitin sulfuric acid, chondroitin 4-sulfuric acid, chondroitin 6-sulfuric acid, hyauronic acid and etc.. These polysaccharides except heparin have repeating unit of mono- or di- saccharide. Cellulose and mannan having the repeating unit of monosaccharide have supporting and

storaging functions, while most of acidic mucopolysaccharides have physiological activity to keep the function of living body. For example, hyauronic acid contributes as lubricants in the corpus vitreum liquid and joint parts. It binds to protein and protects tissues from bacterial attack. According to the recent report, it plays important roles in the cell construction such as angiogenesis (6). Heparin is known to bind to fibronectine, which has a function to regulate a cell attachment by its conformational change(51). It inhibits of serine protease activity working with antithrombin III to prevent the blood coagulation(7). Though chitin is useless for animals, animals have enzymes to hydrolyse chitin and chitosan as one of the functions of body protection since chitin is a component of bacterial cell wall as described above. So most of the invading bacteria die in the animal body. When chitin invades into animal body, it is digested slowly by enzymes in the body and nothing else occurs. This digestive property in animal body is very important for the utilization of chitin.

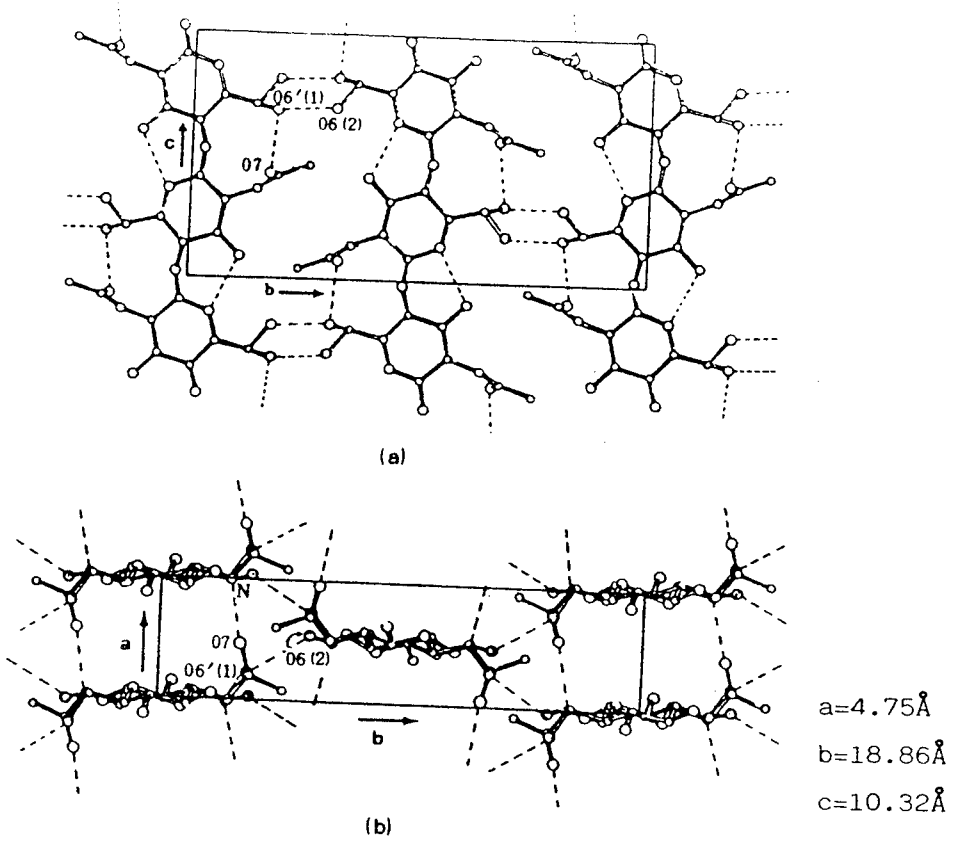
10^{9}-10^{11}t of chitin is produced biologically per year(8), similarly to 10^{11}t of cellulose(9). It has been the cause of environmental pollutions owing to its abandon with attached proteins. Use of chitosan as a coagulant is only one example of its large scale utilization. Thus, the functionalization of chitin has become to the attractive project, also from the standpoint of this environmental cleaning up.

Studies on the functionalization of chitin have been

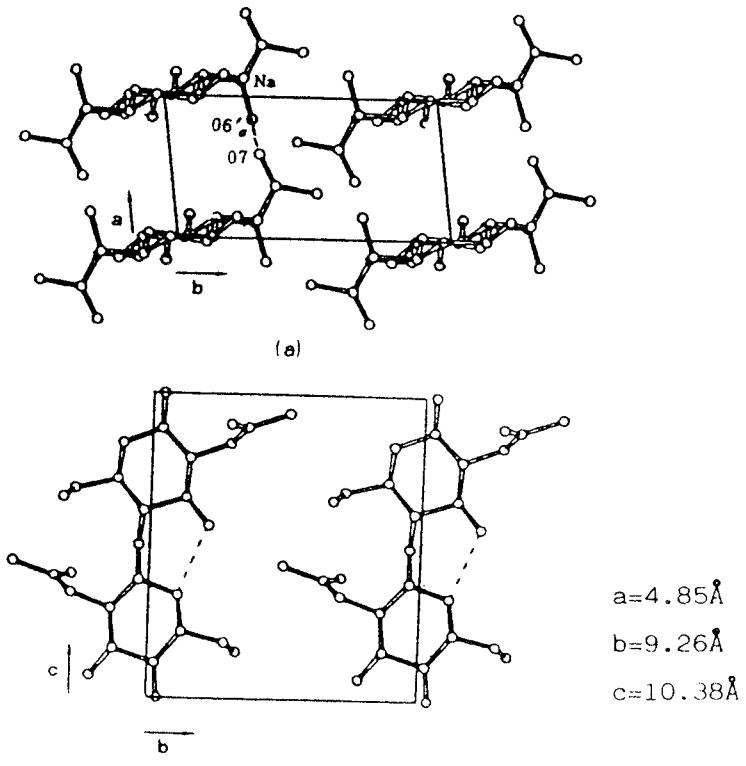
developing on the basis of the ideas that chitin can be converted to the material having physiological activity by chemical modifications, such as sulfonation or carboxylation, since chemical structure of N-acetylglucosamine in the chitin molecule is similar to that of acidic mucopolysaccharides(10-12). Hydroxyl groups at C-1 and C-4 position in N-acetylglucosamine residue of chitin is not reactive because they are used for glycoside linkage. Also C-2 position is not reactive since the amino group of C-2 position is protected by acetyl group. The secondary hydroxyl group at C-3 position and primary hydroxyl group at C-6 position are the only functional group to be regulated on the chemical modification. Thus chitin is regarded as a valuable starting material for the chemical modification.

Three kinds of crystalline structure are known for chitin. Chitin of the largest existence in nature (e.g. crab or shrimp shell) is termed as α-chitin. It has anti-parallel orientation of molecular chains. Chitin in Loligo Pen is termed as β-chitin which has parallel orientation. The mixture of these two forms is in stomach wall of squid, and is called as γ-chitin(19). α-Chitin forms rigid crystalline structure and β-chitin forms soft one, its difference was reported by X-ray crystallography to come from the difference of the number of hydrogen bonding in a unit crystal lattice. Figure of crystalline structure and the unit crystal lattice constant are shown in Fig.1(20,21).

α-Chitin is rhombic system of which space group is $P2_1 2_1 2_1$. An unit lattice contains two N-acetylglucosamine



Crystalline structure of α-chitin ; (a) bc-side (b) ab-side



Crystalline structure of β-chitin without water ; (a) ab-side (b) bc-side

Fig.1 Crystalline structure of chitins from X-ray diffraction

units in two species of chains on each direction of anti-parallel, and the chitin conformation is 2_1 helix structure(20). On the other hand, β-chitin is monoclinic system , space group is $P2_1$ and an unit lattice contains two sugar unit in a chain on repeated parallel direction and its conformation is also 2_1 helix. Hydrogen bonds in β-chitin are formed only by hydroxyl group at C-6 with other molecular chain along *a* axis. α-Chitin has more hydrogen bonds than β-chitin, because these bonds are formed by the hydroxyl group with the adjacent chain of opposite direction along *b* axis in addition to that along *a* axis. Accordingly, α-chitin has rigid structure to form the most packing and stable crystalline structure, while β-chitin is so soft that it swells up in water easily. α-Chitin makes rigid shell together with proteins and calcium carbonate, but β-chitin makes soft bone with large amount of proteins and small amount of calcium carbonate. In both chitin forms, the hydrogen bond within intra residue is between the hydroxyl group at C-3 position and the oxygen atom of hemiacetal at O-5. Since secondary hydroxyl group at C-3 position in N-acetylglucosamine is protected by the hydrogen bond, chitin seems to be an ideal starting material for the structure analysis of polysaccharide and site selective chemical modification.

As one examples, chitin was converted to chitin heparinoid which has the anti-blood coagulant property(13-15). The heparin has the chemical structure as shown in Fig.2(16). In the above mentioned investigations, the purpose was to

obtain the heparin-like physiological activity not by constructing the same chemical structure as heparin by the chemical modification but by regulating the position of substitution with acidic functional groups.

The rigid crystalline structure of chitin interferes the reactivity in chemical modification. The glycoside linkage is destroyed in the acidic condition or acetamide group at C-2 position is hydrolyzed to amino group in the alkaline condition. Thus the yield of reaction was low since the mild condition is always needed. Carboxymethyl-chitin(CM-chitin) was employed as a starting material for the preparation of heparinoid, and was derived by the carboxy-methylation method which was improved by Tokura et. al.(17). This reaction was carried out in the heterogeneous system to introduce the carboxymethyl group to C-6 position at N-acetylglucosaime selectively. Deacetylation of acetamide group does not occur even in the high alkaline concentration because the reaction is carried out at low temperature. Detergent was used to increase the degree of substitution(D.S.). Since CM-chitin obtained was a non toxic material in the immunological property except that it activated peritoneal macrophages in a short period to induce a little mitogenic activity(10,52), CM-chitin was suggested to be the most suitable starting material for heparinoid. Heparinoid activities of chitin heparinoids obtained by the sulfonation of CM-chitin under the several condition were exhaustively investigated. The results suggested that N-O(3,6)-sulfations of partially N-deacetylated 6-O-carboxy-

methyl-chitin(70% deacetylation) induced the highest activity. The product had the activity of one-third of heparin and it was non-toxic. Though sulfated chitosan had toxicities, they were found to disappear by the 6-O-carboxymethylation. It suggests that chitin derivatives are converted to be more biocompatible by the 6-O-carboxymethylation. Accordingly, the mechanism of interaction between 6-O-CM-chitin and biomaterials was focused on. Adsorption property between blood proteins and 6-O-CM-chitin was investigated as a fundamental study to evaluate biocompatibility and the relationship to blood coagulant system(18). As adsorption of fibrinogen, a main factor of blood coagulant, to 6-O-CM-chitin was found to be enhanced in the presence of calcium ion, calcium ion was suggested to play the important role in the interaction between 6-O-CM-chitin and biomaterials.

The purpose of the present study is to clarify the mechanism of interaction between 6-O-CM-chitin and proteins in detail. So the first purpose is to clarify the relationship of 6-O-CM-chitin with calcium ion, especially about the adsorption capacity of calcium ion compared to that of other metal ions. As metal ions, alkali-earth metal ions and transition metal ions, were employed, since their ability of chelation was easily selected owing to the variously occupied *d* orbitals. As there is a difference between α-chitin and β-chitin in the crystal rigidity and amount of inorganic salts contained in nature, it is estimated that CM-chitins derived from both chitins have the different ability in the metal ions adsorption. Therefore the ability for the

adsorption of metal ions, mainly calcium ion, to CM-chitins from both of the α- and β-chitin was examined.

The carboxymethylation described above was found to proceed on the primary hydroxyl group at C-6 position predominantly. Re-carboxymethylation under the same condition resulted in the substitution of secondary hydroxyl group at C-3 position in addition to the C-6 position to give the product of DS>1.0. Effect of the degree of substitution and substituted sites were also investigated. CM-chitins used here are 6-O-CM-chitin and 3,6-O-CM-chitin.

Adsorptions of metal ions to water insoluble 6-O-CM-chitin (flaky CM-chitin) from α-chitin were measured by the frontal affinity chromatography(47-49). The capacity of flaky CM-chitin (natural crystalline structure) to calcium ion seems to be higher than that of other metal ions. On the other hand, the specificity of CM-chitin toward calcium ion seems to have disappeared probably owing to the great enhancement of the adsorption capacities for other metal ions by the stretching process in the spinning of the CM-chitin fiber. These results suggested that the specific adsorption of calcium ion is due to the geometrical arrangement of several functional groups. The participations of acetamide group and primary and secondary hydroxyl groups, in addition to the electrostatic interaction by the carboxyl group, were suggested by the differential IR spectrum of CM-chitin-Ca mixture and CM-chitin only. The change in pH to acidic condition was not enough for the complete release of calcium ion from CM-chitin. It needed chelating reagent such as

EDTA, to release calcium ion. On the other hand, the release of other metal ions was easily performed by the pH shift. It would support that the strong adsorption of calcium ion is due to the CM-chitin-Ca complex formation. When aqueous solution of calcium ion was added to magnesium ion-adsorbed CM-chitin, release of magnesium ion from CM-chitin was observed. It is really an ion-exchange reaction. Moreover, CM-chitin was found to show the ability of selective adsorption toward calcium ion in Ca^{2+}-Mg^{2+} mixture. The specificity of CM-chitin from α-chitin toward calcium ion seems to relate to its existence with a large amount of calcium carbonate in nature. Therefore, CM-chitin from β-chitin, which exists with a small amount of calcium carbonate, is expected to show the smaller adsorption capacity to calcium ion. Water soluble CM-chitins,in which freedom on molecular motion increased, were applied to the investigation for the adsorption ability. Effect of the degree of substitution on the adsorption property was also investigated with 3,6-O-CM-chitin.

In low concentration of metal ions, all CM-chitins were found to have the high affinity to calcium ion among several metal ions. Especially, in the adsorption of calcium ion to 6-O-CM-chitin from both of α- and β-chitin, negative cooperativity was not shown in Scatchard plot. But that was shown for the other metal ions. These might suggest that CM-chitin from β-chitin also obtained the high affinity for calcium ion. The reason might be as follows, 6-O-CM-chitin from β-chitin forms the same local chemical and geometrical

structure as that of 6-O-CM-chitin from α-chitin by the carboxymethylation owing to the adsorption mechanism by the all functional groups in N-acetylglucosamine residue. These phenomena would be the functionalization of chitin because the specificity to calcium ion was given to β-chitin by the carboxymethylation. As water-soluble 6-O-CM-chitin was confirmed to maintain the specificity to calcium ion, the adsorption mechanism of calcium ion was investigated by FT-IR spectra in solution compared to that to non-specific Ni ion. The adsorption of calcium ion was suggested to be contributed by carbonyl groups, primary and secondary hydroxyl groups. On the other hand, that of nickel was found to be contributed by only carbonyl groups. Therefore, nonspecific adsorption of other metal ions also seem to be the simple electrostatic interaction by carbonyl groups. 6-O-CM-chitin from β-chitin and all 3,6-O-CM-chitin were found to form gel, followed by precipitation, in the presence of transition metal ions. As interchain cross-linking is necessary for gel formation, transition metal ions seemed to induce the cross-linking between CM-chitin chains. This phenomenon suggests that transition metal ions bind to carboxyl groups in the wide range of polymer chains to bundle several chains. The crystalline structure was found to regulate the function of CM-chitin because loose crystalline structure of β-chitin was maintained in its derivatives owing to the distribution of carboxymethyl group in the wide region of molecules. It clearly showed the difference between the calcium ion binding and the

adsorption of other metal ions. Calcium ion binds to CM-chitin tightly in the local specific area, and other metal ions are supported by only carboxyl groups in the wide region of polymer chains. 6-O-CM-chitin adsorbed Fe^{2+} keeping the soluble state, while adsorbed Fe^{3+} to form gel. The adsorption site seems to increase by the increase in the charge of adsorbed metal ion. 3,6-O-CM-chitin from both structure also formed the gel and precipitate on the adsorption of barium ion, which had the largest ion radius among alkali-earth metal ions. Gel formation and precipitation on the adsorption of metal ions to 3,6-O-CM-chitin suggest that interchain cross-linking occurred by the adsorption of metal ions owing to the high charge density of carboxymethyl group. Especially gel formation proceeds smoothly in the presence of metal ions with large ion radius. However, 3,6-O-CM-chitin did not show the specificity to calcium ion. It might be suggested that one of the calcium adsorption site in CM-chitin molecule was blocked by re-carboxymethylation. Thus the mechanism of calcium ion adsorption to CM-chitin was elucidated, and functionalization of chitin was found to be regulated by the crystalline structure and the degree of carboxymethylation.

As CM-chitin has the specificity to calcium ion, it would be important to analyze the surface properties of CM-chitin-Ca complex in order to investigate the interaction of CM-chitin with biomaterials. The second purpose of this study is the investigation on the surface properties of CM-chitin-Ca complex.

On this study, adsorption of several optically active amino acids to the surface of the complex was analyzed considering the stereo geometry. The property of the surface can be estimate by analyzing the differences in the affinity for the various amino acids, since amino acids have hydrophilic α-amino and α-carboxyl groups in the main chain and hydrophilic or hydrophobic functional groups in the side chain, depending on the kind of amino acid. The interaction of CM-chitin with proteins may be presumed by the profiles of adsorptions of amino acids which are the basic components of proteins.

On the flaky CM-chitin having natural structure, the adsorption capacities of amino acids was shown as Lys>Phe>Glu, independently on the presence or absence of calcium ion. The adsorption of *D,L*-Lys and *D,L*-Glu reduced to 1/10 and 1/5 respectively, by the chelation of calcium ion. On the other hand, the adsorption of *D,L*-Phe enhanced about 50 times. Similar results were obtained also for water soluble 6-O-CM-chitin from α-chitin. The adsorption of neutral amino acids having the alkyl side chain was found to increase linearly with the increment of the hydrophobicity of the side chain, while the adsorption of phenylalanine having the aromatic side chain increased more strikingly with the increase of the hydrophobicity. As the contribution of other factor was bigger than that of the hydrophobicity on the Phe adsorption, contribution of benzyl group, of which electron state is different from that of alkyl groups, should be very large. When aromatic ring forms the

metal ion complex, π electron plays a role as a ligand to form π complex. The adsorption of phenylalanine seems to be contributed by the similar mechanism. These adsorption phenomena seem to be similar with the calcium binding mechanism of calmodulin, a famous calcium binding protein. The binding sites of metal ions in calmodulin molecule are formed by "E-F hand", which has repeating unit of Asp-Gly and two α-helix composed of non-polar amino acids and Phe exists in the adjacent position. In this case, the binding of calcium ion has influence upon the benzyl group of Phe side chain(51). This mechanism might explain the adsorption of phenylalanine on to CM-chitin-Ca^{2+} complex.

The side chain of Phe was found to be important for the adsorption to CM-chitin-Ca complex. But that is not enough to clarify the mechanism of amino acids adsorption. Ionic binding of α-amino group and especially α-carboxyl group, which is dissociated in neutral pH, should be examined from the standpoint of geometric specificity. Though it is suitable for the adsorption experiment to protect the functional groups of main chain (α-position), non-polar protective groups are not suitable because they have small solubility in water and such a non-polar material can not be a model of proteins. To investigate the effects of main chain functional group on the adsorption of side chain, the difference in the adsorption capacity was examined by regulating the distance between main chain and side chain. The adsorption of peptide was investigated using glycine as a spacer. The adsorption studies for peptides would also

give us some informations about the interaction between CM-chitin and proteins.

Since the adsorption capacity for H-Gly-Phe-Gly-OH is almost same as that for H-Phe-Gly-OH, the α-amino and the α-carboxyl side of the Phe residue do not seem to affect on the adsorption of Phe so much. The increase of the adsorption capacity for the peptides in the presence of calcium ion was similar as in the case of Phe adsorption. This would suggest a contribution of only benzyl group for the adsorption of Phe in the peptide. These mechanism seems to be explained using a model that the side chain of Phe justly incorporated in the adsorption site formed by the calcium adsorption, and main chain of the peptide is exposed on the surface of polymer chain.

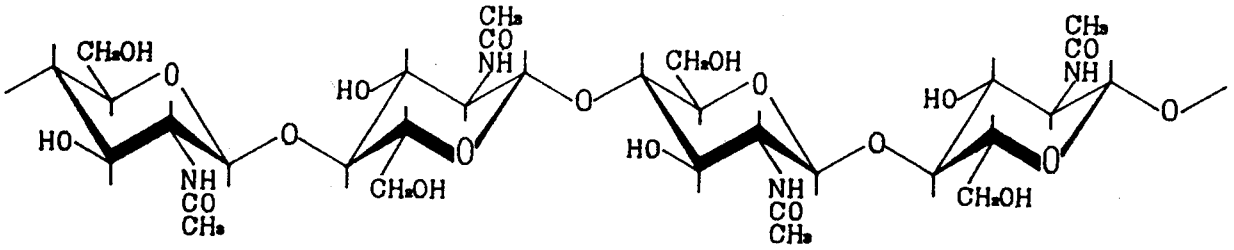
The function given by the carboxymethylation is thought to be developed to biomimetic material to utilize the high biocompatibility described before and biodegradability, which is higher than chitin(23). Taking these properties into account, model synthesis for sustained release system of drug without side effects was planned. The adsorbed molecule would be released with the digestion of CM-chitin in animal body, and CM-chitin would disappear in the metabolism system without affection to the body. It was planned to obtain fundamental informations about sustained release from the rate of lysozyme hydrolysis, rate of molecular weight decreasing and the relationship between the adsorption capacity of Phe and the molecular weight of CM-chitin.

6-O-CM-chitin was found to be hydrolyzed to 1/10 of original molecular weight in 24 hrs by the lysozyme concentration of the same level as in human tear. It was hydrolyzed finally to 2-3 residue oligomers in 96 hrs. The adsorption of Phe decreases by the reduction of the molecular weight. These results suggest that Phe adsorbed to CM-chitin-Ca complex is released by the decrease of retaining ability for Phe due to CM-chitin hydrolysis with the glycosidase such as lysozyme. 3,6-O-CM-chitin was not hydrolyzed by lysozyme, because it seems not to be recognized by lysozyme due to the substitution at C-3 position. Only 6-O-CM-chitin was suggested to be suitable for the carrier of sustained release.

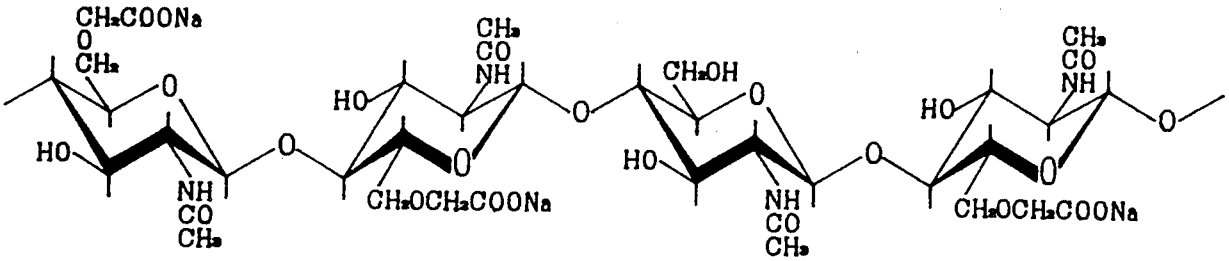
6-O-CM-chitin was reported to be utilized as a carrier for inclusion type sustained release by the addition of ferric ion to form gel(53). The release of doxorubicine, a anti-cancer drug, from the gels was found to increase by the lysozyme digestion in a time-dependent manner. All these results, that CM-chitin-Ca complex can be used as a polymeric drugs and CM-chitin adsorbed metal ions specifically, would indicate the excellent use fullness of CM-chitin.

The results and discussions in detail was described in the following three chapters. Chapter I described the preparation of various CM-chitins, their identification and their digestion by lysozyme. Chapter II described about the adsorption of metal ions and the adsorption of amino acid in order to analyze the surface of metal ion-CM-chitin complex. Chapter III described about the adsorption of

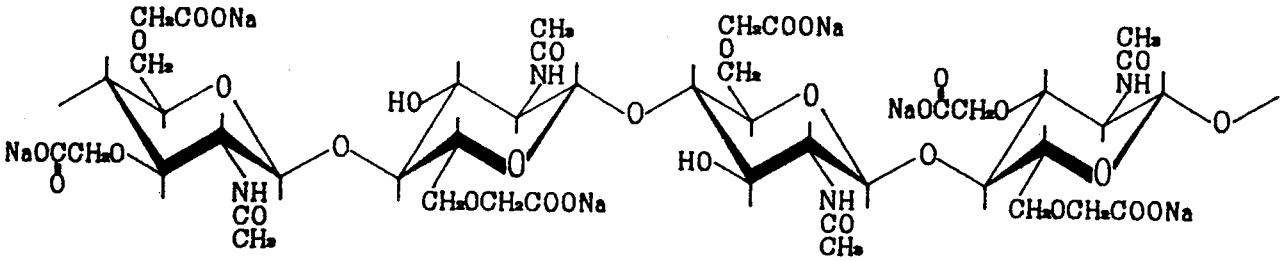
peptide for cleaning up mechanism of amino acids adsorption and discussed about the possibility of utilization of CM-chitin as polymeric drug.



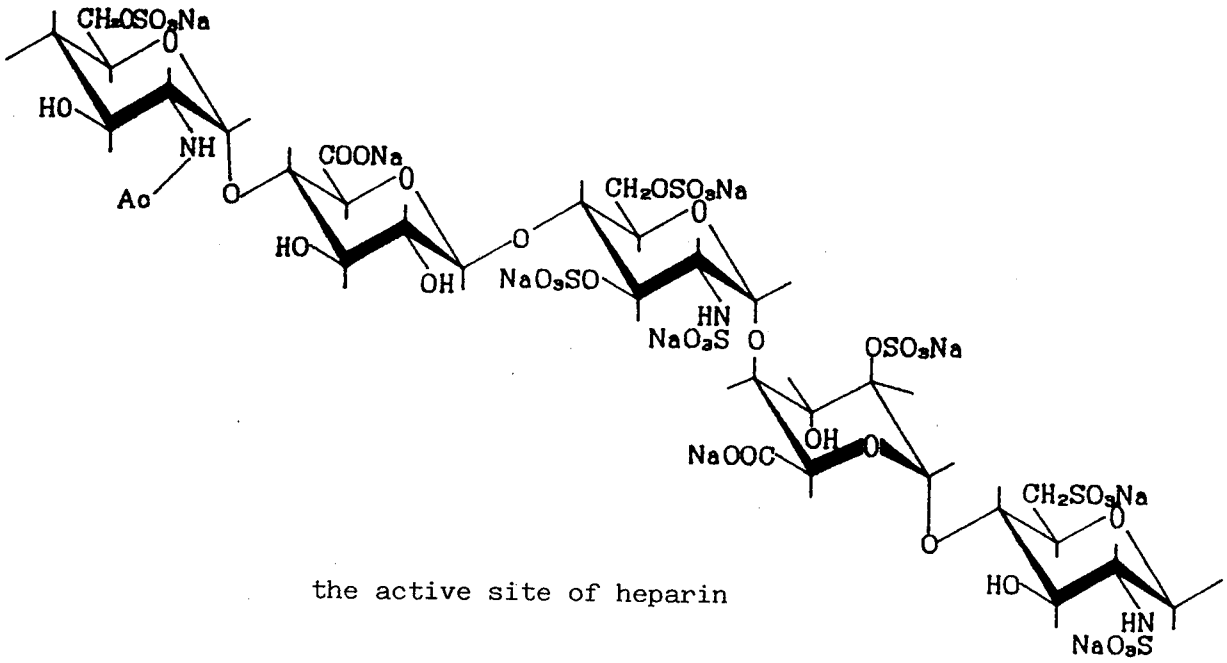
chitin



6-O-CM-chitin



3,6-O-CM-chitin



the active site of heparin

Fig.2 Chemical structures of chitin , CM-chitins and the active site of heparin

Chapter 1.

