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CALCIUM-MEDIATED ADSORPTION OF NEUTRAL AMINO ACIDS TO CARBOXYMETHYLATED CHITIN

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ABSTRACT

Among various divalent metal ions, calcium has been found to be adsorbed tightly onto carboxymethylated chitin. The adsorption was completed not only by induced carboxyl groups but also by the support of acetamide, as well as primary and secondary hydroxyl groups. Although the adsorption capacity for transition metal ions was enhanced appreciably by regeneration into fibrous form, only that of calcium ion, among alkali-earth metals, was at the same level as that of transition metals. Since little effect was shown on the adsorption of phenylalanine by the blocking of α-amino and α-carboxyl groups of *L*-Phe, and since *D*-Phe was so a little adsorbed, the chiral specific adsorption of phenylalanine might be supported by mediation of calcium ion and by the contribution of hydrophobicity of the β-phenyl group.

INTRODUCTION

Carboxymethylated (CM) chitin, which is derived from chitin, a supporting mucopolysaccharide of crusteceans, has been reported to show various properties in the biomedical field [1-5]. The adsorption of fibrinogen, one of the blood proteins, has been reported to be greatly increased by the presence of calcium ion [6], and quite strange adsorption behavior of CM-chitin

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has been observed for calcium ion, which was only desorbed by the use of chelating reagents such as EDTA or EGTA. Hence, it was intended to investigate the mechanism of binding of CM-chitin onto fibrinogen through calcium ion. In addition, the adsorption mechanism of calcium ion, among alkali-earth metals, was at the same profile as that of transition-metal ions on CM-chitin subjected to stretching in a spinning process. The geometrical arrangement of acetamide at the C-2, primary hydroxyl at the C-6, and secondary hydroxyl groups at C-3 positions of *N*-acetylglucosamine (GlcNAc) residue was suggested to support the specific adsorption of calcium ion by FT-IR measurements. On investigation of calcium-mediated binding of various amino acids, the amount of adsorption of several neutral amino acids was enhanced by the presence of calcium ion, and phenylalanine was found to have the highest affinity toward the Ca-CM-chitin complex. On the other hand, calcium ion inhibited the adsorption of basic and acidic amino acids for CM-chitin.

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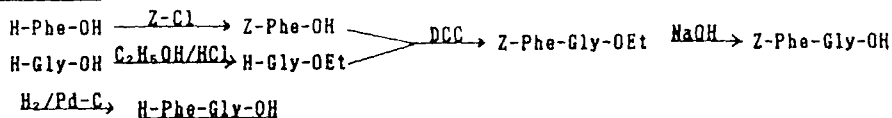
EXPERIMENTAL

CM-chitin was prepared by the method reported previously [7] from chitin which was extracted from queen crab shells according to the method of Hackman [8] and powdered to 30-45 mesh in the case of slight carboxymethylation (water insoluble). A chitin powder of 60-120 mesh was used to prepare the highly substituted CM-chitin (water soluble) by using various concentrations of sodium hydroxide. The degree of carboxymethylation (*DS*) was estimated by elemental analysis and potentiometric titration. CM-chitin fiber (water insoluble) was prepared by the method reported previously [9] and was cut to 2-3 mm lengths for use in column chromatography.

The adsorption of divalent cations on CM-chitin was measured by titration of the eluted ions with EDTA, using Dotite indicators [10]. The CM-chitin columns (2.3 × 23.5 cm) were packed with 22.5 g CM-chitin powder (30-45 mesh) or 7.9 g of CM-chitin fiber which was loaded after swelling in water. The flow rate of 0.05 *M* aqueous solution of divalent cations was 0.5-0.8 mL/min for insoluble CM-chitins, and capacities were estimated according to a previous paper [11]. The adsorption capacities for water-soluble CM-chitin (highly substituted) were calculated from the amount of free divalent ions after equilibrium dialysis of the CM-chitin and divalent cation mixture against deionized water for 48 h.

Various peptides were synthesized by liquid-phase condensation by using the dicyclohexyl carbodiimide (DCC) and mixed anhydride methods (see Scheme 1). The adsorption capacities of amino acids and peptides were esti-

H-Phe-Gly-OH



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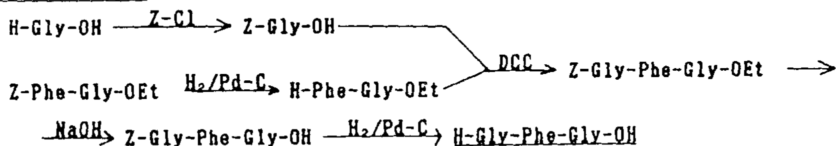

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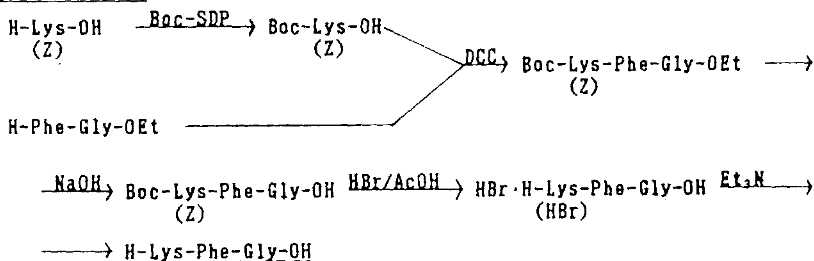