Preparation of various CM-chitins and its identification

This chapter will describe the preparation of variously substituted 6-O-CM-chitins and 3,6-O-CM-chitins and discuss the reactivity of chitin molecule by the difference of crystalline structure. The susceptibility of CM-chitin to lysozyme and its products will also be discussed.

EXPERIMENTAL

MATERIALS

α-chitin, which is usually derived from Crab or Shrimp Shell, was prepared from Queen Crab Shell according to the method of Hackman(25) and powdered to 45-60 mesh before use. β-chitin was prepared from Squid Bone(Loligo Pen) by the similar procedure as those for α-chitin except shorter reaction time, but it took only one-fifth of treatment time in the alkaline and acidic condition because of lower amounts of calcium carbonate and various proteins than those of α-chitin.

Monochloroacetic acid, dichloroacetic acid and other reagents of reagent grade were obtained from Wako Pure Chemical Industries Ltd., and were used without further purification. Hen egg white lysozyme was purchased from Seikagaku Kogyo Co. Ltd.(50000units/mg, 6times recrystallized).

PREPARATION OF 6-O-CM-CHITIN

6-O-CM-chitins(below D.S. 1.0) were prepared by the method reported previously(17) from α or β-chitin.

In the case of water insoluble 6-O-CM-chitin, 1g of chitin powder was mercerized in 4mL of 20% aqueous NaOH solution (containing 0.02% sodium dodecyl sulfate) for an hour and kept in -20℃ to be alkali-chitin. The alkali-chitin was thawed in *i*-propanol at room temperature followed by the neutralization with the addition of monochloroacetic acid. Crude CM-chitin thus obtained was washed by deionized water

to remove salt completely and dried in air to give pure 6-O-CM-chitin.

Water soluble 6-O-CM-chitin was prepared by the same method as above with 40%(W/W)NaOH aqueous solution. Purification was performed by the dialysis of crude CM-chitin against distilled and then deionized water to remove salt followed by concentration of inner solution to a small volume. Pure CM-chitin was obtained by the lyophilization or precipitation with acetone. Degree of substitution of CM-chitin was controlled by alkaline concentration on the process of mercerization.

PREPARATION OF 3,6-O-CM-CHITIN

One gram of 6-O-CM-chitin(D.S. 0.74) was dissolved in 4mL of 50%(W/W)NaOH solution and kept in -20℃ overnight. It was thawed in 5mL *i*-propanol at room temperature. Alkali-CM-chitin was neutralized by the addition of monochloroacetic acid(4g) with vigorous stirring to give crude 3,6-O-CM-chitin. This crude product was dissolved in 45mL of distilled water and saturated $BaCl_2$ solution was added to precipitate the highly substituted 3,6-O-CM-chitin. After precipitate was collected by the centrifugation, it was dissolved in aqueous EDTA solution and dialyzed against deionized water to remove salts completely. Supernatant of the centrifugation was dialyzed against the distilled water, the EDTA was added and dialyzed against deionized water. Both inner solution was concentrated, then product was obtained by the precipitation with acetone or

lyophilization.

Table 1. Yield of 3,6-O-CM-chitin

	yield	yield(%)	D.S.
3,6-O-CM-chitin precipitated by Ba^{2+}	0.55g	43%	1.7
CM-chitin not precipitated by Ba^{2+} *	0.30g		below1.2

Yield(%) was estimated from D.S.. *: It was included by the lowly substituted one, and it was re-carboxymethylated to give 3,6-O-CM-chitin of D.S.1.53.

MEASUREMENT OF INFRA-RED ABSORPTION SPECTRA

Infra-red absorption spectrometry of CM-chitin was measured using JASCO A-302 type infra-red spectrophotometer to identify the functional groups.

POTENTIOMETRIC TITRATION

After 0.05g of CM-chitin(COOH type) was dissolved in 30mL of aqueous 0.1M NaCl, 3mL of aqueous 0.1M NaOH was added and the resulting solution was treated with nitrogen gas for 30 min. Then the solution was titrated by 0.1M HCl aqueous solution using TSC-10A automatic titrator(TOA Electronic Ltd.) under the nitrogen atmosphere at room temperature.

125MHz-^{13}C-NMR MEASUREMENT

^{13}C-NMR measurement for 3,6-O-CM-chitin was achieved in D_2O at the concentration of 33mg per mL with dioxane as the outer standard at 60℃ using JEOL JNM GX-500 spectrometer.

LYSOZYME SUSCEPTIBILITY OF VARIOUS CM-CHITINS

The lysozyme susceptibility of CM-chitin was determined viscometrically as a standard indicator for biodegradability with an Ubbelhode type viscometer at 30℃. One mL of lysozyme solution (final concentration, 0.63μM) was applied to 10mL of CM-chitin solution(1.0mg/mL) in 10mM Tris-HCl buffer(pH 7.4), and the rate of hydrolysis by lysozyme was measured according to the method of Hamaguchi and Funatsu(26).

PREPARATION OF CM-CHITIN OLIGOMERS

CM-chitin oligomers were prepared by the hydrolysis with lysozyme and the rate of lysozyme accessibility was also studied.

A 500mL of 6-O-CM-chitin solution(1% of CM-chitin in phosphate buffer, M/15, pH6.2) was hydrolyzed by the 2500 units/mL of lysozyme, Similar level to that in human tear, in which the lysozyme concentration is higher than that in other organs of human body(27). Moreover, lysozyme of the same concentration was added every day for 9 days at 37℃ to maintain constant enzymatic activity and to simulate the physiological condition. To investigate the compositions of CM-chitin oligomer, a part of reaction solution was taken after 7, 24, 72 and 96 hours. 20% aqueous trichloroacetic acid was added to give 5% of final concentration to

inactivate and precipitate lysozyme. After the precipitate was removed by centrifugation, trichloroacetic acid was removed from a supernatant by the extraction with ether. The supernatant was concentrated and slowly added to acetone. The precipitate was collected by centrifugation. Molecular weight(M.W.) of CM-chitin oligomers obtained in several reaction was measured by the gel permeation chromatography using Sephadex G-50 gel. As there are no suitable standard materials for indicating M.W., molecular weight was estimated both by the method of titration of reducing end(28) and the method of HCl-Indole(29). Titration of reducing end in saccharides gives the number of molecular chains and the method of HCl-Indole gives the number of total N-acetylglucosamine residues in CM-chitin oligomer. Molecular weight was calculated by applying following equation

$$M.W. = \frac{\text{number of CM-GlcNAc residues}}{\text{number of molecular chains}} \times R.W.$$

where R.W. was residual molecular weight estimated from the degree of substitution of CM-chitin.

RESULTS AND DISCUSSION

PREPARATION OF 6-O- AND 3,6-O-CM-CHITIN

6-O-CM-chitin was prepared from alkali-chitin, which was derived by mercerization and keeping at -20℃, and monochloroacetic acid in the heterogeneous reaction system by the Williamson reaction. The infra-red spectra of the products were shown in Fig.3. Since the new absorption at 1735cm^{-1} due to the stretching of carbonyl group appeared with increase of the degree of carboxymethylation, it was suggested that the carboxymethyl group was introduced in chitin molecule. The carboxymethyl group was suggested to be substituted at only primary hydroxyl group of C-6 position in the N-acetylglucosamine residue by ^{13}C-NMR investigation(17).

The degree of carboxymethylation was found to depend on the concentration of sodium hydroxide used, but not be above 1.0 under this condition, in which the reaction was proceeded without removal of excess alkaline to avoid deacetylation (50).

These results would indicate that carboxymethylation occurs at C-6 position predominantly under this reaction condition. Since chitin molecule forms hydrogen bonding between acetamide group at C-2 position and secondary hydroxyl group at C-3 position in N-acetylglucosamine residue and between its bonding and primary hydroxyl groups at C-6 position through water molecule(30), and forms hydrogen bonding between hydroxyl group at C-3 and hemi-

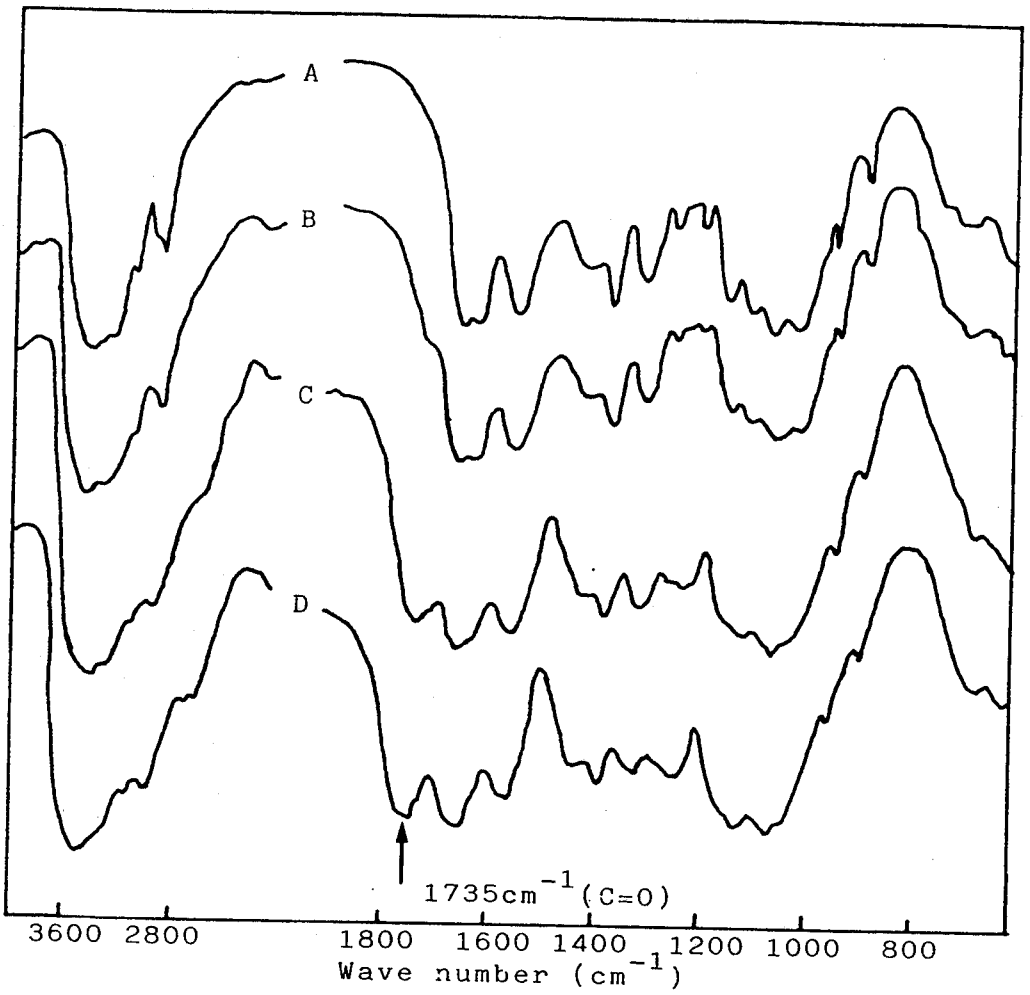


Fig.3 Infrared spectra of chitin and CM-chitins

A;chitin, B;D.S. 0.08, C;D.S. 0.7, D;D.S. 0.8

acetal oxygen atom (O-5) as described by Sundarajan et. al.(31), secondary hydroxyl group at C-3 position is protected by a few hydrogen bonding. As hydroxyl groups at c-3 position is still protected by the hydrogen bonding under the heterogeneous chemical modification and primary hydroxyl group is more reactive than secondary hydroxyl group, carboxymethylation of C-6 position occurs more predominantly than that of C-3 position.

3,6-O-CM-chitin was obtained by the carboxymethylation of mercerized 6-O-CM-chitin under the similar condition as those for 6-O-CM-chitin. 125MHz-^{13}C-NMR spectrum for 3,6-O-CM-chitin was shown in Fig.4. Good separation was observed between the chemical shifts at C-3 and at C-5, which were not separated in 6-O-CM-chitin, and chemical shifts of C-3 itself also separated. Though two kinds of chemical shifts for carbonyl group were observed on 6-O-CM-chitin in low magnetic field, 3,6-O-CM-chitin had several chemical shift for carbonyl. It would suggest the introduction of carboxymethyl groups also to C-3 position in addition to C-6 position.

This reaction was carried out in homogeneous system, in which 6-O-CM-chitin was once dissolved in aqueous sodium hydroxide solution. Owing to the destruction of the crystalline structure, especially by the blocking of hydrogen bonding by carboxymethylation at C-6 position, C-3 position is estimated to become reactive.

However, it was impossible to substitute only C-3 position under this reaction condition, because the hydroxyl group at

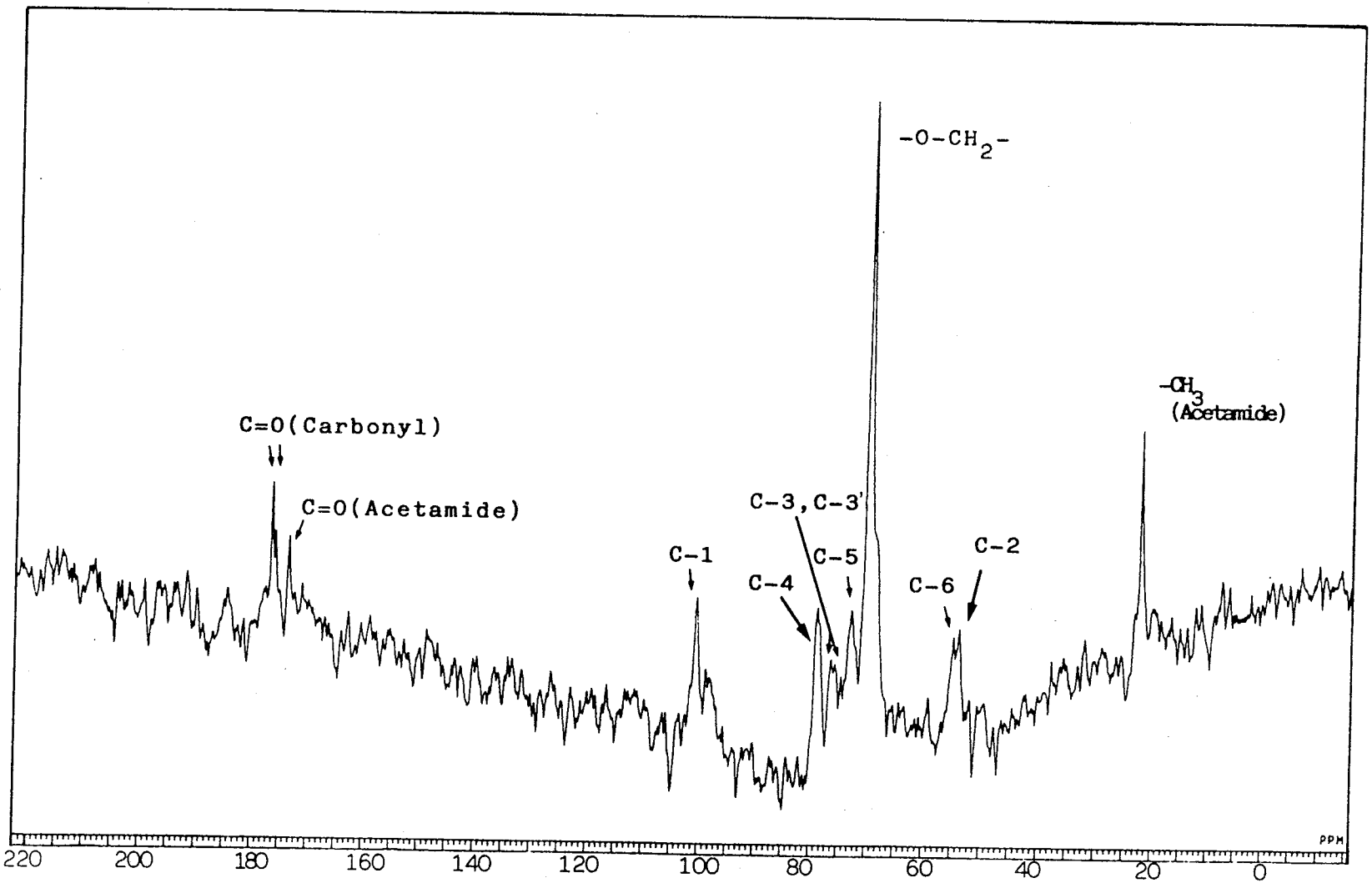


Fig.4 125MHz-^{13}C-NMR spectrum of 3,6-O-CM-chitin in D_2O at 60 °C

C-6 position should be protected before carboxymethylation. Even if this hydroxyl group can be protected, the molecular weight would decrease on the process of deprotection and complete deprotection could not be carried out due to the steric hindrance of CM-chitin. It was concluded that the substitution to only C-3 position was very difficult so far.

Preparation scheme for 3,6-O-CM-chitin was summarized in Fig.5 together with the outline of preparation reported previously by Okimasu(32).

The preparation of CM-chitin by Okimasu was carried out in homogeneous reaction directly from chitin. Sodium monochloroacetate was added to alkali-chitin solution which was prepared by dispersing chitin in alkaline ice-water mixture by vigorous stirring. In this reaction condition, CM-chitins of low substitution such as D.S. 0.2-0.25 are obtained, and random substitutions at C-6 and C-3 position occur. Further, the reaction is contaminated with N-carboxymethylation owing to deacetylation during the reaction. This procedure is unsuited for the purpose of present study which is to obtained highly substituted 3,6-CM-chitin.

3,6-O-CM-chitins derived from β-chitin easily by twice carboxymethylation from β-chitin had D.S. 1.5-1.7. But these from α-chitin had about D.S. 1.2. This result might be from the difference between the crystalline structures of α- and β-chitin. Increase in the degree of substitution for the preparation of 3,6-O-CM-chitin from α-chitin was achieved by the following improvement. i)Saturated sodium hydroxide

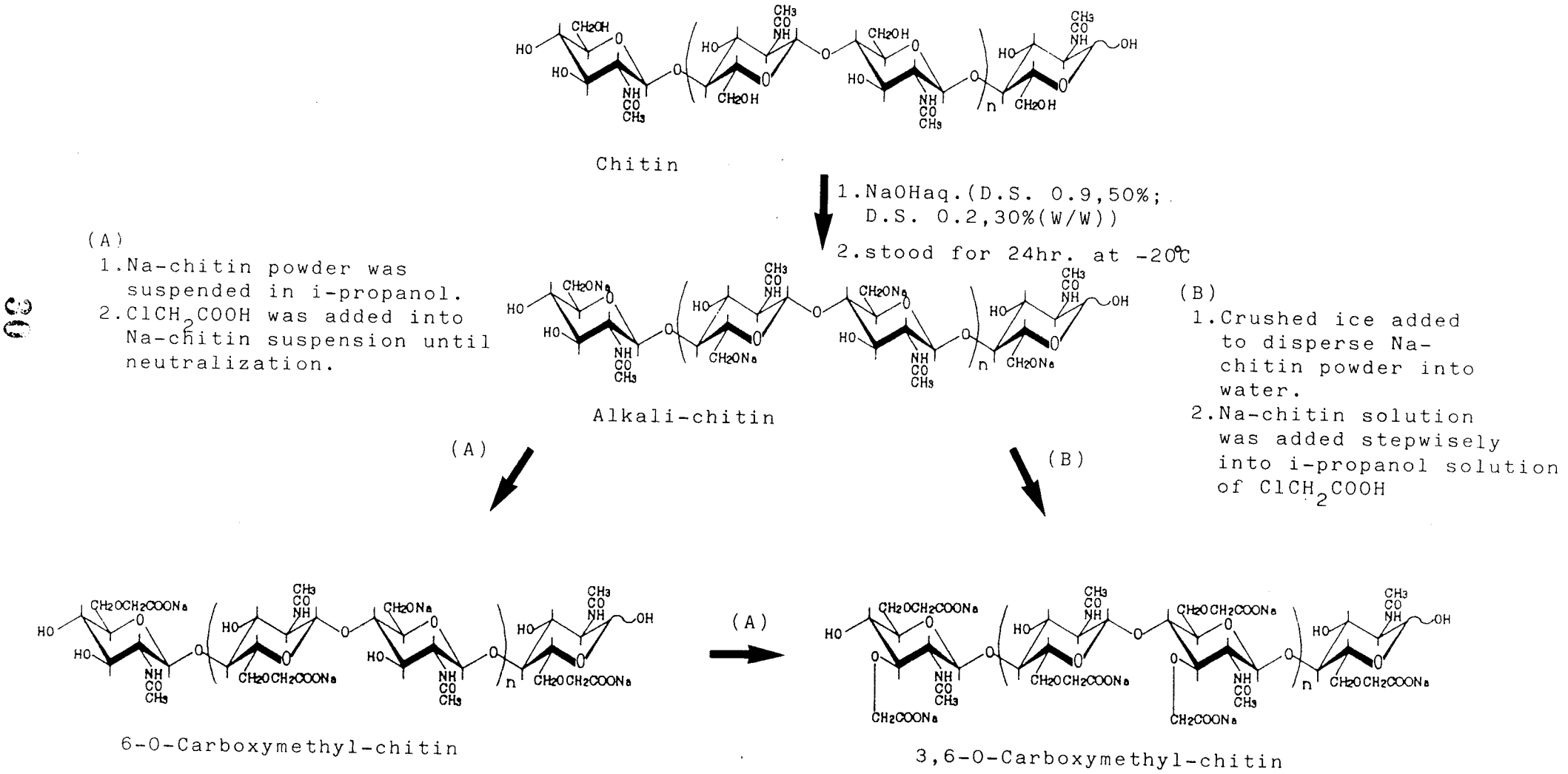


Fig.5 Synthetic route of various CM-chitin.

solution was used on the process of mercerization ii)When the neutralization in the re-carboxymethylation reaction was finished, more sodium hydroxide was added in order to proceed the reaction by re-neutralization with monochloro-acetic acid. iii)Detergent such as C_7F_5COOH with higher polarity than SDS was used in order to polarize the hydroxyl group on CM-chitin.

These data show the difference in the reactivities derived from difference in the crystalline structures. α-Chitin has rigid crystalline structure. β-Chitin has soft and loose crystalline structure, and high affinity toward water. This difference can be interpreted by the fact that α-chitin forms much hydrogen bondings per a unit lattice of crystal in anti-parallel chain orientation, while β-chitin forms less hydrogen bondings in parallel chain orientation This difference was strictly represented in the process of chitin paper preparation and its properties. The compli-cated process was necessary for the preparation of α-chitin paper. α-Chitin is dissolved in formic acid and regenerated by the spinning to the simple fibers and these are converted to non-knitting cloth with binder. In contrast, β-chitin's sheet was made up without binder owing to high self attachment ability. Since hydrogen bondings in β-chitin do not completely cross-link in nature, new hydrogen bonds would be generated on the process of drying up. Properties of these sheet were shown in Table II. Rigidity and stiffness of β-chitin were apparently lower than those of α-chitin from crab shell. The softness of β-chitin would be

Table II Physical properties of chitin sheets from Loligo Pen and Crab shells compared with others.

Samples	Basis weight (g/m^2)	Young's modulus (GPa)	Rigidity (E'I)	Stiffness (E'I/W)	Breaking length (Km)	Bursting factor	Tearing factor	Permeability O_2 (ml/m^2 24hr)	Permeability moisture (g/m^2 24hr)	Colony formation
Nonwoven Sheet from Loligo Pen	11	9.3	49	4	6.6	5.3	39	—	7800	-
	22	11.3	271	12	6.9	6.9	37	30	6600	-
	33	16.6				3.0		13	4400	-
	44	8.9	738	17	6.3	7.4	40	35	6100	-
from Crab shells	31	4.3	7600	243	4.1	2.1	96	free	free	+
from collagen	27	3.6			4.9	3.2		free	free	+
Fiber paper from Crab shells	85	5.1	5620	66	3.0	1.0		free	free	-
Bacterial cellulose	11	50.2	195	17	7.9	5.5		free	4600	-
pulp sheet(KP)	50	8.0	2720	36	7.4	5.9	139		4400	+
News paper MD	47	8.6	3150	67	4.6	1.5	42			+
CD	47	1.4	523	11	2.0	1.6	67		6900	+
Kevler sheet	50	4.2	2730	55	2.1	1.7	211		7100	+
Glass Fiber sheet	50	9.6	140000	2800	6.3	4.6	376		8300	+

the reflection of the crystalline structure.

Thus, CM-chitin of the high degree of substitution can be prepared easily from β-chitin because of looser protection of the hydroxyl groups by the hydrogen bondings.

ESTIMATION OF DEGREE OF CARBOXYMETHYLATION

The results of potentiometric titration were shown in Fig.6. The degree of substitution was estimated from the difference of the consuming HCl volume between the titrated midpoint for NaOH only and that of CM-chitin in sodium hydroxide solution. The titration for the Na content in CM-chitin-Na salt was consistent with the result of former titration. The degree of substitution obtained by these results and result of elemental analysis were as follows and various CM-chitins used in this study were listed in Table III.

Low substituted 6-O-CM-chitin

D.S. 0.1 from titration

Elemental analysis

Found			Calcd.		
C(%)	H(%)	N(%)	C(%)	H(%)	N(%)
46.02	6.43	6.58	45.18	6.52	6.43

Calculated on the assumption that the chitin molecule contains 1/2 H_2O per N-acetylglucosamine residue.

3,6-O-CM-chitin

D.S. 1.36 from titration

Found			Calcd.		
C(%)	H(%)	N(%)	C(%)	H(%)	N(%)
42.23	5.58	4.73	42.90	5.91	4.67

Calculated on the assumption that 3,6-O-CM-chitin molecule contains 1.0 H_2O per N-acetylglucosamine residue.

Table III. Degree of Substitution for Various CM-chitins.

Carboxymethyl-chitins	Degree of Substitution
6-O-CM-chitin from α-chitin	0.1, 0.53, 0.72,0.88
6-O-CM-chitin from β-chitin	0.74
3,6-O-CM-chitin from α-chitin	1.36
3,6-O-CM-chitin from β-chitin	1.70

Degree of Substitution: number of carboxymethyl group per N-acetylglucosamine residue.

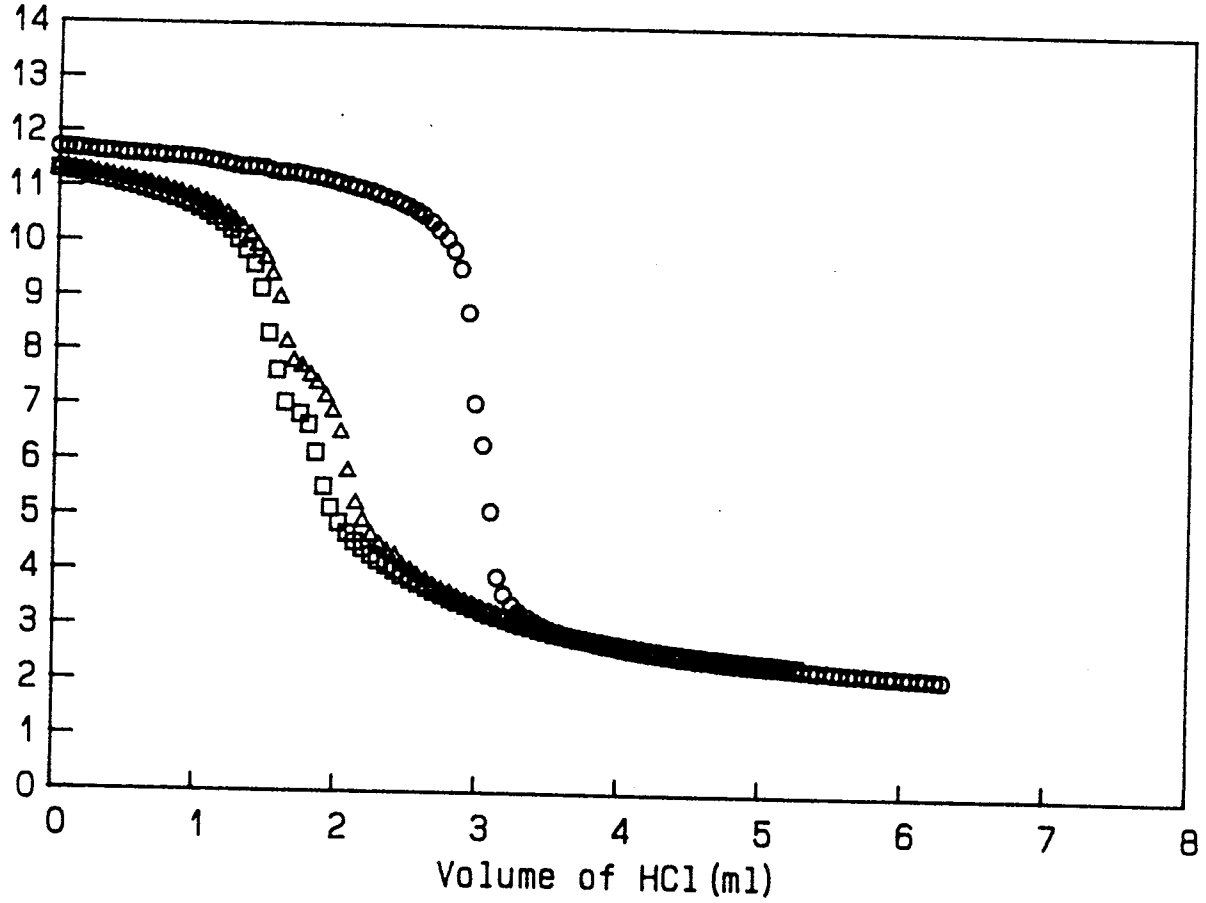


Fig.6 The potentiometric titration of CM-chitins

○,Blank(0.1M NaCl and 0.01M NaOH); □ ,CM-chitin(D.S.0.63); △ ,CM-chitin(D.S.0.49)

After 0.05g of CM-chitin(COOH type) was dissolved in 30mL of 0.1M NaCl aqueous solution, 0.1M NaOH aqueous solution was added to that solution to replace oxygen gas in the solution by nitrogen gas. Then the solution was titrated by 0.1M HCl aqueous solution using TSC-10A automatic titrator(TOA Electronic Ltd.) under the nitrogen atmosphere at room temparature.

DIGESTION OF CM-CHITIN BY LYSOZYME

The biodegradability and the biocompatibility are very important matter on applying CM-chitin to animal body. The susceptibility of CM-chitin to lysozyme was investigated on the basis that chitin is hydrolyzed by chitinase or muraminidase such as lysozyme. The decrease of molecular weight of various CM-chitins was measured by the reduction of viscosity as shown in Fig.7.

The susceptibility of 6-O-CM-chitin (below D.S. 1.0) increases remarkably with the increase in the degree of substitution. But 3,6-O-CM-chitin of D.S. 1.2 is hardly hydrolyzed by lysozyme.

Chitin is hydrolyzed by lysozyme very slowly. In the case of CM-chitin the enhance of the susceptibility seems to be interpreted by increase of the encounterring frequency with lysozyme. This would be due to the higher freedom of CM-chitin molecule, which was introduced by the destruction of crystalline structure. Increase of the binding ability with lysozyme, due to the electrostatic interaction of CM-chitins having negative charge, would be an another reason of the high susceptibility(34).

Decrease of susceptibility by the substitution at C-3 position seems to be related to the substrate specificity of lysozyme. Though the substitution of C-6 position did not affect on the susceptibility by lysozyme, the susceptibility disappeared by the substitution at C-3 position in addition to C-6 position. These phenomena may suggest the importance of C-2 and C-3 positions in N-acetylglucosamine residue on

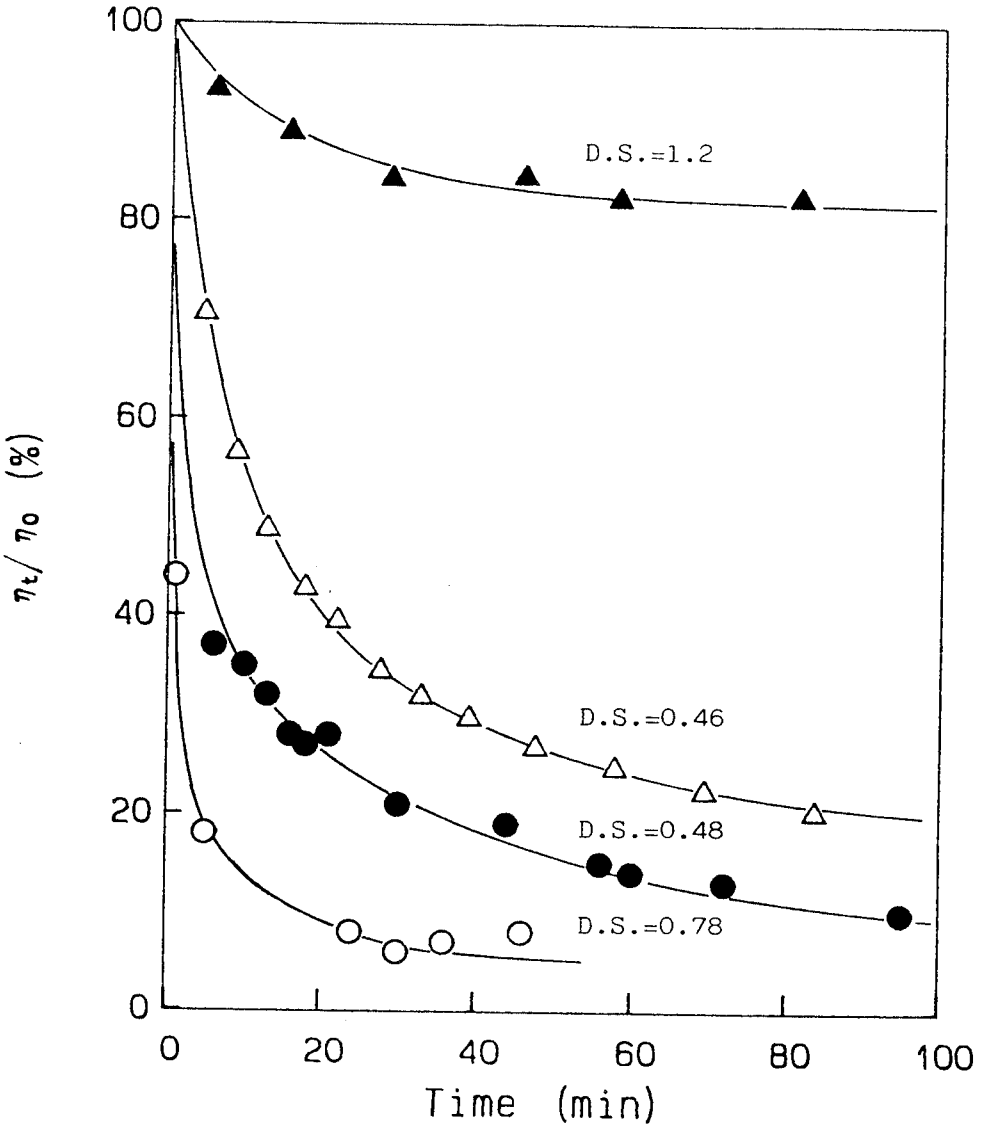


Fig.7 Time course of CM-chitin hydrolysis by lysozyme

The lysozyme susceptibility of CM-chitin was determined as a standard indicatior for biodegradability with an Ubbelhode type of viscometer at 30C. One mL of lysozyme solution (final concentration,0.63 M) was applied to 10mL of CM-chitin solution(1.0mg/mL) in 10mM Tris-HCl buffer (pH7.4), and the rate of hydrolysis by lysozyme was measure according to the method of Hamaguchi and Funatsu.

the binding mechanism of lysozyme, though lysozyme has been always mentioned to recognize the acetamide group at C-2 position. The low susceptibility of 3,6-O-CM-chitin would be explained by the following reasons. i)Most lysozyme can not bind to 3,6-O-CM-chitin because of the substitution of the binding site. ii)Even if 3,6-O-CM-chitin binds to lysozyme, CM-chitin has too negative charge comparing to 6-O-CM-chitin to dissociate from lysozyme and can not proceed to the catalytic process.

More exhaustive investigations will be necessary on the relationship of the function of lysozyme and susceptibility of CM-chitin.

6-O-CM-chitin is biocompatible material immunologically except the activation of peritoneal macrophage to induce the mitogenic activity in a short period. Its high biodegradability was also suggested in this study. Hence, 6-O-CM-chitin would be utilized as polymeric drug such as a carrier of sustained release of drug. 3,6-O-CM-chitin also, which is not a biodegradable material but a biocompatible material, seems to be suitable as a biomedical material owing to its high negative charge.

PREPARATION OF CM-CHITIN OLIGOMER

The relationship between the reaction time and the molecular weight of CM-chitin oligomers was shown in Fig.8 for the hydrolysis of 6-O-CM-chitin(D.S.0.72) by lysozyme.

6-O-CM-chitin(M.W.=30,000 estimated by the titration of reducing end) was hydrolyzed to M.W.=5,000 after 7 hrs, to

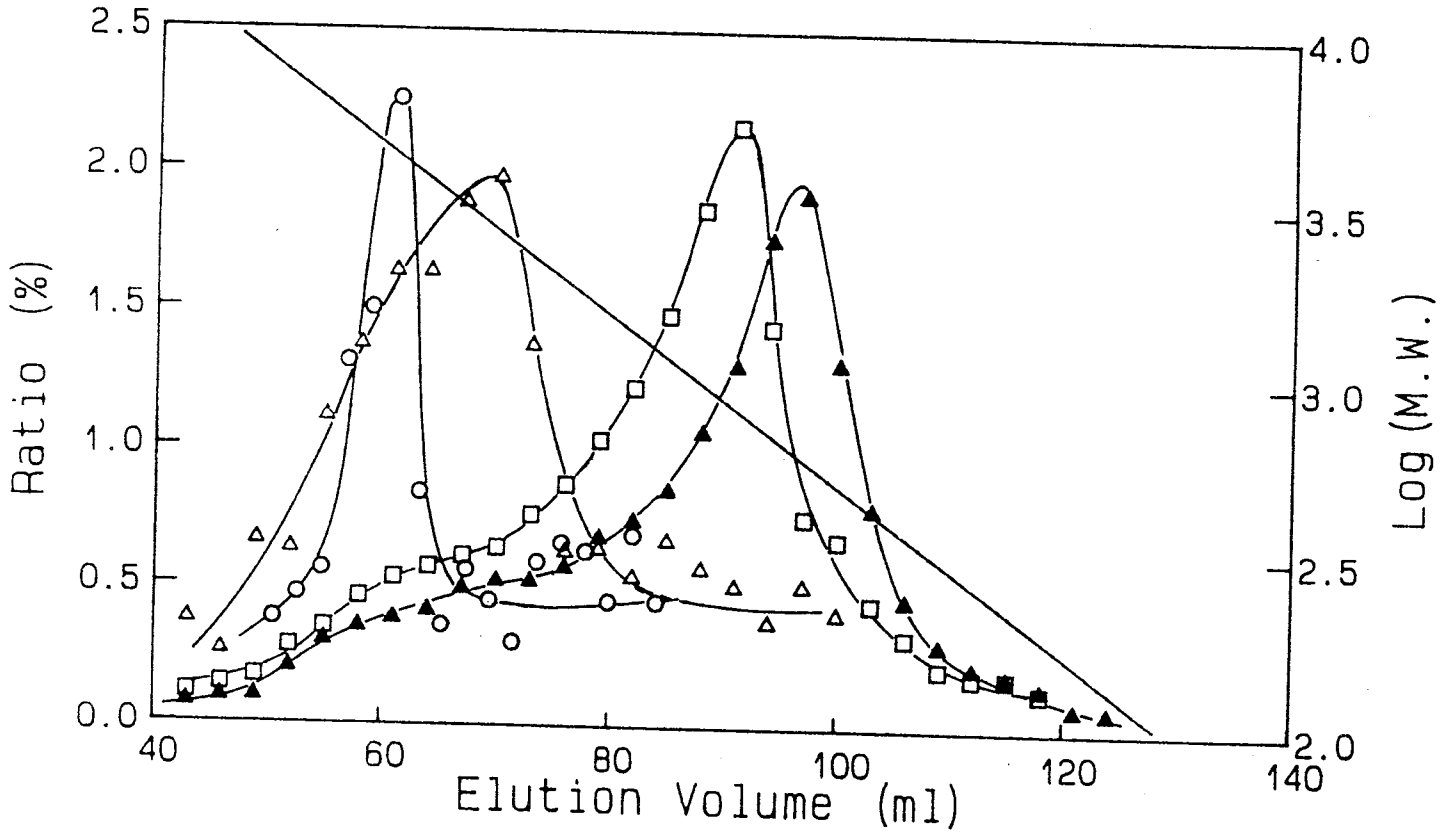


Fig.8 Gel permeation chromatogram of 6-O-CM-chitin hydrolysate by Lysozyme.

500mL of 1.0% 6-O-CM-chitin(D.S. 0.8) solution in phosphate buffer pH6.0 was hydrolysed by stepwise addition of lysozyme(1250 x 5 unit/mL).

—○—: for 7h.

—△—: for 24h.

—□—: for 72h.

—▲—: for 96h.

Ratio(%) = (number of chain)/(number of total residue) x 100

3,000 after 24 hrs and to 630(i.e. oligomer composed of 2-3 residues) after 96 hrs.

Though this result seems to be different from that from viscosity measurement, the result from the latter may represent the beginning reaction of hydrolysis by the endo type enzyme and the present result would represent the precious molecular weight of hydrolysate in the low molecular weight region.

The experiment described here was carried out in the same lysozyme concentration as in human tear. In the serum condition, which has 1/3 activity as in tear, M.W.=3,000(11 residue oligomer) to be would be attained after 72 hrs and 2-3 residue oligomer after 12 days. For reference, the amount of lysozyme in several organs was shown in Table IV.

Table IV Lysozyme content and specific activity

Human serum	0.3～4.2mg/L
Human saliva	3.7～625mg/L
Human tears	800～2500units/ml
Rabbit tears	10～170mg/L

Human lysozyme has approximately fourfold higher specific activity than Hen Egg White Lysozyme.

CONCLUSION

This chapter described about the site selective chemical modification of chitin to 6-O-CM-chitin under heterogeneous condition and 3,6-O-CM-chitin under homogeneous condition. The reactivity in this chemical modification was discussed in connection with crystalline structure of chitin. Comparing to α-chitin, β-chitin had higher reactivity and was modified easily to give CM-chitin of the high degree of substitution owing to the low crystallinity due to a few hydrogen bondings.

Although 6-O-CM-chitin was found to have the high susceptibility to lysozyme, proceeding of the substitution to C-3 position reduced the susceptibility remarkably.

6-O-CM-chitin was found to be hydrolyzed by lysozyme to 1/6 of the original molecular weight by 7 hours and to oligomers composed of only 2-3 residues by 92 hrs.

Chapter2.

Adsorption of metal ions to CM-chitin and surface properties of the complex.

In this chapter, the adsorption of metal ions to various CM-chitins and the surface properties of metal ions-CM-chitin complex will be discussed through the investigation of the adsorption of amino acids having optical isomers.

EXPERIMENTAL

PREPARATION OF CM-CHITIN FIBER

CM-chitin fiber was prepared by the method previously reported(35).

One gram of CM-chitin was thoroughly swollen in 20mL of formic acid(99.7%) and kept at -20℃. After thawing at room temperature, formic acid and dichloroacetic acid were added to obtain viscous solution as a spinning solution(final concentration: CM-chitin, 5.7%(W/V); formic acid, 94.7% (W/V); dichloroacetic acid, 5.7%(1mL)). This solution was filtered through the flannel to remove the insoluble part, and the bubbles in the spinning dope was removed by the gentle suction.

CM-chitin dope was spun into 1st coagulant (acetone) through platinum nozzle(0.1mmφ, 50holes), and 50% aqueous ethanol solution was used as a second coagulant. Resulting filaments were stretched in air at the ratio of 1.13 by the different roller speed.

PREPARATION OF 6-O-CM-N-ACETYLGLUCOSAMINE

(6-O-CM-chitin monomer)

Acetolysis(55); 5.8g of 6-O-CM-chitin oligomer derived by lysozymic hydrolysate of 6-O-CM-chitin was added to 100mL of acidic mixture (acetic anhydride : glacial acetic acid : sulfuric acid = 2 : 1 : 0.03) to react with vigorous stirring for 40 h at room temperature The resulted solution

was poured into saturated aqueous sodium acetate solution at 0℃ and was be stirred for 4 hours. Crude product was extracted by chloroform from that solution. Chloroform component was washed by cold aqueous sodium bicarbonate and cold water successively, then water was removed by the addition of sodium sulfate anhydrous. The solution was concentrated to syrup. After the syrup was dissolved in benzene, the solution was applied to the silica gel column to purify the product which was not adsorbed the silica gel. Its purity was identified by the thin layer chromatography(TLC).

Selective saponification of O-acetyl groups(56); 0.52g of 1,3,4-O-acetyl-6-O-CM-N-acetylglucosamine was dispersed in the distilled methanol(without water), 4.1mL of methanolic sodium methoxide(0.2g of metalic sodium was dissolved in 25mL of methanol) was added to that methanol solution to react for 4 hours till single spot of TLC was obtained. 6mL of H type of Amberlite IR-120 swollen thoroughly in water was added to the solution, then the mixture was filtered to remove ion-exchanger. The filtrate was evapolated to dryness to give product. Then further purification was carried out in the re-precipitation of methanolic solution by ether.

Elemental analysis; Found: C, 43.55%; H, 6.05%; N, 5.09%; Calcd.: C, 43.01%; H, 6.09%; N, 5.02%.

THE ADSORPTION OF DIVALENT CATIONS ON LOW SUBSTITUTED 6-O-CM-CHITIN

The adsorption of divalent cations on lowly substituted 6-O-CM-chitin was estimated by the method of frontal affinity chromatography(47-49) using the titration of eluted ions with EDTA, using Dotite indicators(36,37). The columns(2.3×23.5cm) were packed with 22.5g of CM-chitin powder(30-45mesh) or 7.9g of CM-chitin fiber which were loaded after swelling in water. The flow rate of 0.05M aqueous solution of divalent cations was 0.5-0.8mL/min and adsorption capacities were estimated according to a previous paper(38).

THE ADSORPTION OF METAL IONS TO VARIOUS WATER SOLUBLE CM-CHITINS

Ten mL of 0.2% CM-chitin aqueous solutions were mixed with various concentration of aqueous solutions of metal ions and the mixed solution was shaken for 24 hours (final CM-chitin concentration=0.1%). After the solutions were dialyzed against 1.0 L of deionized water for 48 hours where the equilibrium dialysis was achieved the concentration of inner and outer solutions were measured by ICP-AES(Inductively Coupled Plasma - Atomic Emission Spectroscopy, SPS1100, Seiko Electric Co. Ltd.). The concentrations were determined by the working straight line which were obtained by applying commercial standard solution. Since spray volumes from neplizer was deviated owing to the viscosity of polymer-metal ions mixture solution, the deviation was corrected by the calibration of viscometric effect.

The adsorption capacities were calculated by the

difference between the concentration of inner solution and that of outer solution.

When aqueous solutions of metal ions were added to CM-chitin solution to form gel or precipitate, the adsorption capacities were estimated by the difference between the concentration of applied metal ions and of supernatant after removal of precipitate by the centrifugation.

FT-IR MEASUREMENT

FT-IR measurements of water soluble 6-O-CM-chitin-Ca^{2+} and CM-chitin-Ni^{2+} complex in which free ions were removed by dialysis, were carried out by the multiple internal reflection method in H_2O using a ZnSe plate and by the transmission method in D_2O using a KRS-5 cell with a Nicolet 5DXB FT-IR spectrophotometer.

AMOUNT OF CALCIUM ION RELEASE FROM 6-O-CM-CHITIN BY PH SHIFT

This measurement was carried out according to the Batch method. Briefly, 0.3g of low substituted 6-O-CM-chitin was immersed in 20mL of 0.05M $CaCl_2$ aqueous solution with shaking for 24hours at room temperature and the complex was washed thoroughly by water to remove free calcium ion. The amount of released Ca^{2+} was estimated from the supernatant concentration following to put CM-chitin-Ca complex into various concentration of aqueous HCl solution of Ca^{2+} ion with shaking for 24h.

SELECTIVE ADSORPTION ON 6-O-CM-CHITIN FROM CA-MG MIXTURE SYSTEM

Adsorption capacities of Ca^{2+} or Mg^{2+} to water insoluble 6-O-CM-chitin were measured by Batch method in the Ca-Mg buffer solution(0.05M Tris-HCl, pH 7.4) under several ionic strength.

Briefly 2.0g of CM-chitin was added to 50mL of 0.05M $CaCl_2$ and $MgCl_2$ mixture in buffer solution(0.05M Tris-HCl buffer, pH7.4) and NaCl was added to adjust the ionic strength. Then calcium ion concentration in the supernatant was titrated by EDTA using BT(Eriochrome Black T) for both metal ion concentration at pH 10, using NN(2-Hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthoic acid) at higher than pH 12 to precipitate $Mg(OH)_2$.

ADSORPTION OF AMINO ACIDS TO CM-CHITIN

D,L-Amino acids aqueous solution were passed through the column of low substituted CM-chitin until saturation, and it was then washed with deionized water. The *D,L*-Lys and *D,L*-Glu were eluted with 0.5M aqueous NaCl solution. *D,L*-Phe was eluted by 0.1M aqueous EDTA solution at room temperature.

Amino acid-water soluble CM-chitin mixture was dialyzed against deionized water to remove free amino acids, and then adsorbed amino acids were released by pH shift(pH 1.0), and it was redialyzed against deionized water.

The adsorption capacities of amino acids were estimated from UV absorption(Hitachi 139 or U-3200 type spectrophotometer) for phenylalanine and fluorescence intensities of *o*-

phthalaldehyde derivatives of other amino acids using 340nm as excitation and 455nm as emission wavelength with Hitachi 650-40 type fluorescence spectrophotometer(40).

X-RAY SMALL-ANGLE SCATTERING(SAXS) FROM CM-CHITIN-METAL ION SOLUTION

Preparation of CM-chitin was made with procedures mentioned above. The degree of substitution of CM-chitin was 0.9. CM-chitin was used without further fractionation to obtain monodisperse saccharide. The viscosity averaged molecular weight was about 7×10^4(54). The aqueous solutions of $FeCl_2$ and $CaCl_2$ were added to the CM-chitin solution. The experimental concentration 1.9×10^{-2}g/mL was set for higher than concentration c* to prevent the free rotation of the stretched chains. Thus solutions were dealed only with semi-dilute solutions. But these solutions were prepared in the condition of open system, so $FeCl_2$ solutions were easily oxidized by oxygen gas in air to form gel.

Measuring of gel formation by Fe^{3+}, Fe^{2+} or Fe^{3+} mixed CM-chitin solutions were prepared in a dry box under the nitrogen flow to prevent the oxidation.

The SAXS profile was recorded using SAXES installed at the Photon Factory of the National Laboratory for High-Energy Physics, Tsukuba and the High-Brilliance X-ray laboratory of Hokkaido University. The measurements were made at room temperature.

RESULTS AND DISCUSSION

ADSORPTION OF METAL IONS

The adsorption capacities of various divalent metal ions to water insoluble 6-O-CM-chitin (low degree of substitution) were plotted against ionic radii of metals as shown in Fig.9.

Since the adsorption capacity of calcium ion to flaky CM-chitin, which kept the natural crystalline structure of chitin, seems to be higher than those of other metal ions, especially much higher than that of cadmium ion having the similar ionic radius, the mechanism of the adsorption of calcium ion seems not to be explained by the simple relation between the size of adsorption site and the volume of ion to be occupied. On the other hand, the capacities of transition metal ions to CM-chitin fiber were increased in which CM-chitin was firstly dissolved in formic acid following to be orientation by the stretching process in the spinning , but those of alkali-earth metal ions were reduced except calcium ion. These results were not found the specificity of calcium ion such as that of flaky CM-chitin. These result suggest that the specificity of calcium ion seems to ascribe to the geometrical arrangement of functional groups of flaky CM-chitin.

The adsorption capacities of various metal ions to water soluble CM-chitin having the various degree of substitution were shown in Table V.

The adsorption of calcium ion to 6-O-CM-chitin seems not

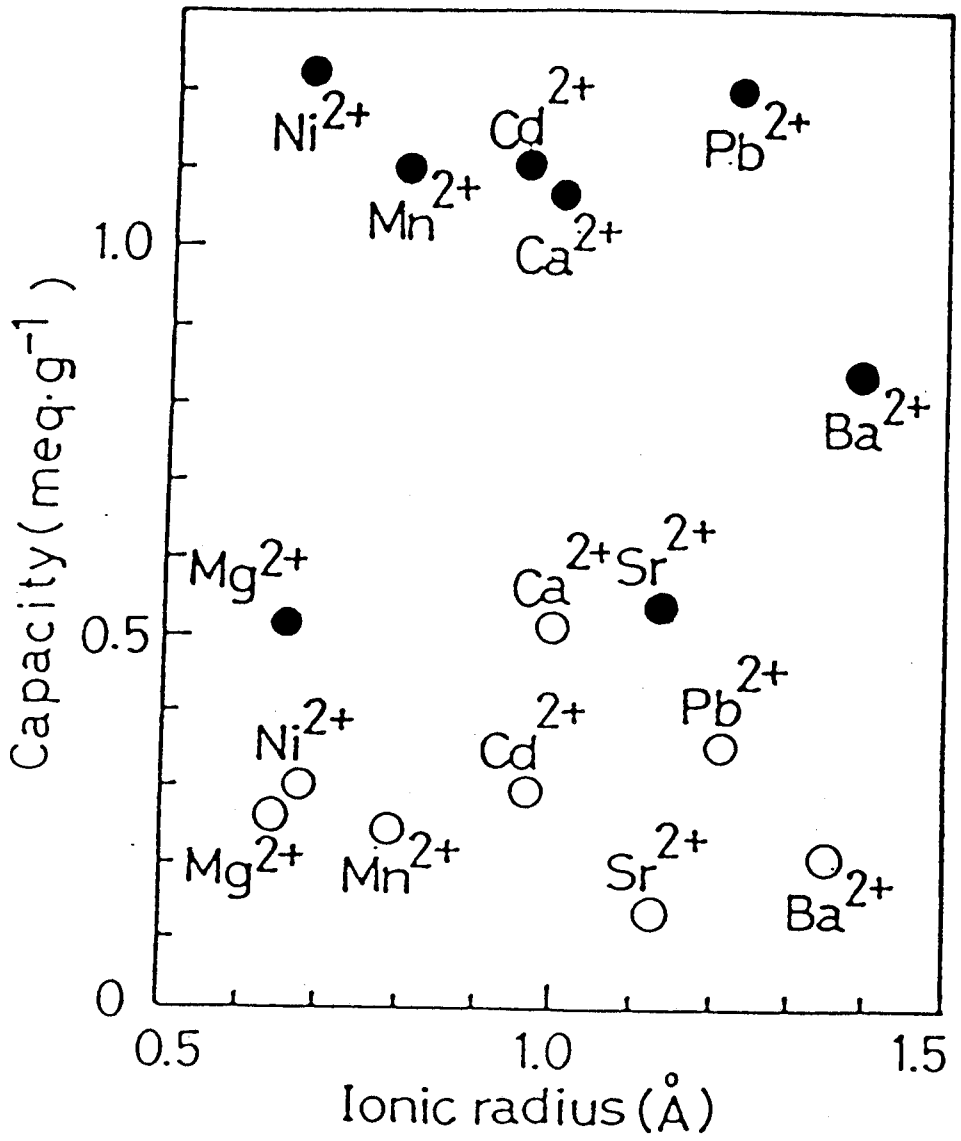


Fig.9 Relationship between ionic radius and adsorption capacities of CM-chitin(D.S.=0.1) for metal ions

(O)Poorly substituted CM-chitin(flake)

(●)Fibrous CM-chitin

Table V Adsorption capacity(m.eq/g) of metal ion to various CM-chitins

Loligo Pen (β)						Queen Crab (α)			
CM-chitin	6-O-CM-chitin		3,6-0-CM-chitin			6-O-CM-chitin		3,6-0-CM-chitin	
D. S.	0.74		1.7			0.58	0.88	1.36	
Polymer	0.1%		0.25%	0.1%		0.1%		0.1%	
Metal Ion	10mM	100mM	10mM	10mM	100mM	10mM	100mM	10mM	100mM
Mg^{2+}	1.7	1.4	2.6	3.1	1.4	1.7	2.1	3.0	4.0
Ca^{2+}	1.7	1.2	2.6	3.2	1.3	1.6	2.5	2.6	3.4
Sr^{2+}	1.5	1.6	2.4	2.6	1.2	1.5	2.1	2.7	3.2
Ba^{2+}	1.5	1.1	2.4	2.7	▼ 7.9	1.6	2.2	2.9	▼10.5
Mn^{2+}	1.5	2.0	2.6	3.0	▼16.4	1.6		3.1	3.5
Ni^{2+}	▼ 2.0	▼ –	▼2.2	3.6	▼ 7.3	1.7	2.2	3.1	▼ 4.1
Cd^{2+}	▼ 2.1	▼ 5.8	▼4.3	▼ 3.6	▼12.0	1.8	2.5	▼4.0	▼ 9.3
Cu^{2+}	▼11.3	▼ –	▼3.4	▼13.3	▼ 2.2	▼1.8	2.1	▼2.3	▼ 3.3
Pb^{2+}	▼ 4.0	▼ 2.8	▼3.6	▼ 5.6	▼21.3	▼1.9		▼2.4	▼ 8.8
trivalent									
Fe^{3+}	▼ 1.5	▼ –	▼5.9	▼ 6.1	▼ 7.1	▼0.6	▼4.5	▼7.3	

to be more specific for other metal ion, but 3,6-O-CM-chitins were found to form gel followed by precipitation with addition of transition metals and also with the addition of barium ion among alkali-earth metal ions. However,in the case of barium ion of high concentration such as 100mM was required to form gel. As the metal ion induced precipitation of 3,6-O-CM-chitin depends mainly on the higher adsorption capacity for metal ions such as Ba^{2+} or Pb^{2+} and the metal ion adsorbed per a carboxyl group was over 1.0, the inclusion of free metal ion in the precipitates is assumed in addition to the positive adsorption effect by adsorbed ions. 6-O-CM-chitin from β-chitin formed also gel followed by precipitation with increase of apparent adsorption capacities in the presence of transition metal ions, though low apparent adsorption capacity was observed for α-chitin derivative by transition metal, even if it forms gel. So precipitation mechanism seems to ascribe to crystalline structure of chitin molecule. Breaking down rigidity of polysaccharide chain seems to contribute to the reactivity on the chemical modification and to adsorption properties of these derivatives, because some features of crystallinity has been assumed to be kept to hold a geometrical arrangement of functional groups even in water soluble derivatives.

The adsorption capacities in low metal ion concentration was measured to estimate the binding constants to clarify the adsorption properties. As it was very difficult to measure precious polymer concentration and free metal ions

in the case of precipitation due to forming incomplete precipitation, only alkali-earth metal ions and manganese ion among transition metals were applied, which were not formed precipitation at low metal concentration. The adsorption capacities are shown in Table VI and Scatchard plots were carried out from those results as shown in Fig.10.

The capacities of various CM-chitins for calcium ion seems to be higher than those of other metal ions at even very low ion concentration i.e. 0.5mM. Though Scatchard plot for all metal ions seems to give a curved line except calcium ion suggesting negative cooperative effect in all CM-chitins, 6-O-CM-chitin from β-chitin gives a straight line for calcium ion and that from α-chitin shows almost straight line. These results seem to suggest that the specificity of calcium ion for low substituted 6-O-CM-chitin is maintained even in water soluble one, especially the specificity is conspicuous in low concentration of calcium ion. Though binding constant on the calculation of Scatchard plot indicating the negative cooperative effect might involved deviation, the binding constants were estimated arbitrarily values from Scatchard plot in the region of 1.5-5.0mM by the first regression straight line to compare the binding ability listed up in Table VII. As all binding constant lay the range from 10^5 to the first half of 10^6, the binding ability of every metal ion seems similar in this region. It might suggest to be similar binding mechanisms of metal ions indicating non specific negative co-

Table.VI Capacities of metal ions to 6-O-CM-chitin at low concentration.

Applied conc.	Species of CM-chitin	Mg2+	Ca2+	Ba2+	Mn2+
0.5mM	6-O-CM-chitin (α)	0.82	0.88	0.83	0.72
	6-O-CM-chitin (β)	0.87	0.91	0.74	0.76
	3,6-O-CM-chitin (α)	0.85	1.00	0.85	0.83
	3,6-O-CM-chitin (β)	0.95	1.05	0.89	0.84
1.0mM	6-O-CM-chitin (α)	1.35	1.16	1.26	1.08
	6-O-CM-chitin (β)	1.51	1.44	1.49	1.35
	3,6-O-CM-chitin (α)	1.50	1.90	1.53	1.75
	3,6-O-CM-chitin (β)	1.62	1.95	1.89	1.72
1.5mM	6-O-CM-chitin (α)	1.54	1.31	1.45	1.26
	6-O-CM-chitin (β)	1.62	1.64	1.80	1.71
	3,6-O-CM-chitin (α)	2.25	2.32	2.20	2.29
	3,6-O-CM-chitin (β)	2.28	2.27	2.54	2.11
2.0mM	6-O-CM-chitin (α)	1.61	1.36	1.68	1.46
	6-O-CM-chitin (β)	1.82	1.80	2.00	1.77
	3,6-O-CM-chitin (α)	2.47	2.94	2.34	2.68
	3,6-O-CM-chitin (β)	2.68	3.12	3.02	2.70
5.0mM	6-O-CM-chitin (α)	1.68	1.52	1.95	1.61
	6-O-CM-chitin (β)	1.97	1.75	2.38	2.06
	3,6-O-CM-chitin (α)	2.93	4.21	2.69	3.02
	3,6-O-CM-chitin (β)	3.06	3.56	3.75	3.13

Capacity:meq/g Ten mL of 0.2%CM-chitin aqueous solutions were mixed in various concentration of aqueous solutions of metal ions and the mixture solution were shaken for 24hours (final CM-chitin concentration, 0.1%). After the solutions were dialyzed against 1.0 L of deionized water for 48 hours where the equilibrium dialysis was achieved completely, the concentration of inner and outer solutions were measured by ICP-AES(Inductively Coupled Plasma - Atomic Emission Spectroscopy, SPS-1100, Seiko Electric Co. Ltd.). The concentrations were determined by the calibrations line which were derived from the commercially standard solution.

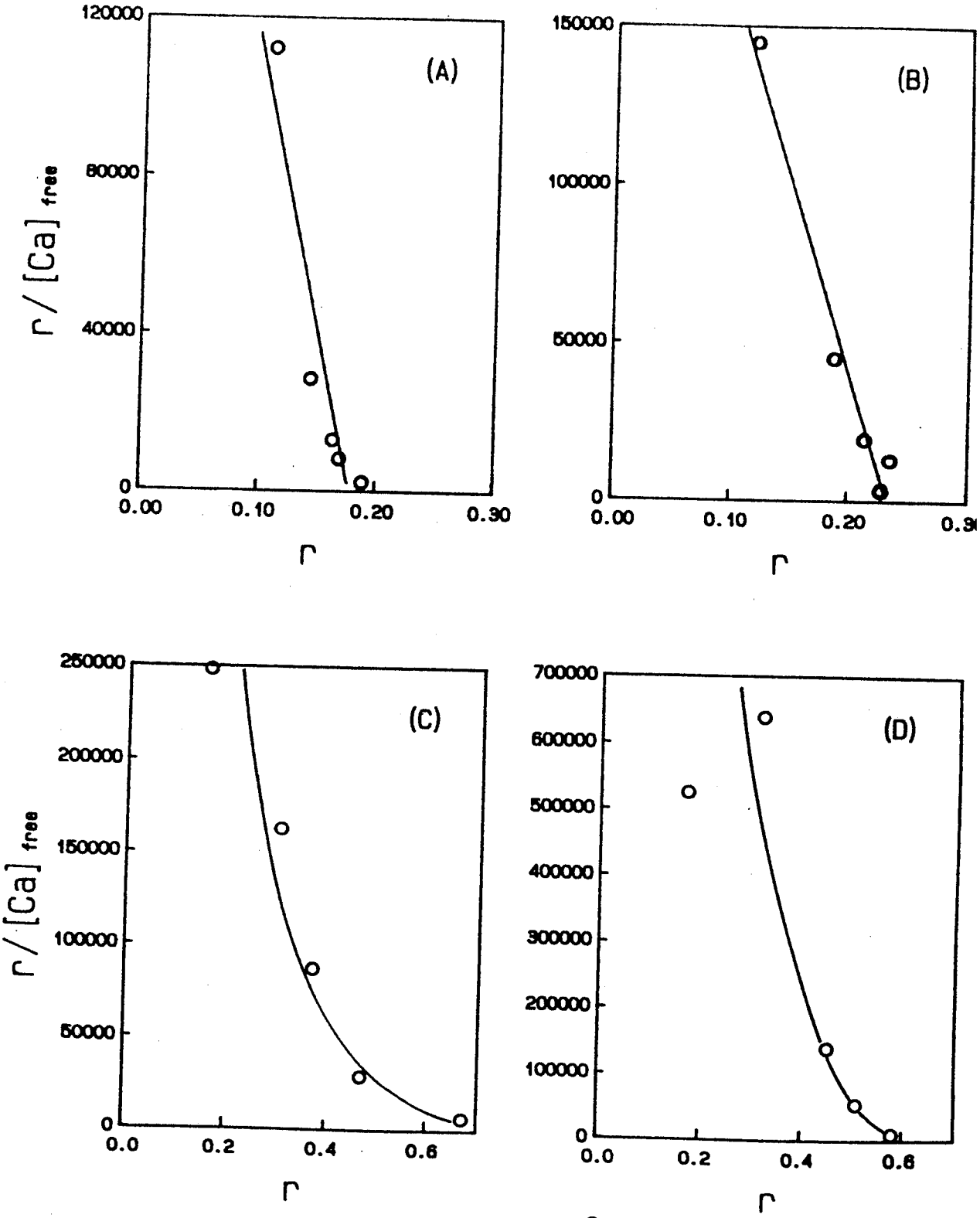


Fig.10-(a) Scatchard plot of Ca^{2+} v.s. CM-chitins

(A): 6-O-CM-chitin from α-chitin

(B): 6-O-CM-chitin from β-chitin

(C): 3,6-O-CM-chitin from α-chitin

(D): 3,6-O-CM-chitin from β-chitin

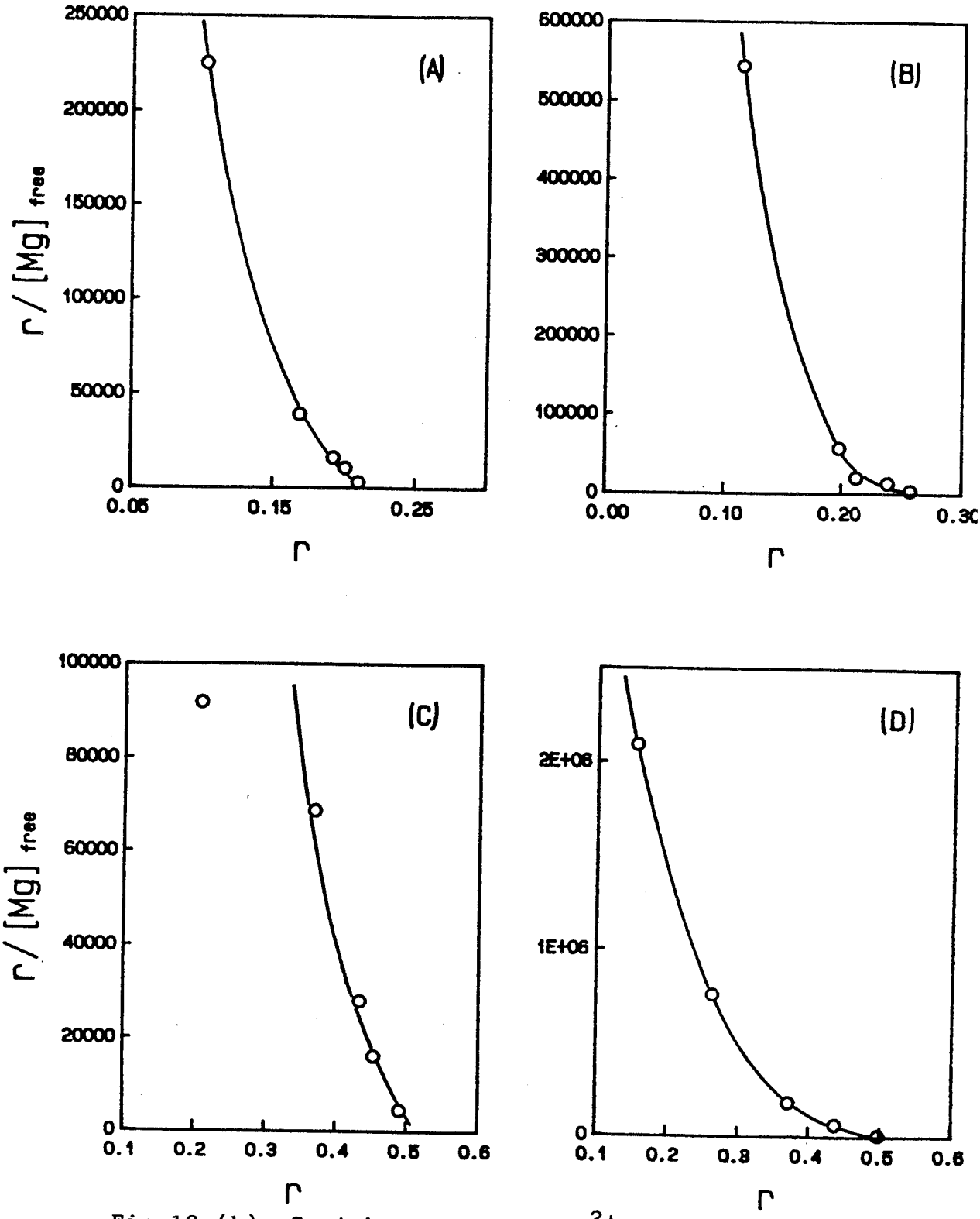


Fig.10-(b) Scatchard plot of Mg^{2+} v.s. CM-chitins

(A): 6-O-CM-chitin from α-chitin

(B): 6-O-CM-chitin from β-chitin

(C): 3,6-O-CM-chitin from α-chitin

(D): 3,6-O-CM-chitin from β-chitin

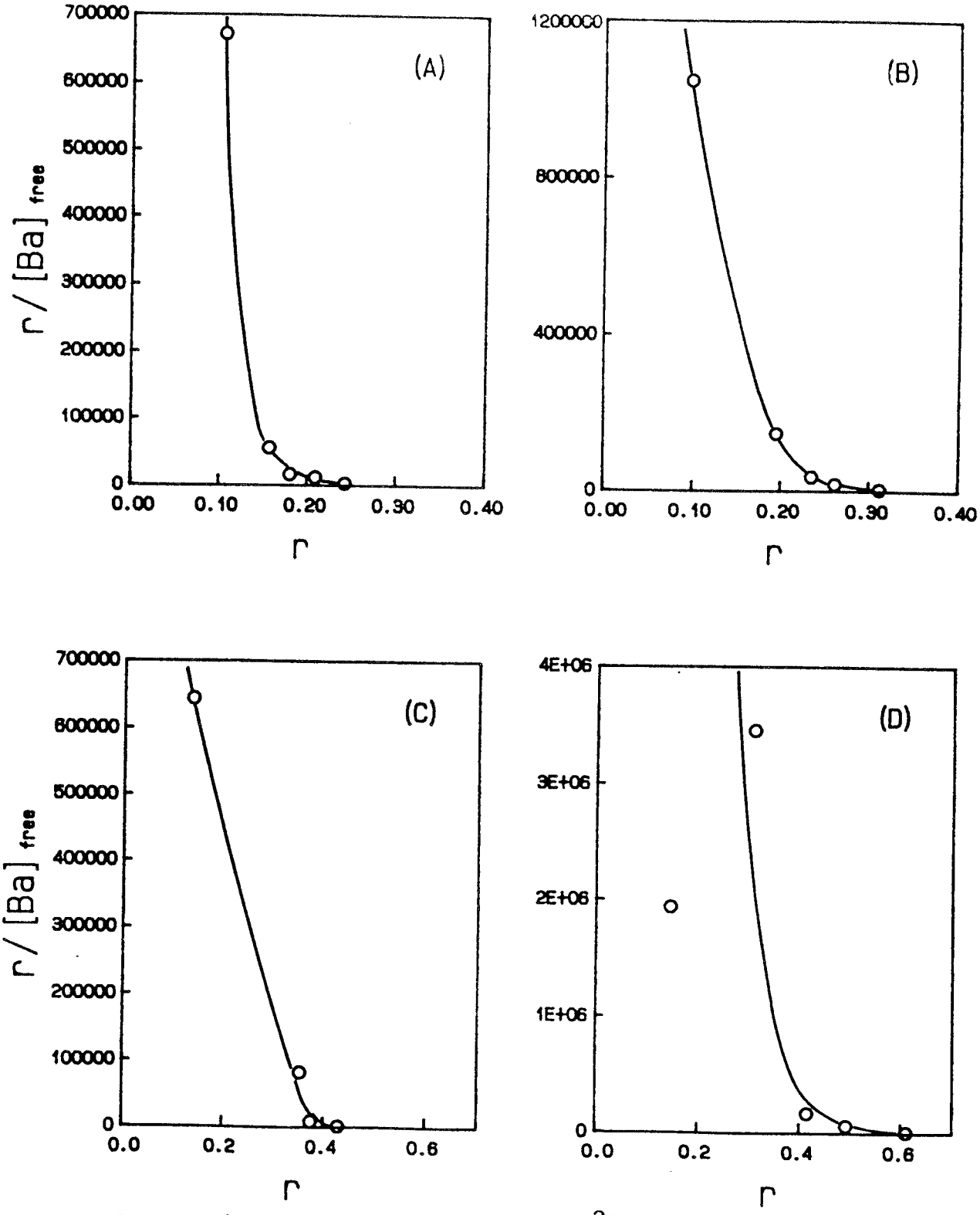


Fig.10-(c) Scatchard plot of Ba^{2+} v.s. CM-chitins

(A): 6-O-CM-chitin from α-chitin

(B): 6-O-CM-chitin from β-chitin

(C): 3,6-O-CM-chitin from α-chitin

(D): 3,6-O-CM-chitin from β-chitin

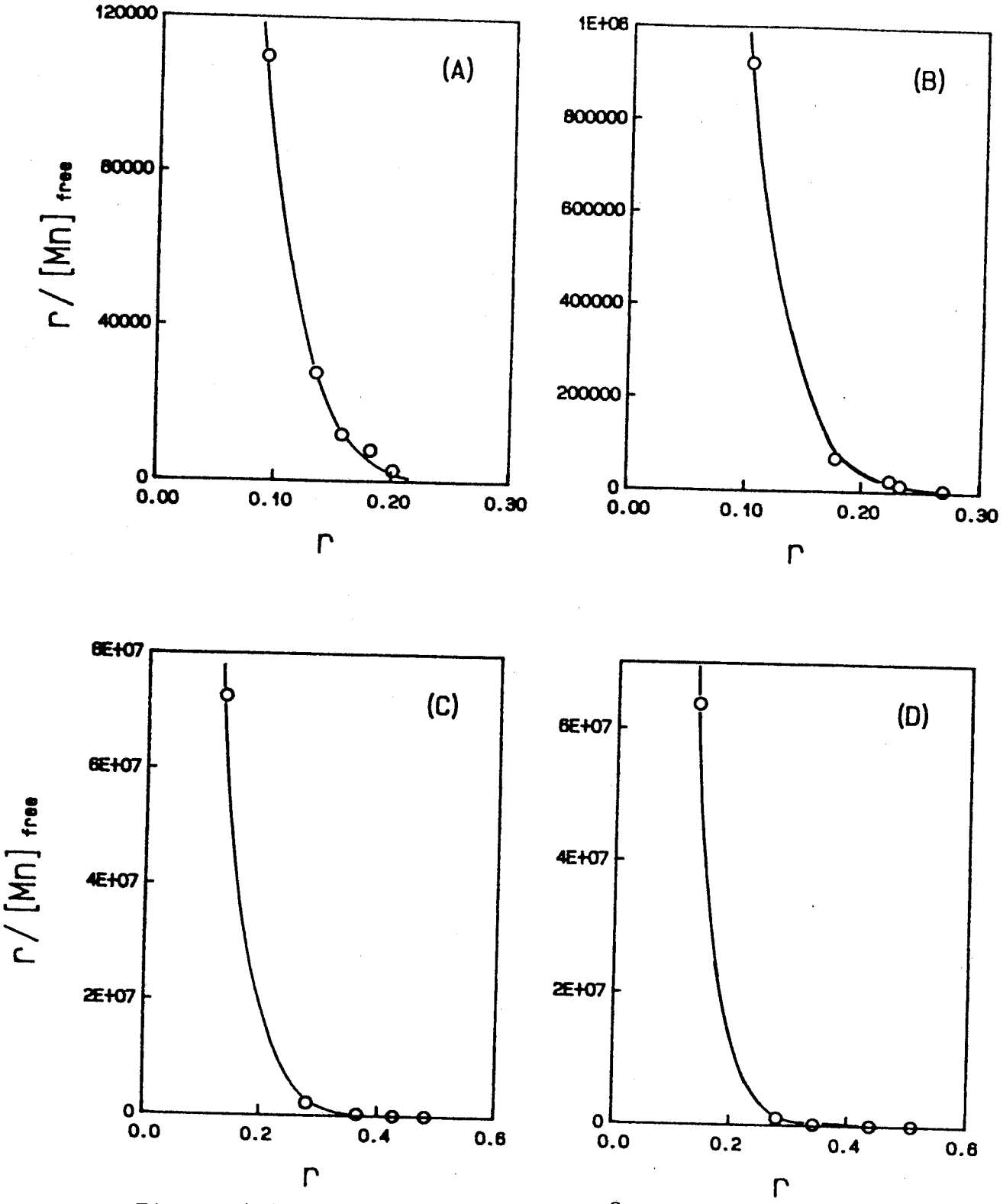


Fig.10-(d) Scatchard plot of Mn^{2+} v.s. CM-chitins

(A): 6-O-CM-chitin from α-chitin

(B): 6-O-CM-chitin from β-chitin

(C): 3,6-O-CM-chitin from α-chitin

(D): 3,6-O-CM-chitin from β-chitin

Table VII. Binding properties of metal ions to anionic chitin derivatives .

chitin deriv.		Mg^{2+}	Ca^{2+}	Ba^{2+}	Mn^{2+}
6-O-CM-chitin from α-chitin	Kd	1.2×10^{-6}	2.5×10^{-6}	4.7×10^{-6}	4.9×10^{-6}
	Ka	8.6×10^{5}	5.9×10^{5}	2.1×10^{5}	2.1×10^{5}
(D.S.0.58)	n	0.2	0.2	0.3	0.2
6-O-CM-chitin from β-chitin	Kd	1.3×10^{-6}	2.5×10^{-6}	2.5×10^{-6}	2.3×10^{-6}
	ka	7.8×10^{5}	4.1×10^{5}	4.0×10^{5}	3.7×10^{5}
(D.S.0.74)	n	0.3	0.3	0.3	0.2
3,6-O-CM-chitin from α-chitin	Kd	2.5×10^{-6}	4.1×10^{-6}	1.1×10^{-6}	3.8×10^{-7}
	Ka	4.0×10^{5}	2.4×10^{5}	8.9×10^{5}	2.6×10^{6}
(D.S.1.36)	n	0.5	0.7	0.4	0.5
3,6-O-CM-chitin from β-chitin	Kd	7.2×10^{-7}	1.0×10^{-6}	1.3×10^{-6}	9.7×10^{-7}
	Ka	1.4×10^{6}	10.0×10^{5}	7.9×10^{5}	1.0×10^{6}
(D.S.1.53)	n	0.5	0.6	0.6	0.5
Succinyl-chitosan	Kd	3.0×10^{-6}	1.1×10^{-6}	6.1×10^{-6}	2.3×10^{-6}
	Ka	3.4×10^{5}	9.2×10^{5}	2.1×10^{5}	4.4×10^{5}
(D.S.0.86)	n	0.3	0.2	0.3	0.2

Adsorption capacities were investigated with equilibrium dialysis. Then binding properties were estimated from scatchard plot.

Kd: dissociation constant($mol\cdot dm^{-3}$), Ka: association constant(M^{-1})

n: number of binding site in a glucosamine derivative residue.

operative.

As nickel ion is adsorbed nonspecifically on 6-O-CM-chitin from α-chitin, a specific geometrical arrangement of functional groups to adsorb calcium ion would suggest by the difference in the FT-IR spectra of CM-chitin-Ca and CM-chitin-Ni complexes (Fig.11). Though the doublet peak due to stretching of carbonyl group is simply decreased by the adsorption of nickel ion, the doublet peak is converted to a singlet and decreased more quickly by the adsorption of calcium ion than by that of nickel. The peak at 1280cm^{-1} due to secondary hydroxyl groups almost disappears in the presence of calcium ion but not by nickel ion. Several peaks in the region of 1000-1100cm^{-1} are also depressed by the adsorption of ions, and nickel ion seems to affect them less than calcium ion, suggesting participation of primary hydroxyl groups to the adsorption of calcium ion.

The participation of amide group in the specific adsorption of calcium ion was also suggested by the IR spectrum of dried CM-chitin-Ca complex(33). The CM-chitin-Ca complex might be specifically composed of several functional groups on N-acetylglucosamine residue as mentioned above.

These results might suggest generally to the difference of contribution of functional groups on the adsorptions of metal ions. The possible explanations for adsorption mechanism might be as follows,

i)As the adsorption of all metal ions except calcium ion is contributed only by carboxyl groups in wide area, one ion

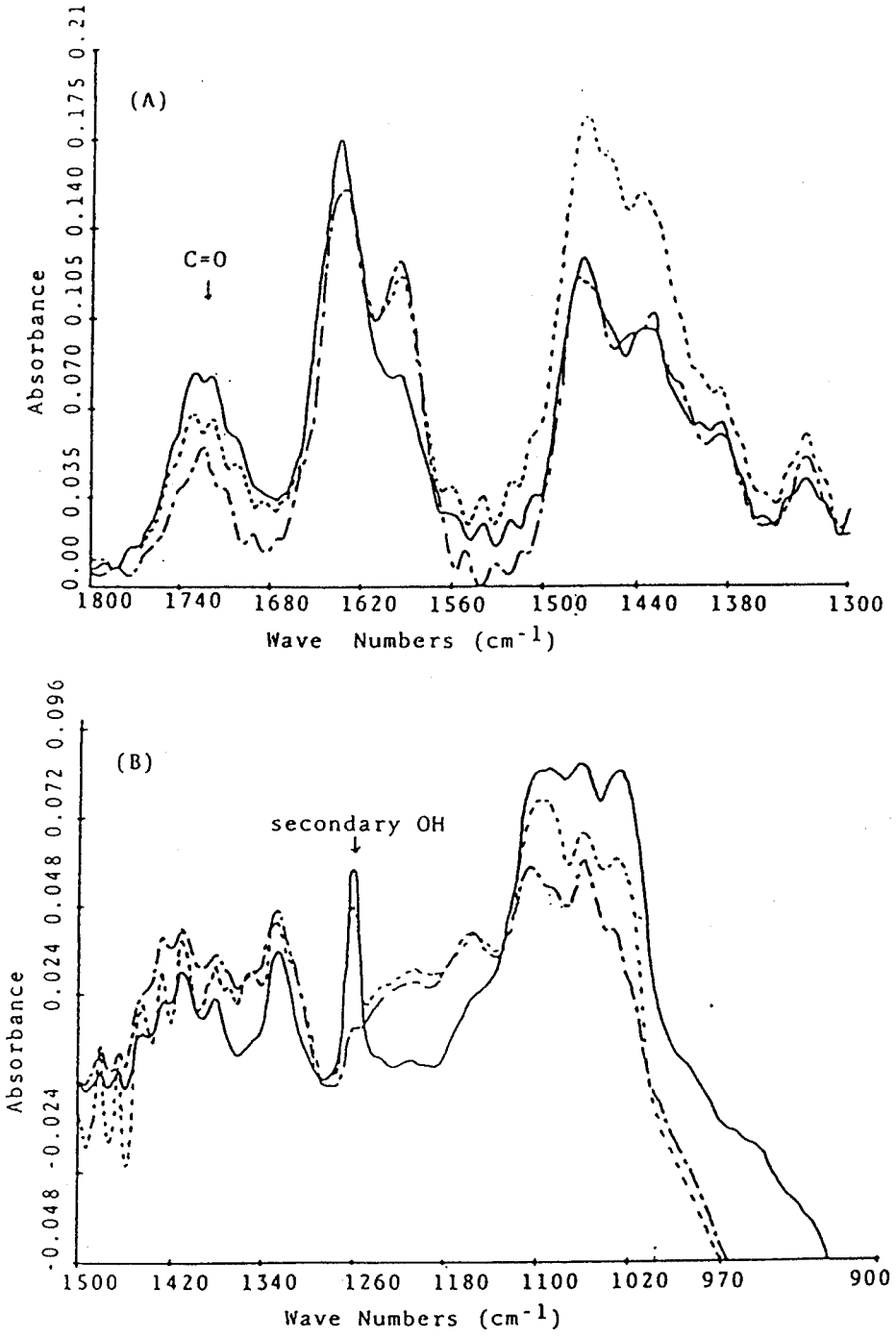


Fig.11 FT-IR absorption spectra of water-soluble CM-chitin(D.S.=0.88) and its metal ion complexes in H_2O and D_2O

(A)In D_2O, (B)in H_2O; (——)CM-chitin only, (--)CM-chitin-Ni^{2+} complex,(-·)CM-chitin-Ca^{2+} complex

molecule occupies a few of carboxyl groups. Therefore, when next ion tries to bind adjacent binding site, the binding seems to be hindered by the repulsion of cations in addition to the depress of charge on CM-chitin. Scatchard plots for nonspecific metal ions suggested negative cooperative effect to adsorb metal ion, because the repulsion phenomena were remarkably observed at higher metal ion concentration.

ii)The adsorption site for calcium ion seems to be formed in very limited domain by intra and inter residual interaction through the electrostatic interaction of two carboxyl groups. Additive contributions are also likely achieved by acetamide group at C-2 position, primary and secondary hydroxyl groups at C-6 and C-3 position, respectively. Binding site seems not to interfere each other as suggesting by the straightness of Scatchard plot.

iii)The specificity of calcium ion to 6-O-CM-chitin from α-chitin seems to concern with the natural composition of functional group of α-chitin. Though chitin has not the binding ability for calcium, chitin maintains calcium ion together with proteins in nature. So, carboxymethyl group seems to play a role as the side chain of proteins to give the adsorption specificity. Since β-chitin exist almost without calcium carbonate and a large of proteins in nature, CM-chitin from β-chitin is

expected to be low binding ability for calcium. However, CM-chitin from β-chitin has the specificity of calcium ion as similar level as that from α-chitin by the similar figure of Scatchard plot and the binding constant.

Though addition of ferric ion to CM-chitin induced gel as shown in Table V, ferrous ion did not bring gel formation. The mechanism of gelation was assumed by the gyration radii of polymer chains with the X-ray small angle scattering comparing to that of CM-chitin-Ca complex in solution. This result would be similar mechanism as that for other gelation such as 3,6-O-CM-chitin gel.

GYRATION RADII OF CROSS-SECTION OF 6-O-CM-CHITIN-METAL IONS COMPLEX BY X-RAY SMALL-ANGLE SCATTERING

In this section, a semi-dilute CM-chitin solution is studied by small-angle X-ray scattering(SAXS) to examine the effect of species of metal ion to CM-chitin.

Fig.12 shows the scattering profile of the CM-chitin solution in salt free system. The profile is characterized by a weak scattering at the limit of $q \to 0$, where $I(q)$ is scattering law and q is scattering vector, and a broad peak at a higher q, whose features depend on whether neither CM-chitin is H-type nor Na-type. These are the typical features in polyelectrolyte solutions. The q_m value at the maximum of the broad peak is about 6.5×10^{-2} Å^{-1} and the monomer concentration of the CM-chitin is 4.7×10^{-5}. The experimental point

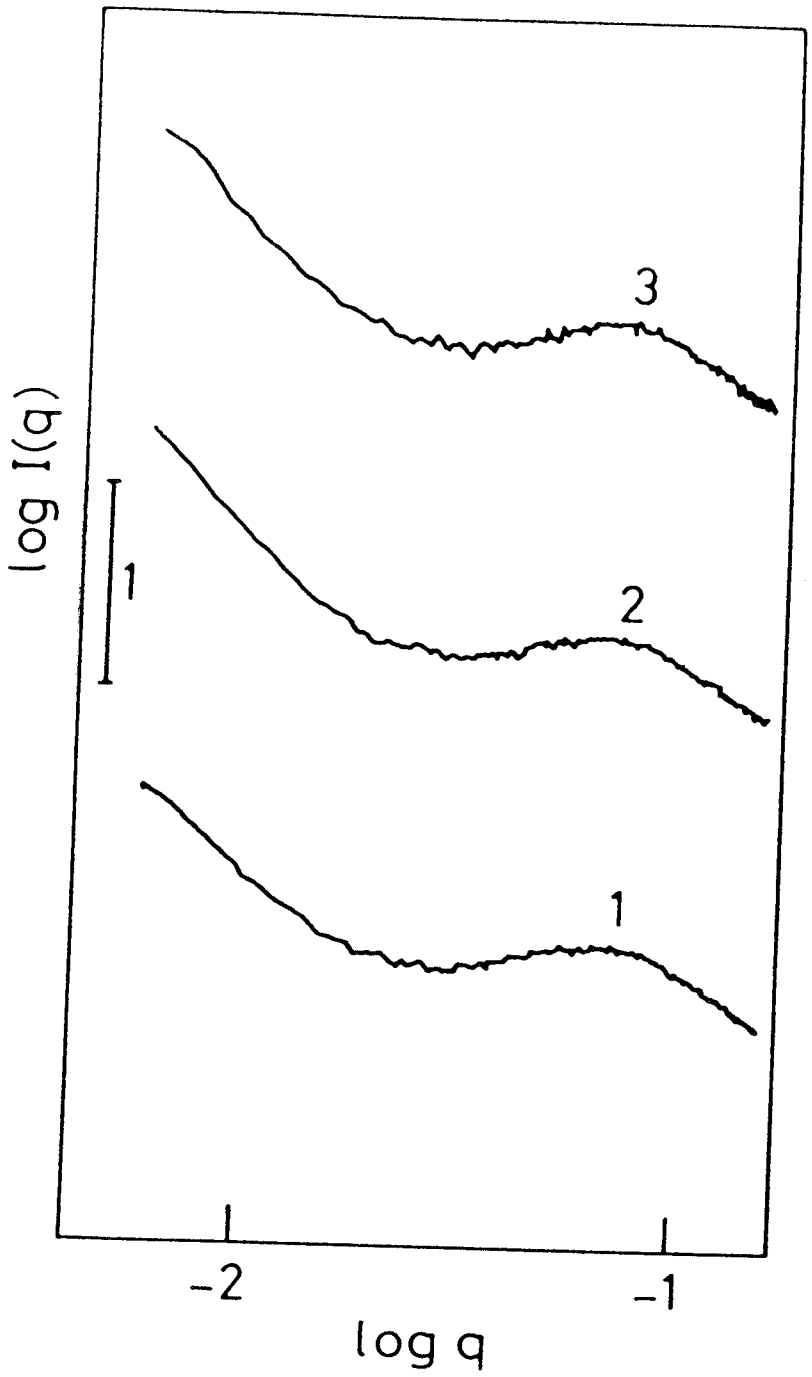


Fig.12 Scattering profile of the CM-chitin solution in salt free

1. H type (CM-chitin concentration :1.9%)
2. Na type (CM-chitin concentration :2.0%, the following is the same concentration)
3. H type

deviate upward from the calculated curve based on the theory of Koyama(43). It means that the radius of gyration of cross-section cannot neglect compared with the total length of complexed molecule.

As the concentration of Ca^{2+} increases, the osmotic pressure is reduced, which is observed as the increases of the forward scattering as shown in Fig.13. The scattering profile at intermediate q is approximated by the scattering function of rod, though the profile deviates at low q. The deviation reflects the effect of the chain flexibility.

The addition of Fe^{2+} causes the changes of whole profile of the scattering profile. The scattering profile is characteristic of that of gel as shown in Fig.14. The scattering exponent is about 2.0 at intermediate q range, whose value agrees well with the theoretical value(44). The sol-gel transition is caused by the cross-link formation according to the oxidation of Fe^{2+} to Fe^{3+} in air during the preparation.

The radii of gyration of the cross-section , R_{app}, of the CM-chitin were estimated from the Guinier plot for the cross-section. R_{app} does not change by the addition of Ca^{2+} but by that of Fe^{3+} oxidized from Fe^{2+} in air. The increase of R_{app} is ascribed in the gel formation as shown in Table VIII(41).

To clarify the mechanism of gelation by Fe^{3+}, it is examined that the q dependence of the scattering law S(q) and its variation of CM-chitin, Fe^{2+} and Fe^{3+}.

Taking into account that the chitin molecule is a stiff

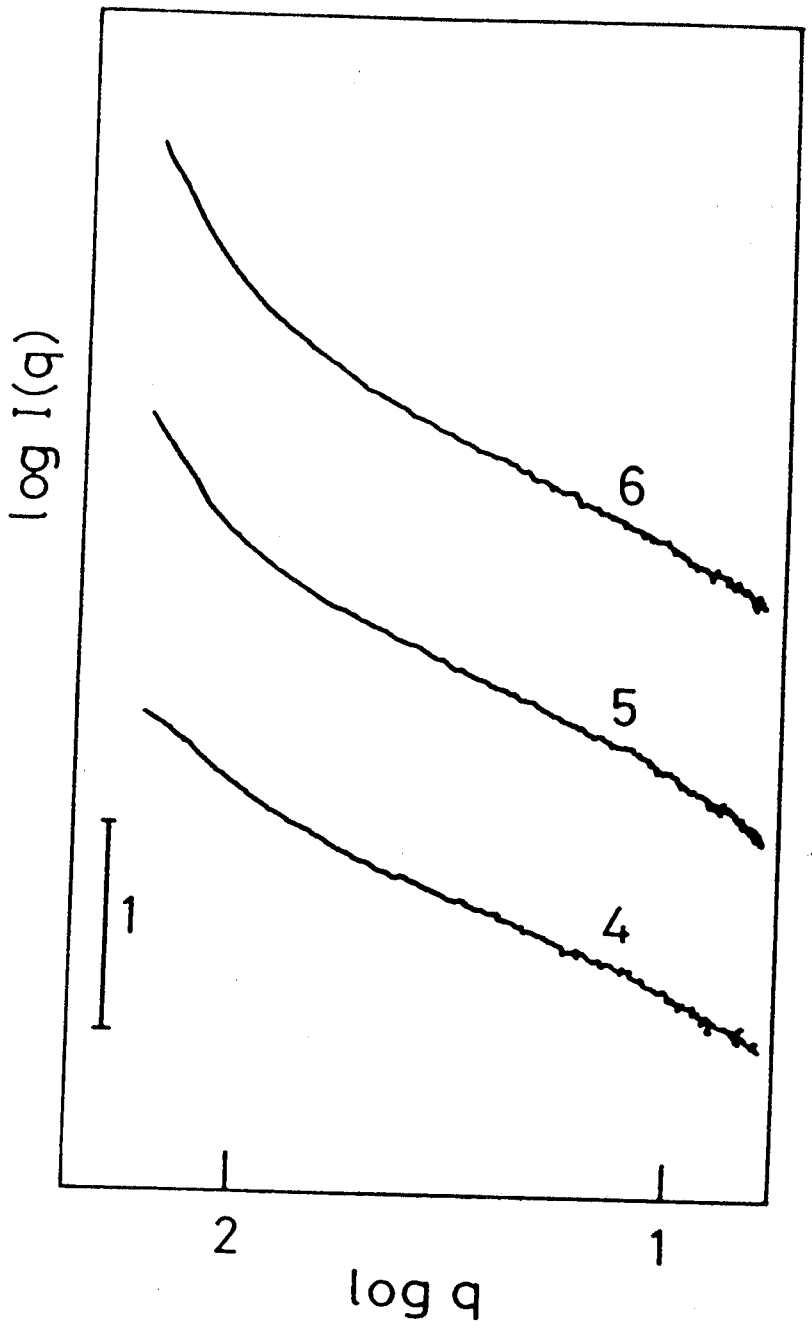


Fig.13 Scattering profile of the CM-chitin solution as a function of Ca^{2+} concentration

4. $CaCl_2$ (0.522)
5. $CaCl_2$ (0.404)
6. $CaCl_2$ (0.306)

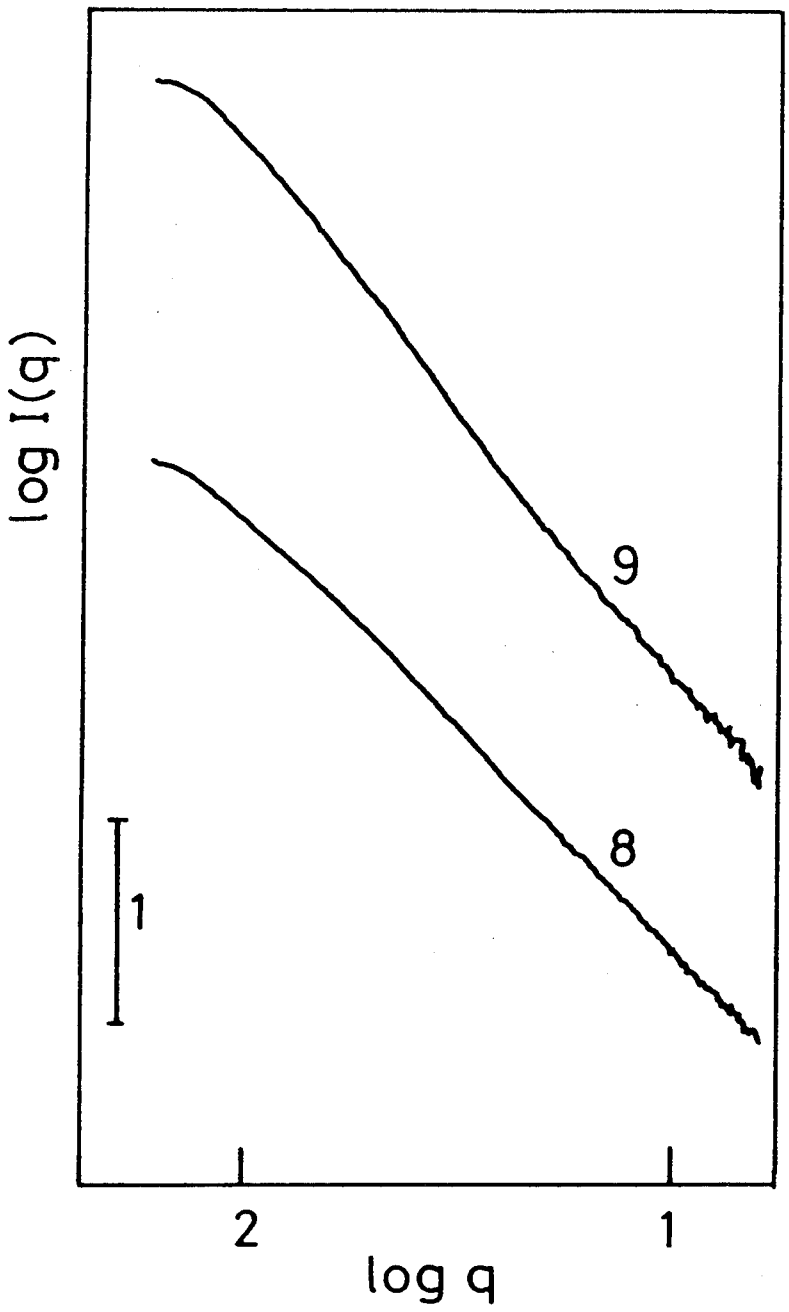


Fig.14 Scattering profile of the CM-chitin solution as a function of Fe^{2+} concentration

8. $FeCl_2$ (0.35)
9. $FeCl_2$ (0.178)

Table VIII Radii of Gyration of CM-chitin-calcium or -ferrous ion complex(Cp=2%)

Sample No.	Ion	Salt conc.	Rc(Å)	Comment
1	H type	0	5	Cp=1.9%
2	Na type	0	4	
3	H type	0	5	
4	$CaCl_2$	0.522	4	
5	$CaCl_2$	0.404	5	
6	$CaCl_2$	0.306	4	
7	$Fe^{3+(*)}$	0.532	-	heterogeneous gel not detectable
8	$Fe^{3+(*)}$	0.35	8	heterogeneous gel
9	$Fe^{3+(*)}$	0.178	9	gel

(*):$FeCl_2$ solution was oxidized to Fe^{3+}

chain, it is assumed that the scattering law at moderate angle is expressed by the equation

$$I(q) = I(0)/(1+q^2\xi^2)^{1/2} \qquad (1)$$

where ξ is the mean distance between the neighbouring chains. Fig.15 shows the plot of the ξ as a function of the concentration of CM-chitin at different concentrations of Fe^{2+} in preparation under the nitrogen atmosphere because of preventing from oxidization by air. The broken line has a negative slope of 1/2. Noting that the ξ corresponds to the inverse of q_m in the Koyama theory(43), this behavior of ξ is within accessible range. Fig.16 shows the plot of ξ as a function of the concentration of Fe^{2+} at different concentrations of CM-chitin. The experimental points are expressed by the broken line with a positive slope of 1/2. Though no prediction is given to the added salt dependence on ξ in Koyama theory, ξ is usually reduced by the reduction of the electrostatic force. This theoretical value contradicts apparently with the experimental result in Fig.16. It is suggested by taking into account that the ξ estimated from eq.1 corresponds to the size of a less local area than the whole size of CM-chitin molecule.

The behavior of the scattering profile at the addition of Fe^{3+} is totally different from that at the addition of Fe^{2+}. The scattering exponent obtained at moderate q is about 2.0, which supports the result above. The radii of gyration of the cross-section R_{app} of the CM-chitin were estimated from the Guinier plot for the cross-section. The R_{app} in Fe^{2+} is comparable to that in Ca^{2+} and the large

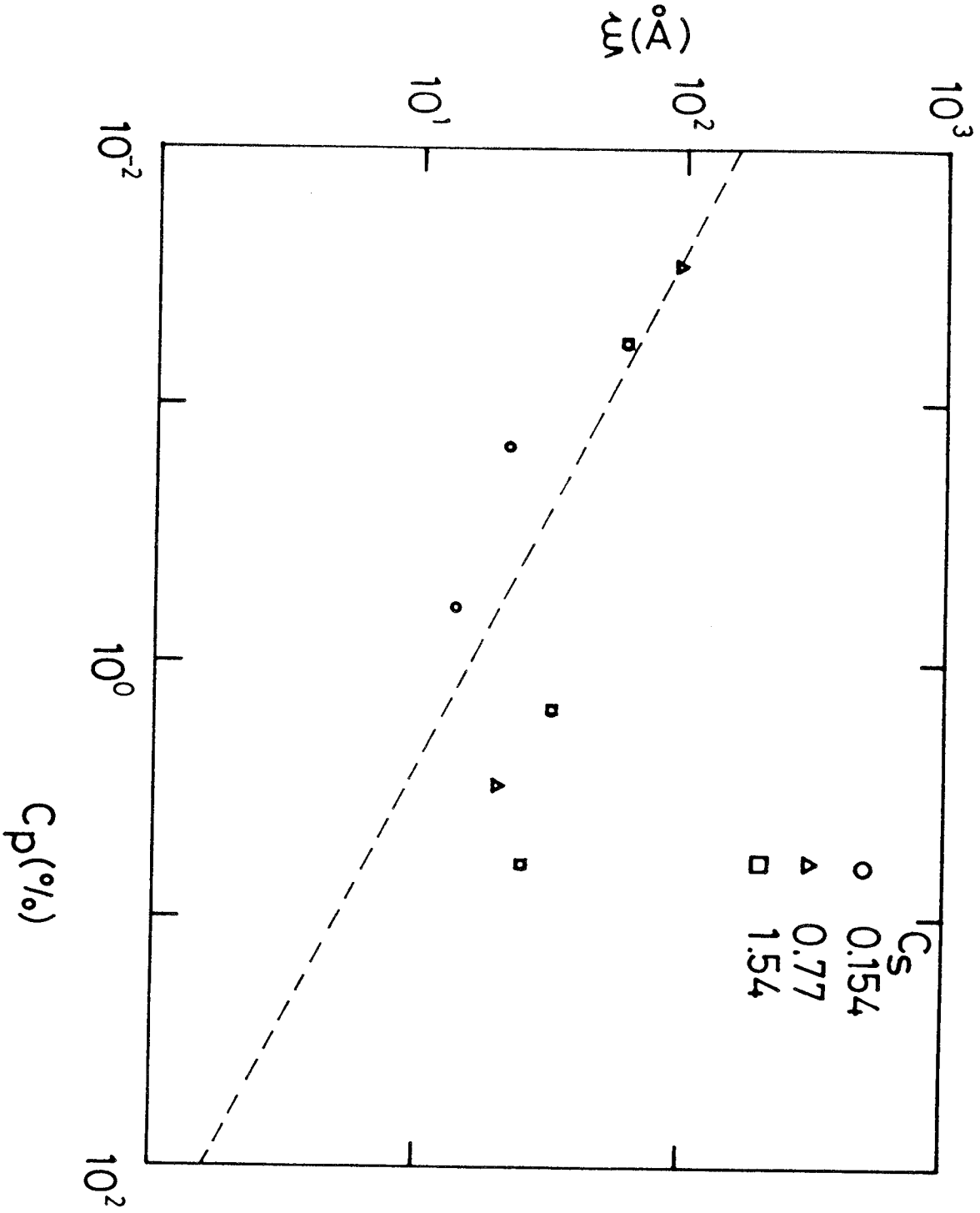


Fig.15 Plot of ξ as a function of the concentration of CM-chitin at different Fe^{2+} concentrations

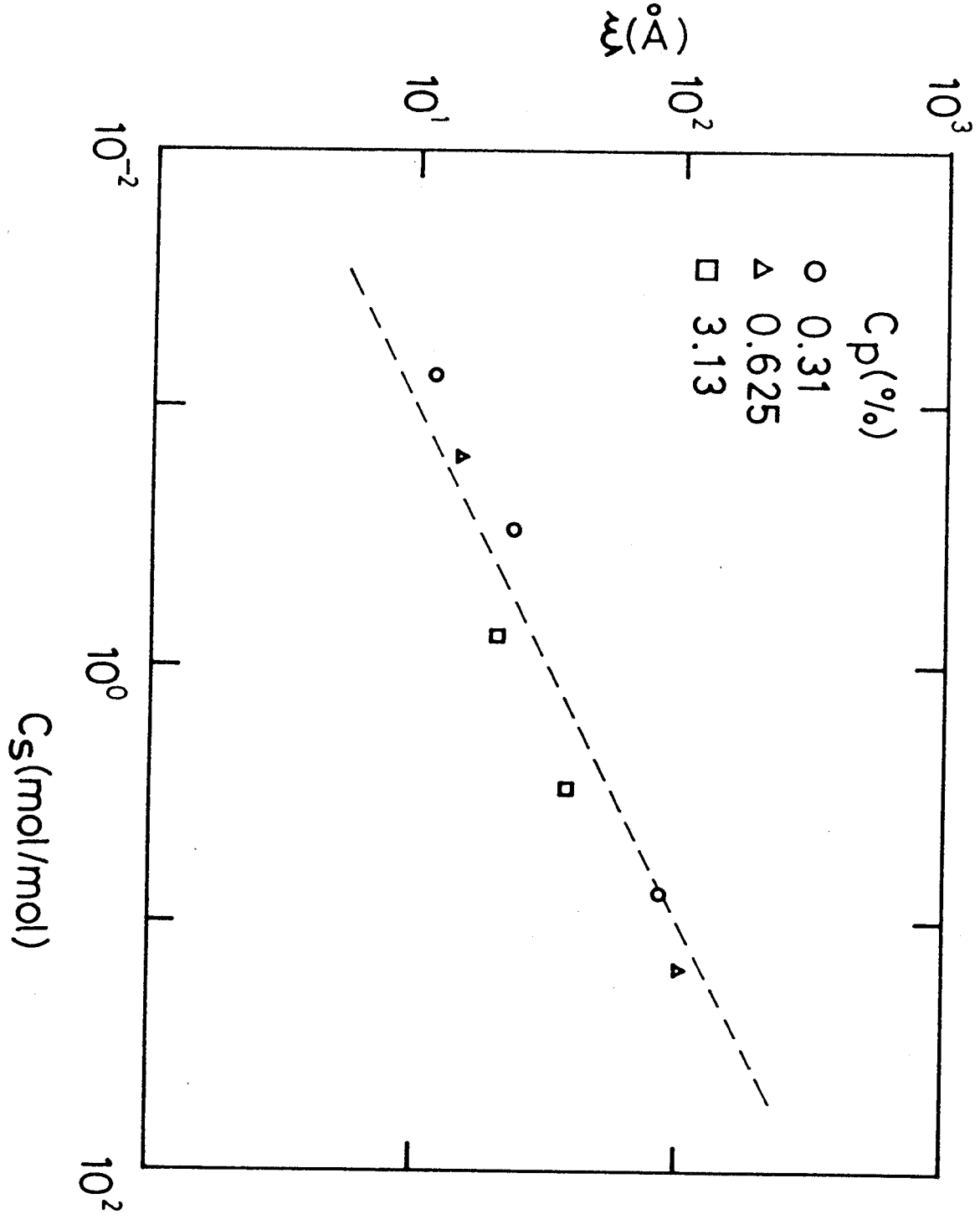


Fig.16 Plot of ξ as a function of the Fe^{2+} concentration at different concentrations of CM-chitin

value of R_{app} in Fe^{3+} is comparable to that in oxidized Fe^{2+}, Fe^{3+}, reported above.

It is concluded that the behavior of scattering profile by the addition of Fe^{3+} is comparable to that of the solution in which Fe^{2+} is oxidized as shown in Table IX(42).

Since 5Å of radius of gyration for cross-section is consistence with 4.8Å of a unit of crystal lattice constant in a axis estimated from X-ray diffraction, calcium ion seems to adsorb CM-chitin between the inter two chains.

6-O-CM-chitin from β-chitin and all 3,6-O-CM-chitin were found to form gel, followed by precipitation, in the presence of transition metal ions. As inter chain cross-linking is necessary for gel formation, transition metal ions seemed to induce the cross-linking between CM-chitin chains similarly to the mechanism of gel formation by the addition of Fe^{3+}. These phenomenon suggests that transition metal ions bind with carboxyl groups around whole area of polymer chains to bundle several chains. The crystalline structure was found to regulate the function of CM-chitin because loose crystalline structure of β-chitin was still maintained in its derivatives owing to the distribution of carboxymethyl group homogeneously along to molecules. It clearly showed the difference between the calcium ion binding and the adsorption of other metal ions. Calcium ion binds to CM-chitin tightly in the local specific area which is formed by two chains or residues, which seems to be two residue, and other metal ions are supported only by carboxyl groups in the wide region of polymer chains.

Table IX Radii of Gyration of CM-chitin Iron Ion Complex

Ion	Cs(mol/mol)	Cp(%)	Rc(Å)
Const. of conc. of additional salts			
Fe^{2+}	0.15	0.15	2.5
Fe^{2+}	0.154	0.625	2.7
Fe^{2+}	0.77	0.03	3.3
Fe^{2+}	0.77	3.13	3.0
Fe^{2+}	1.5	0.06	4.6
Fe^{2+}	1.54	6.25	3.9
Const. of concentration of CM-chitin			
Fe^{2+}	0.077	0.31	2.9
Fe^{2+}	0.3	0.31	3.2
Fe^{2+}	7.7	0.31	2.9
Fe^{2+}	0.154	0.625	2.7
Fe^{2+}	15.4	0.625	6.3
Fe^{2+}	0.77	3.13	3.0
Fe^{2+}	3.0	3.13	3.5
Fe^{3+}	3.0	3.13	-
Fe^{3+}	15.0	0.63	9.5

Cs: salt concentration (salt/CM-GlcNAc)
Cp: polymer concentration
Rc: radii of gyration

A Fe^{2+} adsorbed 6-O-CM-chitin adsorbed keeping the soluble state, while Fe^{3+} adsorbed one forms gel. The adsorption site seems to be increased by the increase of the positive charge for adsorption of metal ion. 3,6-O-CM-chitin from both structure also forms the gel and precipitate by the adsorption of barium ion, which had the largest ion radius among alkali-earth metal ions. Gel formation and precipitation on the adsorption of metal ions to 3,6-O-CM-chitin suggest that inter-chain cross-linking occurred by the adsorption of metal ions owing to the high charge density of carboxymethyl group. Especially gel formation proceeds smoothly by metal ions of large ion radius or multivalent positive charge.

Though the adsorption capacity of calcium ion to 3,6-O-CM-chitin shows the highest value among other ions till 5mM of fairly high concentration, its Scatchard plot indicates negative cooperativity. 3,6-O-CM-chitin did not show the specificity to calcium ion. It might be suggested that one of the calcium adsorption site in CM-chitin molecular was blocked by re-carboxymethylation. Thus the mechanism of calcium ion adsorption to CM-chitin was elucidated, and functionalization of chitin was found to be depend on the crystalline structure and the degree of carboxymethylation.

SPECIFICITY OF CALCIUM ION TO 6-O-CM-CHITIN

All metal ions which adsorbed on 6-O-CM-chitin were released thoroughly by pH shift, but calcium ion was released incompletely by pH shift (88% of total adsorption,

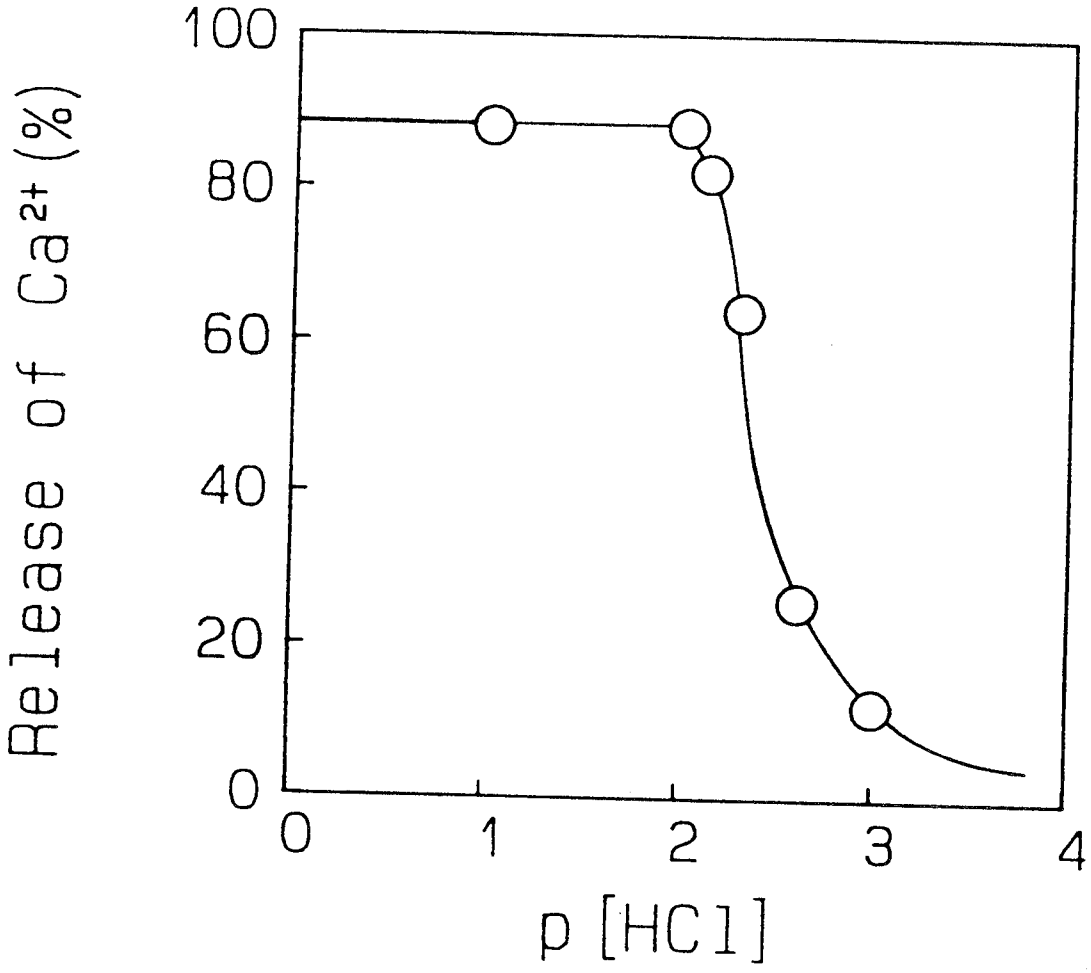


Fig.17 Release of Ca^{2+} from 6-O-CM-chitin

0.3g of low substituted 6-O-CM-chitin was immersed in 20mL of 0.05M $CaCl_2$ aqueous solution under shaking for 24hours at room temparature and the CM-chitin was washed by water to remove free calcium ion. Amount of released Ca^{2+} was estimated from the supernatant concentration after CM-chitin-Ca complex was immersed in several HCl concentration in shaking for 24h.

see Fig.17). For the complete release of calcium ion, chelating reagent such as EDTA was requested. It also suggests the tight adsorption of calcium ion to 6-O-CM-chitin. Other CM-chitins release calcium ion simply by pH shift.

The adsorptions of calcium and magnesium ions to 6-O-CM-chitin were measured by the batch method in the Ca-Mg mixed solution as shown in Table X.

The distribution coefficient for calcium ion, which was estimated from capacity for calcium ion per capacity for magnesium ion, was high value (more than 1.0) in all ionic strength. Especially in the ionic strength of 0.5, the coefficient was as high as 4.3. 6-O-CM-chitin was thus suggested to have the selective adsorption ability toward calcium ion. When magnesium ion adsorbed CM-chitin column was washed out with aqueous calcium chloride solution, the magnesium ion was found to be released from 6-O-CM-chitin completely instead of the adsorption of calcium ion to CM-chitin as reported previously(33).

In the high concentration of magnesium ion, 6-O-CM-chitin-Mg complex is unstable probably owing to the repulsion between the magnesium ions as shown by the negative cooperativity. On the other hand, calcium ion forms a stable complex with CM-chitin. Consequently, the calcium ion forced to push out the magnesium ion from CM-chitin in ion exchange mode. In the equilibrium state also, only calcium ion is adsorbed to CM-chitin similarly.

The present study suggests that 6-O-CM-chitin is higher

Table X Selective adsorption in Ca^{2+}-Mg^{2+} mixture system

I / Ion	0.35(meq/g)	0.50(meq/g)	0.65(meq/g)
Total	0.56	0.48	0.44
Ca^{2+}	0.31	0.39	0.28
Mg^{2+}	0.25	0.09	0.16
D.C	1.2	4.3	1.8

D.C; Distribution coefficience (Capacity Ca/Capacity Mg)
I ; Ionic strength

specific for calcium ion, especially in the low concentration, together with the ion exchange ability.

It is very important to elucidate the property of the surface of CM-chitin-Ca complex to know the fundamental structure and to consider the possibility for utilization as biocompatible polymer. The complex surface was tried to clarify from the standpoint of hydrophobic or hydrophilic. The property was estimated from the adsorption behavior of organic compound to 6-O-CM-chitin-Ca complex, which can be predicted rather simple term of the degree of hydrophilicity or hydrophobicity. Though this method will give the indirect result, the informations obtained from this experiment would be significant.

Amino acids were selected as the molecules to be adsorbed, as amino acids have α-amino and α-carboxyl groups in main chain, and several hydrophobic and hydrophilic functional groups in side chain.

The investigation on the adsorption of amino acids to CM-chitin-Ca complex would give valuable information for the interaction of CM-chitin with a fibrinogen in the presence of calcium ion, which has high affinity to hydrophobic materials(18).

ADSORPTION OF AMINO ACIDS ON CM-CHITIN METAL ION COMPLEX

Since the interaction of 6-O-CM-chitin with fibrinogen, one of the blood proteins, was enhanced in the presence of calcium ion, the CM-chitin-Ca complex is expected to

interact tightly with the hydrophobic domain of fibrinogen (18,22). *D,L*-amino acids having different pI values were used to estimate the adsorption capacity on CM-chitin-Ca complex(low substituted 6-O-CM-chitin), as listed in Table XI. The adsorption of *D,L*-Phe was enhanced about 50 times in the case of calcium chelation compared to CM-chitin without calcium ion.

As the effective number of calcium ion for *D,L*-Phe adsorption is lower than the total number of adsorbed calcium ions, the geometrical arrangement of the functional groups of the CM-N-acetylglucosamine residue would be the driving force for the Phe adsorption, including that with calcium ion. The adsorption of *D,L*-Lys and *D,L*-Glu were reduced to almost 1/10 and 1/5 in the presence calcium ion respectively. The adsorbed phenylalanine was eluted only by EDTA, while the other adsorbed amino acids were eluted by a shift of the ionic strength. Since there was only slight calcium effect on the adsorption of amino acids to fibrous 6-O-CM-chitin, as expected from its adsorption behavior for metal ions, the original binding site for Phe on the CM-chitin molecule seems to have been eliminated by the stretching procedure on the preparation of fiber.

There is also little selectivity for the optical isomer of phenylalanine The adsorption profiles of *D,L*-amino acids for high substituted 6-O-CM-chitin-metal ions complexes from α-chitin were studied using equilibrium dialysis comparing to that of other CM-chitins, as shown in Table XII. The adsorption capacities of 6-O-CM-chitin from α-chitin toward

TABLE XI Adsorption of Amino Acids to Low Substituted CM-Chitin and Fibrous CM-Chitin in the Presence and Absence of Calcium Ion[a]

Amino acid	Ca^{2+} chelate	Capacity, μmol/g	
		Low substituted (DS 0.1)	Fibrous
D,L-Phe	-	0.65	2.0
	+	33	2.0
D,L-Lys·HCl	-	2000	73
	+	300	65
D,L-Glu	-	0.22	0
	+	0.04	0.2

[a]Amino acids were applied to the column to equilibrium, and it was then washed with deionized water. Then *D,L*-Lys and *D,L*-Glu were eluted with 0.5 *M* aqueous NaCl solution. *D,L*-Phe was eluted by 0.1 *M* aqueous EDTA solution at room temperature.

Table XII-a Adsorption of amino acids on water-soluble 6-O-CM-chitin from α-chitin in the presence of several ions

Amino acids	p*I*	Non (μmol/g)	Ca^{2+} (μmol/g)	Mn^{2+} (μmol/g)	Ba^{2+} (μmol/g)	Mg^{2+} (μmol/g)
Nonpolar:						
D,L-Ala	6.0	80	17	13	24	19
D,L-Val	6.0	56	89	21	55	58
D,L-Leu	6.0	27	110	28	47	54
D,L-Phe	5.5	100	390	310	43	34
Neutral polar:						
D,L-Trp	5.9	45	48	48		
Acidic:						
D,L-Glu	3.2	60	32	19	46	39
D,L-Asp	3.0	110	28	26		
Basic:						
D,L-Lys·HCl	9.8	2700	370	370	323	247
D,L-Arg·HCl	10.8	2400	530	490		
D,L-His·HCl	7.6	120	57	30		

Amino acid-water soluble CM-chitin mixture was dialyzed against deionized water to remove free amino acids, and then adsorbed amino acids were released by pH shift(pH 1.0), and it was redialyzed against deionized water to quantify adsorption capacities.

Table XII-b Adsorption of amino acids on water-soluble CM-chitins in the presence of Ca^{2+}

	6-O-CM-chitin from α-chitin		6-O-CM-chitin from β-chitin		3,6-O-CM-chitin from α-chitin	
Amino acids	Non (μmol/g)	Ca^{2+} (μmol/g)	Non (μmol/g)	Ca^{2+} (μmol/g)	Non (μmol/g)	Ca^{2+} (μmol/g)
Nonpolar:						
D,L-Ala	80	17	33	18	34	100
D,L-Val	56	89	22	27	21	13
D,L-Leu	27	110	36	13	17	19
D,L-Phe	100	390	14	21	8	18
Acidic:						
D,L-Glu	60	32	17	20	30	17
Basic:						
D,L-Lys·HCl	2700	370	247	282	262	626

D,L-Phe and *D,L*-Leu are increased about fourfold, and a slight influence on the adsorption of *D,L*-Val was observed among the various amino acids in the presence of calcium ion. *D,L*-Ala adsorption was decreased by calcium. The negative effect was observed for the adsorption of acidic and basic amino acids in the presence of calcium ion. The adsorption capacity of CM-chitin from α-chitin for *D,L*-Phe increased about threefold and hardly any effect was observed on the adsorption of amino acids in the presence of manganese ion. But the adsorptions of neutral amino acids to CM-chitin from α-chitin in the presence of barium ion or magnesium ion did not give the similar phenomena to those of calcium ion and manganese ion. The capacities of Phe in the presence of both ions were lower than that of *D,L*-Val and *D,L*-Leu. However, adsorption capacities for these three amino acids were higher than *D,L*-Ala. It seems that the adsorption site formed by the presence of calcium and manganese ion is different from that by the presence of other alkali-earth metal ions. It will be discuss in detail in next section.

The adsorption profile of amino acids to 6-O-CM-chitin from β-chitin is almost different from that to 6-O-CM-chitin from α-chitin. It seems that the surface property of 6-O-CM-chitin-Ca complex from α-chitin(Ca-6-O-CM-chitin(α) complex) is quite distinguishable from that of 6-O-CM-chitin-Ca complex from β-chitin due to the distribution of carboxyl group and dispersing chains, though the mechanism of calcium adsorption is very similar in both 6-O-CM-chitin

described above.

Since the capacity of neutral amino acids to 3,6-O-CM-chitin decreases with the increase of hydrophobicity of side chain in the absence of metal ions, high negative charge density seems to inhibit the neutral amino acids adsorption to 3,6-O-CM-chitin.

SPECIFIC ADSORPTION OF NEUTRAL AMINO ACIDS TO 6-O-CM-CHITIN-CA COMPLEX

As the affinity of neutral amino acids toward the 6-O-CM-chitin(α)-Ca complex was suggested to depend on the hydrophobicity of its side chain, the adsorption capacity was plotted against hydrophobicity of neutral amino acids according to Nozaki and Tanford(45) as in Fig.18. This data shows that the adsorption capacities of neutral amino acids other than Phe were found to be simply proportional relation to the hydrophobicity of the side chain. The abnormally high adsorption of Phe would be caused by an additive factor, probably by the aromatic hydrophobicity effect.

On the adsorption of neutral amino acids, the adsorption constant depending on temperature was measured at various temperature by equilibrium dialysis to achieve the thermodynamic study of complex formation. The results are listed in Table XIII. The specificity of 6-O-CM-chitin(α)-Ca complex for Phe was confirmed by higher value of $K\alpha$ for Phe comparing that for Val. Adsorptions of non-polar neutral amino acids, Val and Phe, are suggested the conformational

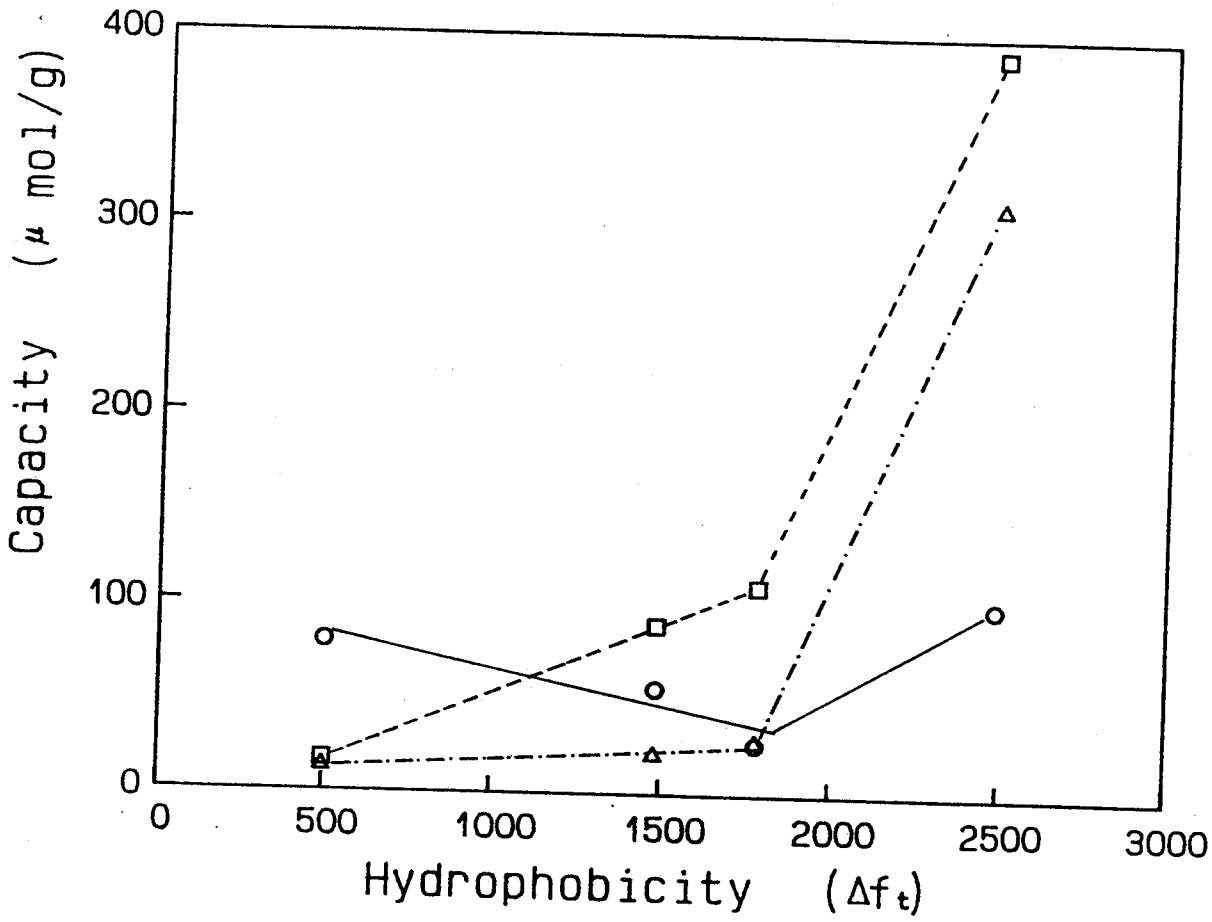


Fig.18 Relationship between adsorption capacities of amino acids for water-soluble 6-O-CM-chitin from -chitin and hydrophobicity of the side chain

(△):In the presence of Ca^{2+}, (□):in the presence of Mn^{2+} , (○):in the absence of metal ion

Table XIII Dependence of association constant on temperature

	T(℃)	$Ka(M^{-1})$	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (calK/mol)
Phe	30	7.2×10^2	-4.0	-18	-48
	40	3.6×10^2	-3.6		
	50	1.1×10^2	-3.0		
Val	30	2.8×10	-2.0	-14	-38
	40	1.0×10	-1.5		
	50	0.7×10	-1.3		
Trp	30	4.7×10^2	-3.7	5.7	31
	40	7.5×10^2	-4.1		
	50	8.3×10^2	-4.2		

Ka: association constant was calculated by Scatchard plot after dialysis equilibrium was carried out in 1mM Tris-HCl buffer(pH7.4, 10mM $CaCl_2$) for 24hours with shaking and concentrations of amino acids were measured by fluorescence spectrometry using *o*-phthalaldehyde method. ΔH was calculated by Van't Hoff plot.

change of CM-chitin-Ca complex to reach ground state, because ΔH for both amino acids show negative value. As it for Phe gives larger ΔH value than that for Val, the CM-chitin-Ca-Phe complex would be formed through the hydrophobic interaction with the conformational change of polymer molecule. Since ΔH for Trp gives positive value because of polar indole ring as side chain and insensitively to the calcium ion, the conformation of CM-chitin-Ca complex might be in unstable state. It suggests that specific domain formed in CM-chitin-Ca complex might just fit for benzene ring but not for more bulky group. than benzene ring.

A preliminary ^{1}H-NMR measurement for CM-chitin(α)-Phe-Ca was carried out by 500MHz-^{1}H-NMR as shown in Fig.19. As chemical shift of meta-proton of phenyl ring seems to reduce and all line width in aromatic ring are broaden, its study also suggests that phenylalanine side chain surely contributes to the adsorption to 6-O-CM-chitin-Ca complex.

The adsorption capacity of benzene to the low substituted 6-O-CM-chitin(from α-chitin) was investigated in several concentration of calcium ion to clarify the contribution of benzene ring on the Phe adsorption. 0.5g of 6-O-CM-chitin was applied to be suspended in 45mL of several aqueous calcium chloride solution. 5mL of saturated aqueous benzene solution was added to the mixture and was shaken for 24h. The concentration of benzene in the supernatant was calculated by the adsorption at 253.7nm using the equation mentioned by Franks et.al.(46), because the concentration of benzene does not obey the equation of Beer-Lambelt. The

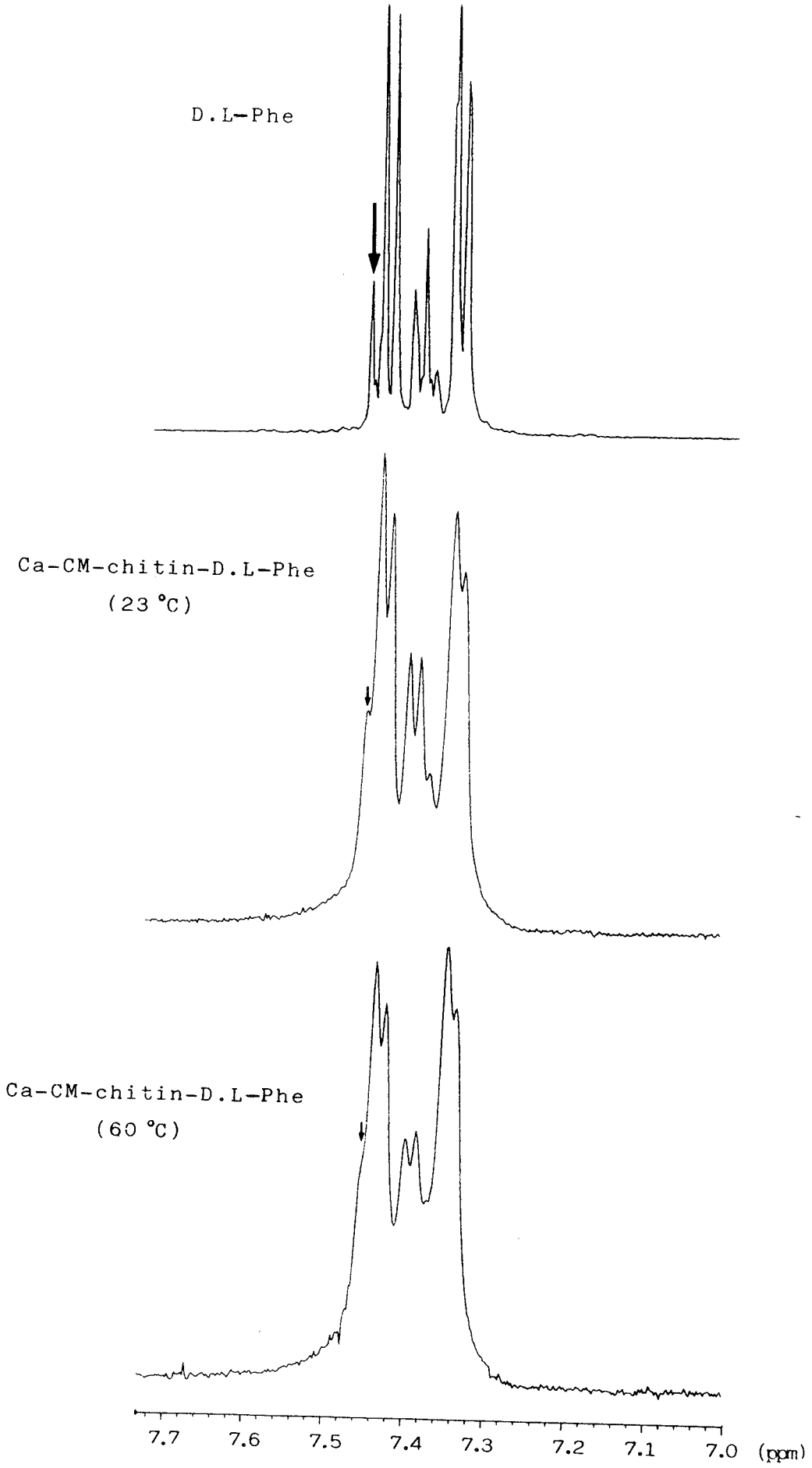


Fig.19 ^{1}H-NMR-spectra of Ca-CM-chitin-D.L-Phe and D.L-Phe in the aromatic region using 500MHz NMR spectrometer

capacity is estimated from the difference between the concentration in deionized water and that in the presence of calcium ion.

The adsorption capacity of benzene to CM-chitin in the presence of calcium ion is higher than that in water. The increased capacity is 28μmol/g in 1mM of calcium ion, and 39 μmol/g in 10mM of calcium ion. This suggests that the capacity of benzene depends on the calcium concentration. As benzene, lacking ionic functional groups, is adsorbed to the CM-chitin-Ca complex, benzyl group in the side chain of phenylalanine would contribute the Phe adsorption to 6-O-CM-chitin(α)-Ca complex. When aromatic ring forms the metal ion complex, π electron induces the formation of π complex for Ca complex. The adsorption of phenylalanine seems to be contributed by the similar mechanism. This assumption would explain fairly well to the higher capacity of Phe for CM-chitin(α)-Mn complex than that of Phe in the presence of other non-specific metal ions, since manganese ion seems to form π complex easily because of insufficient of electron in *d* orbital.

SPECIFICITY OF CM-CHITIN FOR THE OPTICAL ISOMER OF PHENYLALANINE

As the specificity of 6-O-CM-chitin(α)-Ca complex for *D,L*-Phe was found, the affinity of its complex for the optical isomer of phenylalanine was investigated to obtain further information about the geometrical arrangement of the contributed functional group on CM-chitin molecule.

Since the amount of adsorbed *D,L*-Phe was almost three times as much as that of *L*-Phe and a little *D*-Phe was adsorbed to the CM-chitin-Ca complex(as estimated by UV analysis of adsorbed Phe after equilibrium dialysis, as shown in Table XIV, *L*-Phe was suggested to be adsorbed selectively to the CM-chitin(α)-Ca complex without optical resolution. It was assumed that the adsorption site for *D,L*-Phe is both predominant *L*-site and weak *D*-site , which optical isomers were conjugated with respective enantiomer, because the amount of adsorption for *D,L*-Phe is nearly equal to twice as much as the summation of them.

At the same time, optical rotation of the adsorbed mixture solutions were measured. It was observed that the optical rotation for *L*- and *D,L*-Phe adsorbed CM-chitin was higher than that of calculated from the UV absorption analysis. Especially *L*-Phe adsorbed one gives enhanced value sixfold higher than that from UV. This result suggests the induction of the optical rotation by the adsorptions of optical isomers.

The effect of *D,L*-Phe to CM-chitins of various molecular weight on the induction of the optical rotation was investigated by UV and optical rotation to elucidate whether polymeric effect or the chemical structure of CM-N-acetyl-glucosamine residue was ascribed. The experiments were carried out refering with that for carboxymethyl-cellulose (CMC) as shown in Fig.20,21.

The induction of negative optical rotation increases with the decrease of molecular weight. This effect for CM-

Table XIV Induced optical rotation by the adsorption of Phe to Ca^{2+}-CM-chitin in solution

D,L-Phe Adsorbed *D,L*-Phe $\Delta\chi$ = -0.007	 1.2mM estimated from OD_{260} (390 μmol/g) 1.2mM estimated from optical rotation as *L*-Phe
L-Phe Adsorbed *L*-Phe $\Delta\chi$ = -0.013	 0.4mM estimated from OD_{260} (130 μmol/g) 2.3mM estimated from optical rotation
D-Phe Adsorbed *D*-Phe $\Delta\chi$ = 0	 0.1mM estimated from OD_{260} (40 μmol/g)

$\Delta\chi = \chi_{obs} - \chi_{Ref}$, χ_{obs} ;Optical rotation was obtained from Ca^{2+}-CM-chitin and Phe solution

χ_{Ref} ;Optical rotation was obtained from only Ca^{2+}-CM-chitin solution

The concentration of its solution obtained by optical rotation was calulated as the concentration of *L*-Phe.

Water soluble CM-chitin was mixed with amino acids in the presence of Ca^{2+} and then dialyzed against deionized water thoroughly at room temperature.

[*D,L*-Phe]/[COOH] = 0.14, [*D,L*-Phe]/[COOH] = 0.05

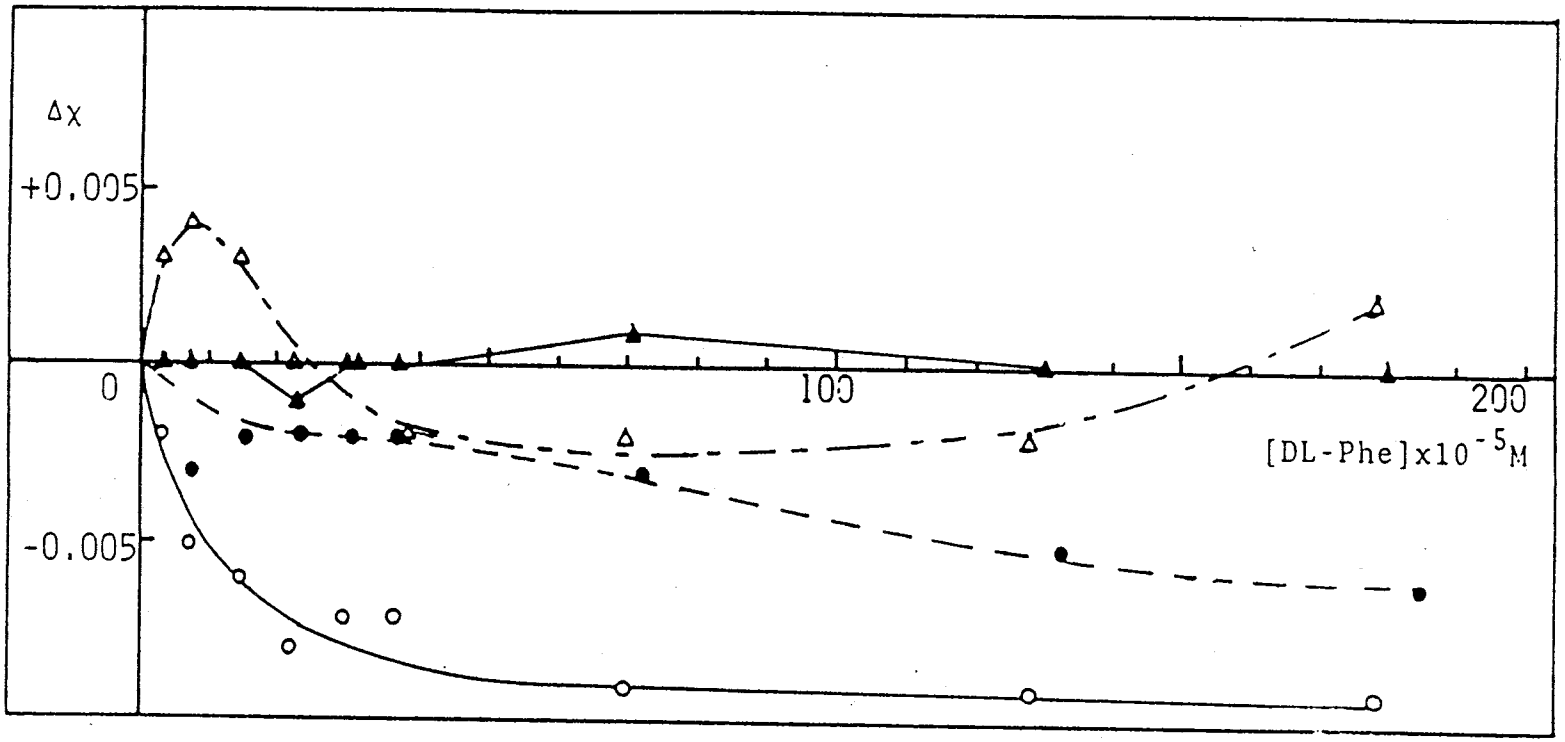


Fig.20 Induced optical rotation on the DL-Phe adsorption to Ca^{2+}-CM-saccharide complexes.

—○—: CM-GlcNAc
-●-: CM-chitin oligomer
-△-: CM-chitin
—▲—: CMC-Na

The concenration of CM-saccharides is 0.2% and the concentration of Ca^{2+} is 25mM.

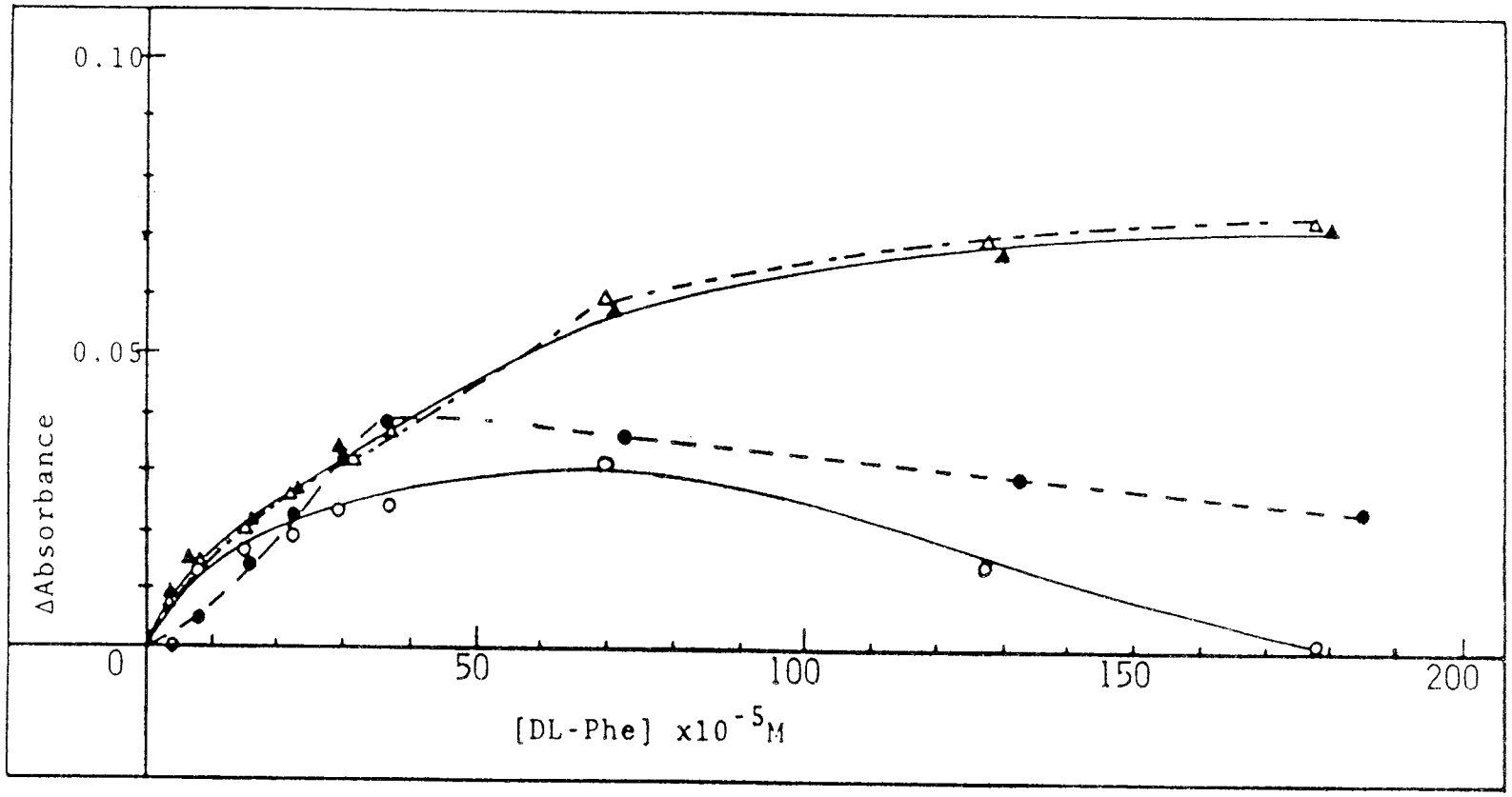


Fig.21 Induced optical density on the adsorption of DL-Phe to calcium-CM-saccharide complexes.

—○—: CM-GlcNAc
-●-: CM-chitin oligomer
-△-: CM-chitin
—▲—: CMC-Na

The concentration of CM-saccharides is 0.2% and the concentration of Ca^{2+} is 25mM.

chitin was observed by the increased concentration of a little over 400μM which is almost saturation point of Phe adsorption to CM-chitin. Its induction value is significant, because the concentration of CM-chitin is lower than that of former experiment. These results would support the induction of optical rotation caused by the tight adsorption of Phe. As *D,L*-Phe does not show optical rotation, the induction of negative optical rotation on the adsorption of D,L-Phe should be caused by CM-chitins molecule.

Since a significant change of cotton effect due to acetamide groups at C-2 position was not observed in CD measurement, the induction phenomena seems to be not by the conformational change of polymer chain but by the configuration change in CM-GlcNAc residue. This results would be interpreted by supporting that configuration change indicates the distortion of pyranose ring, especially in CM-chitin monomer(CM-GlcNAc) owing to little restriction of pyranose ring mobility.

The optical density in the ultra-violet region was enhanced significantly for CM-chitin and CMC. Since this phenomena seem to be induced by a synergy of the weak adsorption of carbonyl group in UV region and the adsorption of Phe including free Phe, it suggests the induction of optical density by the polymeric effect.

These results might indicate that 6-O-CM-chitin from α-chitin has the specificity to optical isomer. It suggests the importance of the regulation experiments for peptides to regulate the chirality.

RELATIONSHIP OF MOLECULAR WEIGHT OF 6-O-CM-CHITIN AND ADSORPTION OF PHENYLALANINE

The adsorption of Phe to 6-O-CM-chitins(α) of several molecular weight were measured to clarify the main reason of the high affinity of 6-O-CM-chitin(α)-Ca complex for Phe.

The adsorption capacity of Phe was estimated by the method of gel filtration on Sephadex G-50 applying the mixture of CM-chitin oligomers, calcium ion and phenylalanine as shown in Fig.22.

The adsorption capacity of Phe reduced with the decrease of molecular weight, but a little increase of the capacity was observed when the oligomer of M.W.=680(2-3 residues) was employed. Since the fraction containing Phe was confirmed to maintain calcium ion by ICP-AES, calcium ion seemed still to contribute to the adsorption of Phe without release. This results clearly indicate the advantage of molecular composition of polymer, that is to say the polymeric effect, of CM-chitin from α-chitin. The crystalline structure of chitin is stable owing to several kinds of hydrogen bonds such as

(acetamide group)NH ····· O=C(acetamide group)

(C-6 position)OH ····· O=C(acetamide group) etc.

As scarce deacetylation occurs in the carboxymethylation reaction, CM-chitin maintains inter chain hydrogen bonds much more than the oligomers. Since 6-O-CM-chitin from

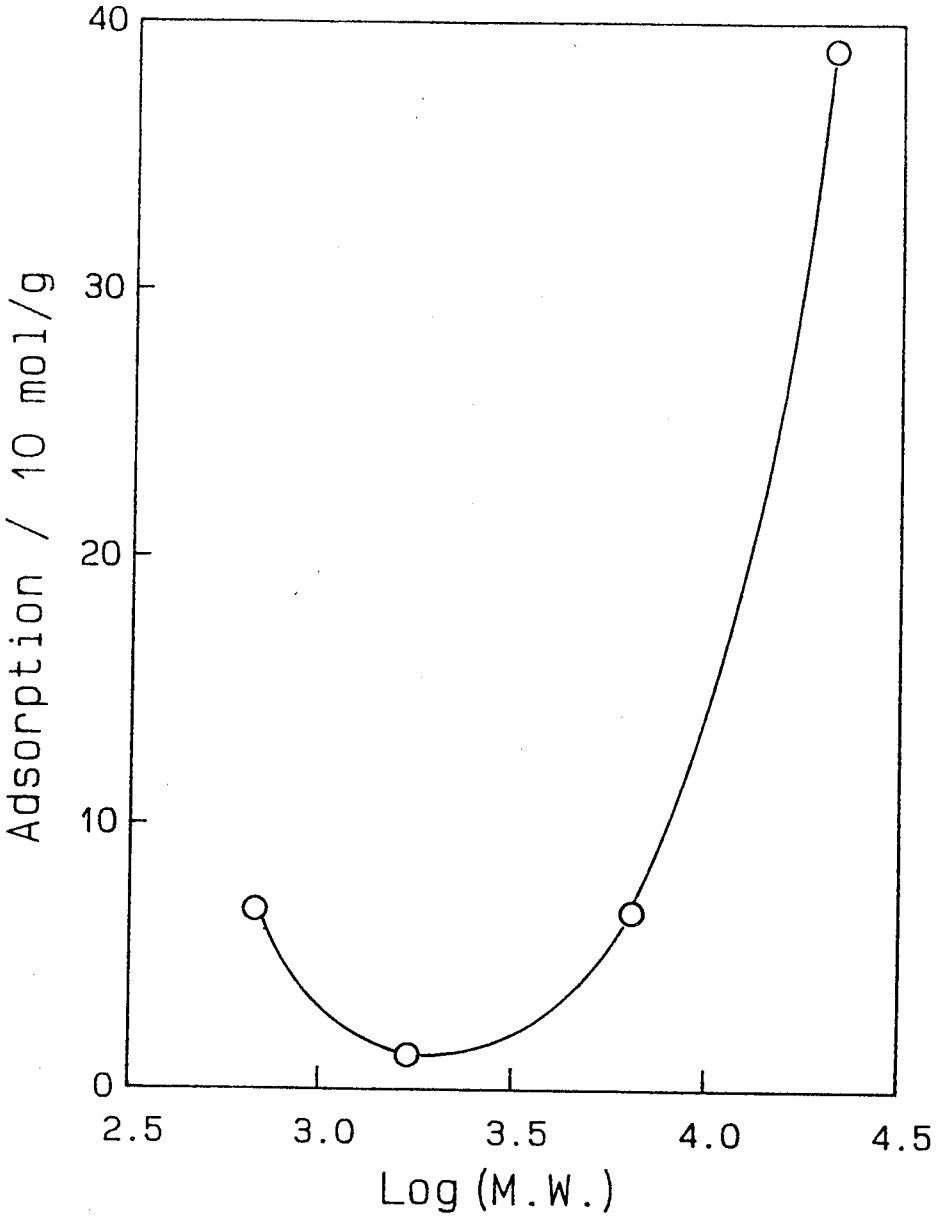


Fig.22 Adsorption capacity of Phenylalanine to Ca-CM-chitin.

The mixture of Phe and Ca-CM-chitin complex was applied to Sephadex G-50 to remove free Phe. The fraction containing Phe was measured the amount of total GlcNAc and Phe by HCl-Indole method and ninhydrin method, respectively.

β-chitin, having loose crystalline structure and widely distributed functional groups, does not show the high affinity for Phe, the adsorption of Phe needs the crystalline structure likely to α-chitin. The mechanism of Phe adsorption is able to be explained by the model that the side chain of Phe justly incorporated into the domain composed by two chains of CM-chitin molecule which forms the adsorption site with calcium ion. When 6-O-CM-chitin chains are hydrolyzed by lysozyme, the stable structure of polymer chains might loosed stable structure and convert to unstable molecular structure. Consequently, the retaining capacity of CM-chitin for Phe was decreased. However, oligomers composed of 2-3 residues are likely to form the most stable complex with Phe through calcium ion resulting a small increase of the capacity.

These phenomena give a fundamental information about the utilization of CM-chitin for the sustained release of drug. When the Phe adsorbed CM-chitin(α)-Ca complex is injected into animal body, polymer chain is hydrolyzed by glycosidases and consequently Phe would be released.

As the retention time for the Phe-contained fraction was shorter than that for CM-chitin itself on the process of gel filtration, apparent molecular weight of oligomer complex increases only threefold of original oligomer. It suggests the formation cross-linkage among CM-chitin oligomers through calcium ion.

STOICHIOMETRIC DISCUSSION

The data described above were treated stoichiometrically to explain more clearly and were summarized in Table XV. It is clear from Table XV that only a part of the bound calcium ion contributes to the adsorption of these neutral amino acids. As the effective number for the *D,L*-Phe adsorption is 13% of bound calcium ions on low substituted CM-chitin, this value is almost the same as the amount of retained calcium ion after procedure such as elution by pH shift. The calcium ions which adsorbed strongly, were found to contribute the adsorption of Phe to CM-chitin. The effective value was 31% in the case of highly substituted CM-chitin. However, the effective number of *D,L*-Phe adsorption on the fibrous CM-chitin is much less in spite of the threefold increase of calcium ion adsorption suggesting non specific adsorption of calcium ion. This result explains reasonably the contribution of geometrical arrangement of the functional groups for the specific adsorption of phenylalanine. On the other hand, the adsorption of lysine seems not to depend on the bound calcium ions but on the simple ionic binding through carboxyl groups. The speculated adsorption mechanism for phenylalanine is discussed in the next section.

MODEL OF 6-O-CM-CHITIN-CA COMPLEX AND 6-O-CM-CHITIN-CA-PHE COMPLEX

In this section, the speculated models to explain the mechanism of the specific adsorption of calcium ion to 6-O-CM-chitin and that of the specific adsorption of Phe to 6-O-

Table XV Summary of unit capacities of 6-O-CM-chitin from α-chitin

Sample	Lowly substituted CM-chitin	Highly substituted CM-chitin	CM-chitin fiber
Degree of substitution	0.10	0.88	0.10
$[Ca^{2+}]/[COO^{-}]$	0.53	0.36	1.08
[*D*,*L*-Phe]/$[Ca^{2+}]$	0.13	0.31	0.008
[*D*,*L*-Phe]/$[Mn^{2+}]$	0.003	0.08	0.01
[*D*,*L*-Ala]/$[Ca^{2+}]$	-	0.01	-
[*D*,*L*-Val]/$[Ca^{2+}]$	-	0.07	-
[*D*,*L*-Leu]/$[Ca^{2+}]$	-	0.09	-
[*D*,*L*-Lys]/$[Ca^{2+}]$	1.15	2.16	0.25
[*D*,*L*-Glu]/$[Ca^{2+}]$	0.0002	0.0026	0.0008

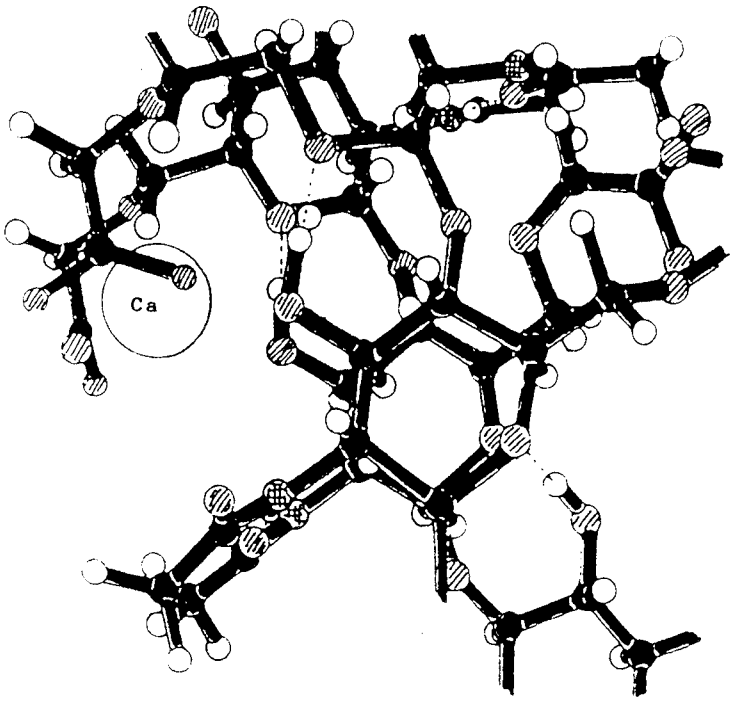
CM-chitin(α)-Ca complex will be proposed.

Considering a number of hydrogen bonds on CM-chitin chains and the result from X-ray small-angle scattering, several functional groups on two polymer chains meet to face to each other by high probability. These functional groups in anti-parallel direction would be able to form the binding site taking suitable distance only for calcium ion adsorption between two chains.

When calcium ion was adsorbed onto CM-chitin, converting the hydrophilic domain in CM-chitin would be converted to hydrophobic one by the neutralization of electric charge. The benzyl group in the side chain of phenylalanine incorporates to that domain just like not to be interfered by the hydrophilic functional groups. It goes so far as to say that α-amino and α-carboxyl groups would form hydrogen bonding with the functional groups in CM-chitin.

On the based on the results and assumption in this chapter, speculative models for CM-chitin(α)-Ca and CM-chitin(α)-Ca-Phe complex are shown in Fig.23.

Ca-6-O-CM-chitin(α) complex



Phe-Ca-6-O-CM-chitin(α) complex

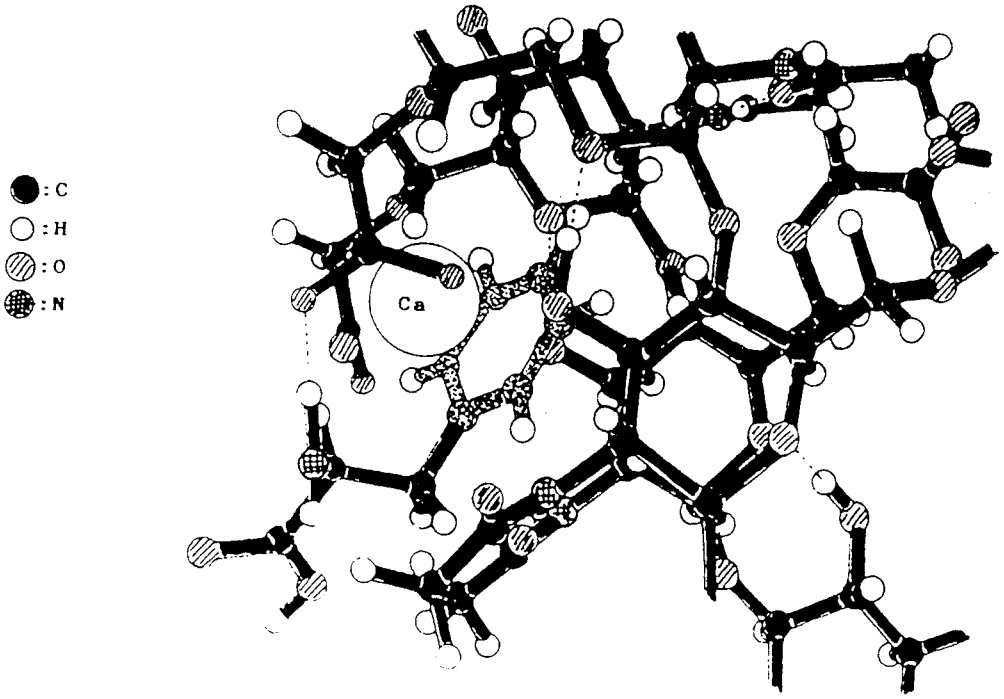


Fig.23 Speculative model of Ca-6-O-CM-chitin (α) complex and Phe-Ca-6-O-CM-chitin (α) complex.

CONCLUSION

On the adsorptions of metal ions to CM-chitins, 6-O-CM-chitin was suggested to have the specificity for calcium ion owing to the selective adsorption ability and the ion-exchange ability. Since 6-O-CM-chitin from β-chitin formed gel followed by the precipitation by the addition of transition metal ions, the carboxymethyl groups would be distributed in the wide region of the molecule maintaining the loose crystalline structure of β-chitin. 3,6-O-CM-chitins also formed gel to precipitate by the addition of barium ion among alkali-earth metal ions in addition to the transition metal ions. These phenomena would suggest the cross-link of CM-chitin chains by the metal ions.

By the investigation for the surface property of 6-O-CM-chitin-Ca complex, it was found to form hydrophobic domain and to interact with benzyl group in the side chain of phenylalanine specifically.

6-O-CM-chitin(α)-Ca complex was found to have the stereo selective adsorption ability to phenylalanine and the retaining the ability by the polymeric effect of CM-chitin.

Chapter 3

Preparation of peptide and its adsorption to CM-chitin

Though the side chain of Phe was found to be important for the adsorption to CM-chitin(α)-Ca complex, the assumed adsorption model is not satisfied to explain the mechanism of amino acids adsorption. Ionic binding of α-amino group and especially carboxyl group, which is dissociated in neutral pH, should be examined from the standpoint of geometric specificity. Though the blocking of α-amino and carboxyl group seems to be satisfactory, non-polar protective groups are not suitable because of less soluble in water and of derivation from protein adsorption. To investigate the additive effects of α- functional group in amino acid to the adsorption of side chain, the difference in the adsorption capacity was examined by regulating the distance between the α-functional groups and those in side chain. The adsorption of peptide was investigated preliminary using glycines for blocking α-functional groups of Phe. The adsorption studies for Phe peptides would also give us some informations about the interaction between CM-chitin and proteins.

EXPERIMENTAL

MATERIALS

Synthesis reagents for the preparation of peptide were purchased from Peptide Institute Inc. and used without further purification. α-Chymotrypsin Type II from Bovine Pancreas was obtained from Sigma Chemical Company (3× crystallize and lyophilized). Lysozyme from hen egg white was obtained from sigma described in chapter 1.

PREPARATIONS OF PEPTIDES

Various peptides were synthesized by liquid-phase condensation by using the dicyclohexyl carbodiimide(DCC) and mixed anhydride methods. Carbobenzoxy chloride(Z-Cl) was applied to protect α-amino groups for Phe and Gly. *t*-Butyloxycarbonyl-4,6-dimethyl-2-mercaptopyrimidine(Boc-SDP) was also applied to protect α-amino group for ε-Z-Lys. Ethyl carboxylate was applied to protect α-carboxyl group in the case of condensation by DCC. The deprotection of Z group was carried out by catalytic hydrogenation on Pd/C until single spot was obtained on TLC in the reaction system or by about 1M of HBr/AcOH for 1h at room temperature, and that of Boc group was carried out by 2M of HCl/dioxane for 1h at room temperature with vigorous stirring. The syntheses schemes of all peptides in detail were shown in Fig.24.

ADSORPTION OF PEPTIDES TO 6-O-CM-CHITIN FROM α-CHITIN

The adsorptions of peptides were measured by the frontal

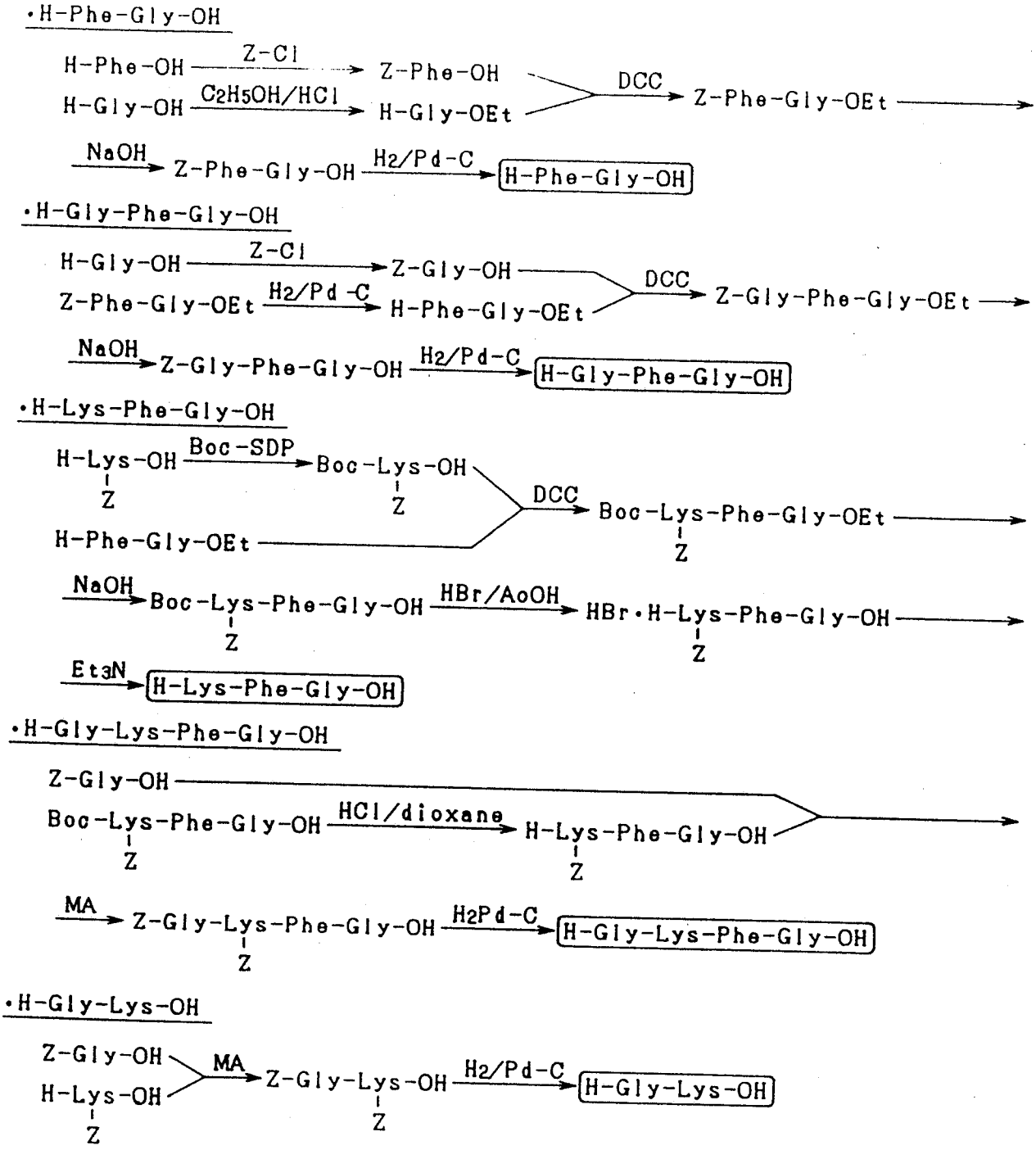


Fig.24 Synthetic route to peptides.

Z: Benzyloxycarbonylgroup. Z-Cl: Benzyloxycarbonyl chloride. Boc: t-Butyloxycarbonyl group. Boc-SDP: S-t-Butyloxycarbonyl-4,6-dimethyl-2-mercaptopyrimidine. DCC: Dicyclohexylcarbodiimide. H-Tyr-Gly-OH, H-Gly-Tyr-Gly-OH, and H-Gly-Tyr-OH were synthesized by methods similar to those for H-Phe-Gly-OH, H-Gly-Phe-Gly-OH, and H-Gly-Lys-OH, respectively.

affinity chromatography(47-49) according to the method in detail described in chapter 1. The adsorption capacities were estimated from UV absorption at 257nm using flow cell and fluorescence intensities of *o*-phthalaldehyde derivatives of amino acids using 340nm as excitation and 455nm as emission wavelengths(see chapter 2).

THE HYDROLYZED PROFILE OF GLY-PHE-*p*-NITROBENZYL ESTER

0.8mL of 2.5mg/mL 6-O-CM-chitin from α-chitin in 30mM of aqueous calcium chloride solution was added to 2mL of 300mM glycylphenylalanine *p*-nitrobenzyl ester hydrochloride (HCl·H-Gly-Phe-ONBzl). The solution was kept in a refrigerator 50min and then stood at room temperature in occasionally shaking to be formed complex and to avoid autolysis of peptide. Then 0.2mL of 0.2mg/mL chymotrypsin or binary enzyme (consisted of 0.2mg/mL chymotrypsin and 1mg/mL lysozyme in 0.03M of calcium chloride solution) was added to the substrate solution. The release of *p*-nitrobenzyl alcohol as a enzymatic hydrolysis product was monitorred at 300nm using Hitachi U-3200 type spectrophotometer.

RESULTS AND DISCUSSION

ADSORPTION OF PEPTIDES TO 6-O-CM-CHITIN

Various peptides were examined to confirm the adsorption sites of amino acids for the CM-chitin-Ca complex(water insoluble), as listed in Table XVI.

Since the adsorption capacity for H-Gly-Phe-Gly-OH is maintained at almost the same level that of H-Phe-Gly-OH and H-Phe-OH, the α-amino and α-carboxyl groups of Phe do not likely to affect the adsorption of Phe significantly. The increase of the adsorption capacity of the peptides containing tyrosin suggest a contribution of aromatic ring to the adsorption. Though there is a positive contribution of the calcium ion on the adsorption of α-N-blocked lysine, a negative effect of calcium is still shown on H-Lys-Phe-Gly-OH and H-Gly-Lys-Phe-Gly-OH. The change of basicity might enhance the calcium effect in spite of a significant decrease in ionic linkages.

These results would suggest the contribution of only benzyl group for the adsorption of Phe even in the peptide form. These results might reinforce to reasonableness of proposed model that the side chain of Phe justly incorporated in the adsorption domain formed by the calcium adsorption, and main chain of the peptide is exposed on the surface of polymer chain.

The functions given by the carboxymethylation are seemed to have right to develop for biomimetic material utilizing its high biocompatibility described in introduction section

Table XVI Adsorption of peptides to low-substituted 6-O-CM-chitin from α-chitin.

Peptide name	Ca^{2+}	Capacity, μmol/g
D,L-Phe	+	33
H-Phe-Gly-OH[a]	-	7
	+	15
H-Gly-Phe-Gly-OH[a]	-	8
	+	12
HCl·H-Tyr-Gly-OH[a]	-	27
	+	32
H-Gly-Tyr-OH[a]	-	23
	+	25
H-Gly-Tyr-Gly-OH[a]	-	22
	+	26
H-Gly-Lys-OH[a]	-	37
	+	46
H-Lys-Phe-Gly-OH[a]	-	230
	+	100
H-Gly-Lys-Phe-Gly-OH[b]	-	90
	+	34

[a] 1g CM-chitin was packed into a column(1.0x10.4cm), and 4.0mM peptide solutions were passed through the column at a rate of about 0.2 mL/min.

[b] Column contained 0.5g CM-chitin;flow rate as above.

and biodegradability, which is higher than chitin described in chapter 1. Taking these properties into account, model synthesis for sustained release system of drug without side effects was planned. The adsorbed molecule would be released with the digestion of CM-chitin in animal body, and CM-chitin would disappear in the metabolism system without any toxicity in animal body. It was examined to obtain fundamental informations about sustained release a synthetic chymotrypsin substrate in two enzyme systems. Its result will be discussed in next section.

HYDROLYSIS OF SYNTHETIC SUBSTRATE CONTAINING PHE

The hydrolysis rate of HCl·H-Gly-Phe-ONBzl, substrate for chymotrypsin, was measured in two enzyme systems, the one was chymotrypsin system and the other was binary enzyme system consisted of chymotrypsin and lysozyme being closer system for physiological condition.

The initial velocities of chymotrypsin hydrolyses of HCl·H-Gly-Phe-ONBzl in the presence of CM-chitin on both systems were higher than that in the absence of CM-chitin as shown in Fig.25. But the initial hydrolysis of the peptidyl substrate in the presence of CM-chitin was settle to extremely slow rate after five minutes. On the other hand, after 72 hours, the hydrolysis in the absence of CM-chitin was continued slowly. These results seem to indicate that CM-chitin accelerate the hydrolyses of chymotrypsin apparently. Since autolysis of α-chymotrypsin is reduced by the presence of calcium ion(57) and CM-chitin-Ca complex had

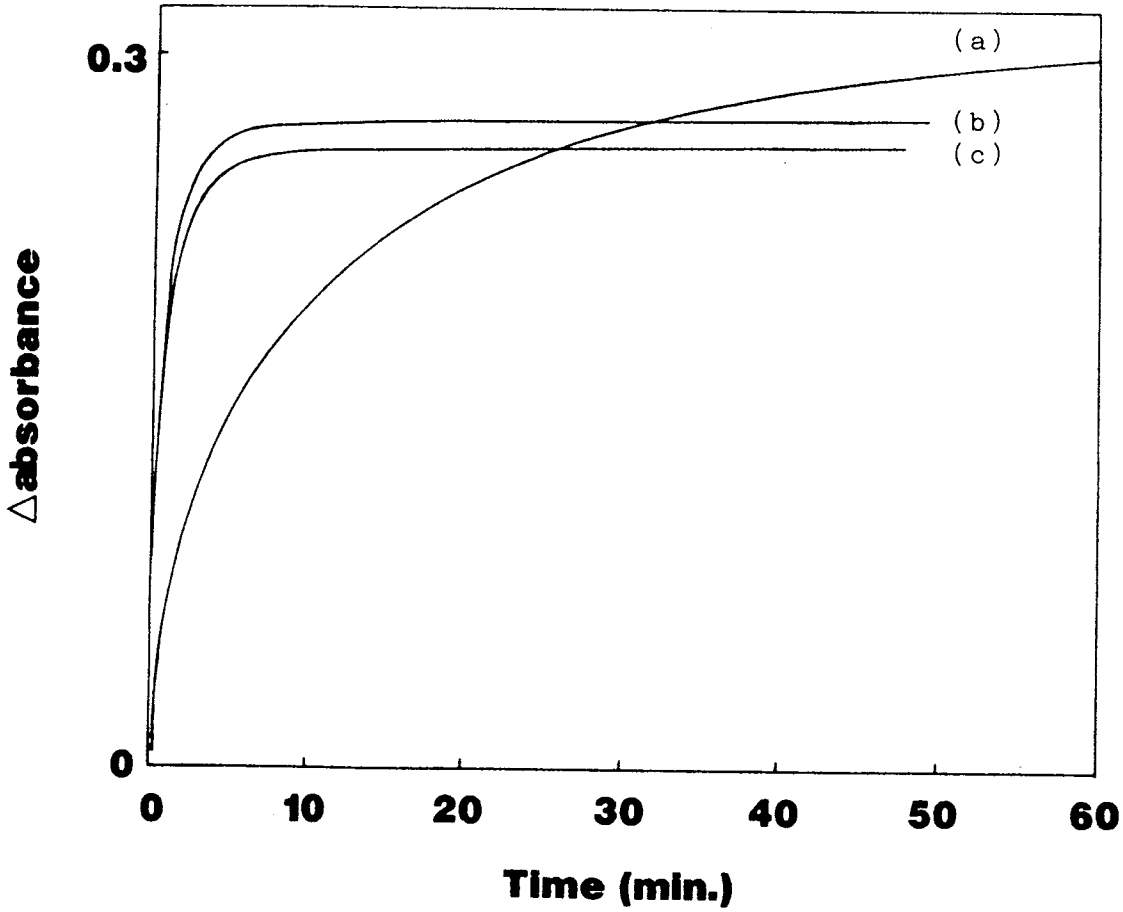


Fig.25 Time course of hydrolyses HCl·H-Gly-Phe-ONBzl by chymotrypsin or binary enzymes (chymotrypsin and lysozyme).

(a) only HCl·H-Gly-Phe-ONBzl as a substrate with chymotrypsin
(b) substrate and CM-chitin with binary enzymes
(c) substrate and CM-chitin with chymotrypsin

been described to adsorb to peptides, it assumes that α-chymotrypsin adsorbs to the surface of CM-chitin through calcium ion to prevent chymotrypsin itself from autolysis by each other, resulting in more activity in the presence of CM-chitin than that in the absent of CM-chitin . The initial stage of hydrolysis in the presence of CM-chitin seems to be caused by the substrate non-adsorbed to CM-chitin. On the calculating the amount of adsorbed component from the difference between the amount of applied substrate and that of non-adsorbed one, the adsorption capacities of substrate show 180 μmol/g equal to 60% of applied amount in both system. The precious adsorbed value obtained from HPLC using GPC column showed the similar value as was almost identical with that from former calculation after separation by ultra filtration of free substrate. CM-chitin seems to protect the substrates containing benzyl group from hydrolysis by chymotrypsin, as the substrate adsorbed CM-chitin was found to be not hydrolyzed in the initial state. Moreover, it seems to show that *p*-nitrobenzyl ester group of chromogenic substrate is not be exposed on the CM-chitin surface but buried in polymer chains, because the substrate adsorbed was not hydrolyzed. It suggests that benzyl derivative might become a ligand to CM-chitin(α)-Ca complex.

However, the hydrolysis in binary system was found to proceed slowly in long period owing to the slow hydrolysis of CM-chitin by lysozyme, though the hydrolysis in single enzyme system (chymotrypsin)was reduced extremely slow rate as shown in Fig.26. The substrate adsorbed CM-chitin is

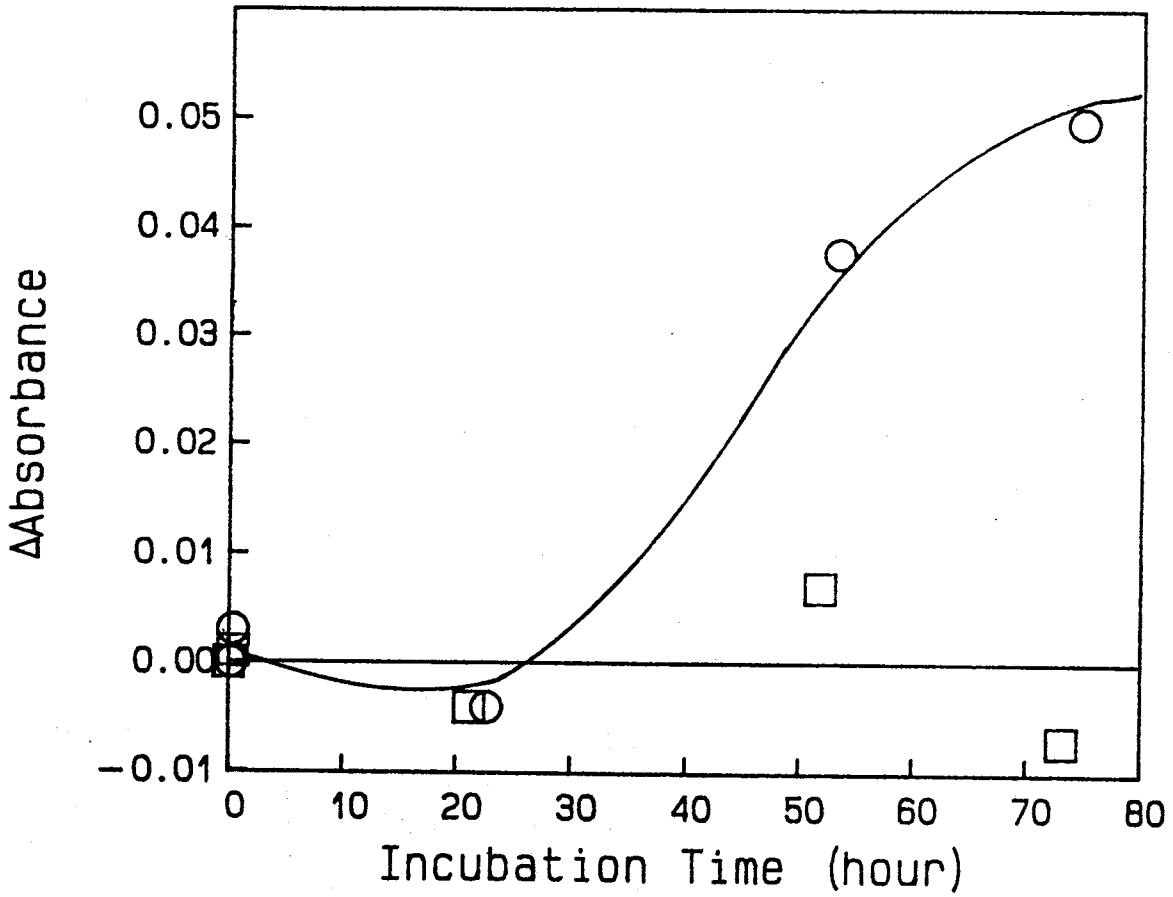


Fig.26 Time course of hydrolysis of HCl·H-Gly-Phe-OBzl(NO_2) by chymotrypsin or binary enzyme system(chymotrypsin and lysozyme).

ΔAbs.=Abs-Abs0; Abs0, after 10min.

(a),-○-, substrate and CM-chitin with binary enzyme system

(b),-□-, substrate and CM-chitin with chymotrypsin

suggested to be hydrolyzed by chymotrypsin following to the digestion of CM-chitin with lysozyme.

These results might suggest that 6-O-CM-chitin(α)-Ca complex is a suitable material for a drug carrier of sustained release. Especially, the complex is a useful carrier to stabilize peptides containing Phe such as peptide hormones during they carrying in the blood to organs. The mechanism of peptide adsorption to CM-chitin seems to be similar as that of phenylalanine, which the side chain of phenyl groups of peptide is burried in the domain formed inter-molecular interaction of polymer chains. It can be explained well the interaction of CM-chitin with blood protein by this mechanism.

6-O-CM-chitin have been also reported to be utilized as a carrier for inclusion type sustained release by the addition of ferric ion to form gel(53). The release of doxorubicine, a anti-cancer drug, from the gels was found to be achieved by the lysozyme digestion in a time-dependent manner. In this chapter these results in that CM-chitin(α)-Ca complex can be used as a polymeric drugs, and CM-chitin as a specific adsorbent for metal ion.

CONCLUSION

The mechanism of the Phe adsorption to 6-O-CM-chitin(α)-Ca complex was found to be a main factor of the contribution by result from the adsorption measurements of peptides containing Phe.

The rate of hydrolysis of peptide adsorbed on 6-O-CM-chitin(α)-Ca complex was found to be much slower than that of peptide itself. When CM-chitin of complex was hydrolyzed by the co-existed lysozyme, adsorbed peptide was released into solution and then suggested hydrolyzed by peptidase.

It suggests that CM-chitin(α)-Ca complex is a useful material as a drug carrier of sustained release to carry peptide hormones containing Phe.

CONCLUDING REMARKS

The results obtained from present investigations are summarized and concluded as follows;

(1)6-O-CM-chitin was prepared by the site selective chemical modification of chitin. Further carboxymethylation of resulting 6-O-CM-chitin gave 3,6-O-CM-chitin. The degree of carboxymethylation was found to be regulated by the crystalline structure of chitin. High degree of substitution of CM-chitin was difficult to prepare from α-chitin, but it was easily achieved from β-chitin.

(2)6-O-CM-chitin from both structural chitins was found to have the specificity for calcium ion among divalent metal ions. 6-O-CM-chitin forms tight complex with calcium ion, and has the selective and ion-exchange abilities for calcium ion. The complex was formed by the contribution of acetamide, primary and secondary hydroxyl groups by the inter N-acetylglucosamine residual support in addition to carboxymethyl groups.

(3)Carboxymethyl groups in 6-O-CM-chitin from β-chitin were estimated to distribute rather homogeneously along of the molecule than those from α-chitin. It ascribes to loose crystalline structure of β-chitin. Unit density of carboxymethyl groups would affect to gel formation velocity

by the addition of transition metal ions.

(4)3,6-O-CM-chitin which high charge density also forms gel by barium ion among alkali-earth metal ions in addition to the transition metal ions.

(5)Fe^{3+} causes the gel formation, induced by the cross-linking of 6-O-CM-chitin chains, owing to the multivalent cationic charge density, but Fe^{2+} dose not induce the gelation.

(6)Ca-6-O-CM-chitin(α) complex was found to have the specificity toward Phe due to the contribution of benzyl group. It might form π-complex. Ca-6-O-CM-chitin(α) complex was found to have the high affinity to the *L*-isomer of Phe. 6-O-CM-chitin from β-chitin and 3,6-O-CM-chitins have low affinity to phenylalanine.

(7)6-O-CM-chitin(α)-Ca complex adsorbs peptides containing Phe. In the complex, the adsorbed peptide is resistant to the hydrolysis by peptidase such as chymotrypsin. This result suggests that the complex is a significantly useful material as a drug carrier of sustained release.

(8)In the adsorption mechanism of phenylalanine and Phe-containing peptide to 6-O-CM-chitin(α)-Ca complex, the side chain of Phe would be burried in the domain formed between CM-chitin chains by the Ca chelation, and the

main chain of peptide is estimated to be exposed on the surface of CM-chitin.

By the present investigations, the process of the generation of CM-chitin-metal ion complex and other property were clarified on the molecular level. The possibility of the utilization of the complex, which has biocompatibility and biodegradability, was also suggested.

ACKNOWLEDGEMENT

I would like to express my special gratitude to professor Seiichi Tokura, faculty of Science, Hokkaido University, for his guidance and encouragements through this work. I was also indebted to Dr. Takashi Komai. I wish to express my appreciation to Dr. Norio Nishi for his valuable advices in the preparation of peptide and encouragement.

I am very thankful to emeritus professor Motozo Kaneko of Hokkaido university, and Dr. Yoshinobu Izumi for their valuable advices in the measurement of X-ray Small-Angle Scattering. I would like to give my thanks to the members in laboratory of polymer chemistry, Faculty of Science, Hokkaido University, for their continuous encouragement and assistance.

Finally, I would like to express my deep sense of gratitude to my mother for taking care of whole my life.

REFERENCES

(1)S. Bartnicki-Garcia, *Ann,rev. Microbiol.*, 22, 87(1968).

(2)Ch. Jeuniaux, "*Chitine et Chitinolyse*", Masson, Paris (1963).

(3)Ch. Jeuniaux, *Bull. Soc. Chim. Biol.*, 47, 2267(1965).

(4)J. F. V. Vincent and J. E. Hillerton, *J. Insect. Physiol.*, 25, 653(1979).

(5)J. E. Denginger and A. Alterman, *Comp. Biochem. Physiol.*, 75A, 421(1983).

(6)D. C. West, I. N. Hampson, F. Arnold anf S. Kumar, *Science*, 228, 1324(1985).

(7)R. D. Rosenberg and P. S. Damus, *J. Biol. Chem.*, 248, 6490(1978).

(8)M. V. Tracey, *Rev. Pure. Appl. Chem.*, 7, 1(1957).

(9)"*Encycolopedia of Biotechnology*, Ed. S. Hukui and H. Saito." CMC,p-645(1986).

(10)K. Nishimura, S. Nishimura, N. Nishi, I. Saiki, S. Tokura and I. Azuma, *Vaccine*, 2, 93(1984).

(11)K. Nishimura, S. Nishimura, N. Nishi, F. Numata, Y. Tone, S. Tokura and I. Azuma, *ibid.*, 3, 379(1985).

(12)K. Nishimura, S. Nishimura, H. Seo, N. Nishi, S. Tokura and I. Azuma, *ibid.*, 5, 136(1987).

(13)S. Nishimura, N. Nishi, S. Tokura, W. Okiei and O. Somorin, *Carbohydr. Res.*, 156, 286(1986).

(14)S. Nishimura and S. Tokura, *Int. J. Biol. Macromol.*, 9, 225(1987).

(15)S. Nishimura, N. Nishi and S. Tokura, *Int. J. Biol.*

Macromol., 9, 305(1987).

(16)Lindahl, *Carbohydr. Res.*, 100, 393(1982).

(17)S. Tokura, N. Nishi, A. Tsutsumi and O. Somorin, *Polym. J.*, 15, 485(1983).

(18)S. Nishimura, Y. Ikeuchi and S. Tokura, *Carbohydr. Res.*, 134, 305(1984).(19)K. M. Rudall, *J. Polym. Sci., Part C*, 28, 83(1969).

(20)R. Minke and J. Blackwell, *J. Mol. Biol.*, 120, 167(1978).

(21)K. H. Gardoner and J. Blackwell, *Biopolymers*, 14, 1581(1975).

(22)M. Okano, S. Nishiyama, I. Shinohara, T. Akaike and Y. Sakurai, *Kobunshi Ronbunshu*, 36 209(1979).

(23)S. Tokura, N. Nishi, S. Nishimuran and O. Somorin, *Sen-i Gakkaishi*, 39, 507(1983).

(24)S. Tokura, S. Baba, Y. Uraki, Y. Miura, N. Nishi and O. Hasegawa, *Cabohydr. Polym.*, in submission.

(25)R. H. Hackman, *Aust. J. Biol. Sci.*, 7, 168(1954).

(26)K. Hamaguchi and M. Funatsu, *J. Biochem.(Tokyo)*, 46, 1659(1959).

(27)F. H. Adler, "*Physiology of the Eye*", Mosby St. Louis, p-19(1965).

(28)T. Imoto and K. Yagishita, *Arg. Biol. Chem.*, 35,1154(1971).

(29)N. Ohno, I. Suzuki and T. Yadomae, *Carbohydr. Res.*, 137, 239(1985).

(30)D. Carlstrom, *J. Biophys. Biochem, Cytol.*, 3, 669(1957).

(31)P. R. Sundarajan and R. H. Marchessault, *Adv. Carbohydr. Chem. Biochem.*, 35, 377(1978).

(32)T. Okimasu, *J. Agr. Chem. Soc. Japan.*, 32, 303(1958).

(33)M. Takai, Y. Shimizu, J. Hayashi, M. Ogawa, Y. Uraki, S. Tokura, T. Koroyama, M. Satake and T. Fujita, *Abstracts of 39th Nippon Mokuzaigakkai*, p-302(1989).

(34)H. Sashiwa, Y. Uraki, H. Saimoto, Y. Shigemasa and S. Tokura," *Chitin and Chitosan*", Ed. P. Sanford et. al., Elsevier, p-265(1989).

(35)S. Nishimura, N. Nishi, S. Tokura, W. Okiei and S. Somorin, *Int. J. Biol. Macromol.*, 7, 100(1985).

(36)H. N. Elsheimer, *Talanta*, 14, 97(1967).

(37)P. W. Reeder, *Anal. Chem.*, 28, 1026(1956).

(38)S. Tokura, S. Nishimura and N. Nishi, *Polym. J.*, 15, 597(1983).

(39)Y. Uraki and S. Tokura, *J. Macromol. Sci.-Chem.*, A25, 1427(1988).

(40)M. Roth, *Anal. Chem.*, 43, 880(1971).

(41)Y. Izumi, M. Kaneko, S. Tokura and T. Ueki, *Rep. Prog. Polym. Phys. Jpn.*, 31, 575(1988).

(42)Y. Izumi, T. Matsuo, Y. Uraki, M. Kaneko and S. Tokura, *ibid*, 31, 577(1988).

(43)R. Koyama, *Macromolecules*, 19, 178(1986).

(44)J. E. Martin and A. J. Hurd, *J. Appl. Cryst.*, 20, 61(1987).

(45)Y. Nozaki and C. Tanford, *J. Biol. Chem.*, 246, 2211(1971).

(46)T. Franks, M. Gent and H. H. Johnson, *J. Chem. Soc.*, 2716(1963).

(47)K. Kasai and S. Ishii, *J. Biochem.*, 77, 261(1975).

(48)M. Nishikata, K. Kasai and S. Ishii, *J. Biochem.*, 82, 1475(1977).

(49)K. Kasai and S. Ishii, *J. Biochem.*, 84, 1061(1978).

(50)S. Tokura, N. Nishi, S. Nishimura, Y. Ikeuchi, I. Azuma and K. Nishimura, "*CHITIN, CHITOSAN, AND RELATED ENZYMES*", Ed. by J. P. Zikakis, Academic Press, p-303(1984).

(51)E. G. Ankel, G. Homanberg, N. M. Tooney and C. S. Lai, *Arch. Biochem. Biophys.*, 244, 50(2986).

(52)S. Nishimura, N. Nishi, S. Tokura, K. Nishimura and I. Azuma, *Cabohydr. Res.*, 146, 251(1986).

(53)K. Watanabe, I. Saiki, Y. Uraki, S. Tokura and I. Azuma, in submission.

(54)Y. Inoue, S. Tokura and M. Kaneko, *Rep. Proc. Polym. Phys. Jpn.*, 25, 759(1982).(55)P. Perchemlides, O. Osawa, E. A. Davidson and R. W. Jeanloz, *Carbohydr. Res.*, 3, 463(1967).

(56)J. Conchie, G. A. Levvy and C. A. Marsh, *Advances in Carbohydr. Chem.*, 12, 157(1957).

(57)F. C. Wu and M. Laskowski, *Biochem. Biophys. Acta.*, 19, 110(1956).