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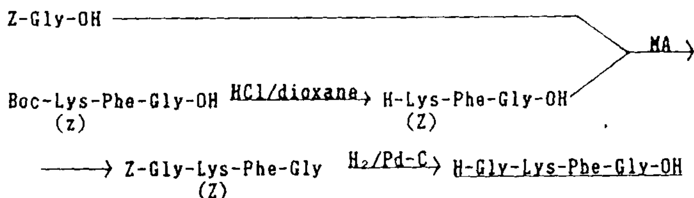
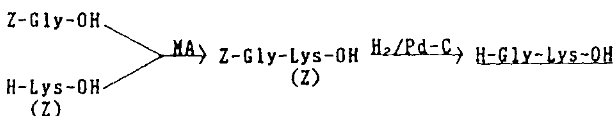


H-Gly-Lys-OH



SCHEME 1. Synthetic route to peptides. Z: Benzyloxycarbonyl group. Z-Cl: Benzyloxycarbonyl chloride. Boc: *t*-Butyloxycarbonyl group. Boc-SDP: *S*-*t*-Butylcarbonyl-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.

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mated from UV absorption at 257 nm and fluorescence intensities of *o*-phthal-aldehyde derivatives of amino acids using 340 nm as excitation and 455 nm as emission wavelengths [12].

FT-IR measurements of the water-soluble CM-chitin-Ca complex 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 in a Nicolet 5DXB FT-IR spectrophotometer.

RESULTS AND DISCUSSION

Adsorption of Metal Ions

The capacity of CM-chitin of low substitution to calcium ion seems to be higher than that of other metal ions (see Fig. 1). The adsorption mechanism of calcium ion might be rather different from that of other ions, because only calcium ion was not eluted by a pH shift. On the other hand, calcium ion bound to *fibrous* CM-chitin was eluted by a pH shift as were the other metal ions. 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. But the adsorption capacities for the other alkaline-earth metal ions are still low level.

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A highly substituted CM-chitin was applied to understand these effects on the specific adsorption, as shown in Fig. 2, since this water-soluble CM-chitin is expected to be more flexible to form a complex with metal ion in water than one of low substitution. But the adsorption profile for metal ions by highly substituted CM-chitin was observed to be similar to that of low substitution. Though the increased adsorption capacities are likely due to the increase of the carboxyl content, the unit capacity for calcium ion ($[Ca^{2+}]$/ $[COO^-]$) is reduced from 0.53 to 0.36, as seen in Table 5. This reduction of unit capacity seems to suggest that the primary hydroxyl group at the C-6 position of the GlcNAc residue contributes to the specific adsorption of calcium ion together with the introduced carboxyl groups. Since the adsorption behavior of metal ions is independent of the ionic radius and is altered by the stretching procedure, the specific adsorption of calcium ion seems to be due to the geometrical arrangement of several functional groups which are held by the rigid backbone of the CM-chitin molecule through hydrogen bonds even in aqueous solution.

As nickel ion is adsorbed nonspecifically on CM-chitin, functional groups causing the calcium adsorption would be suggested by the difference in the

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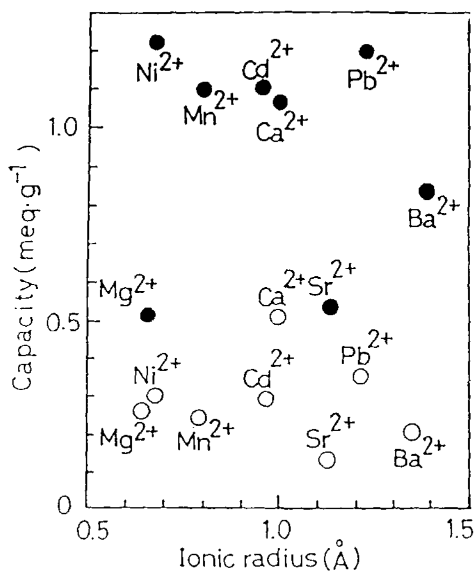


FIG. 1. Relationship between ionic radius and adsorption capacities of CM-chitin (DS = 0.1) for metal ions. (○) Poorly substituted CM-chitin (flake). (●) Fibrous CM-chitin.

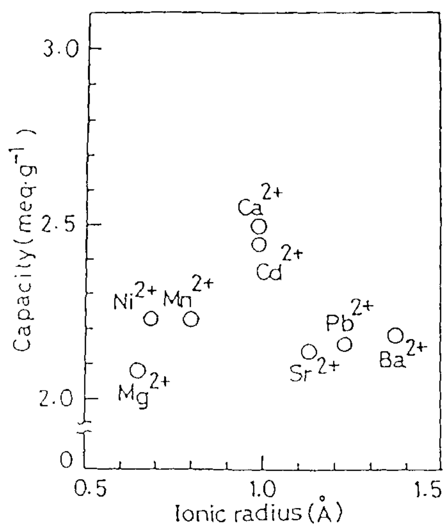
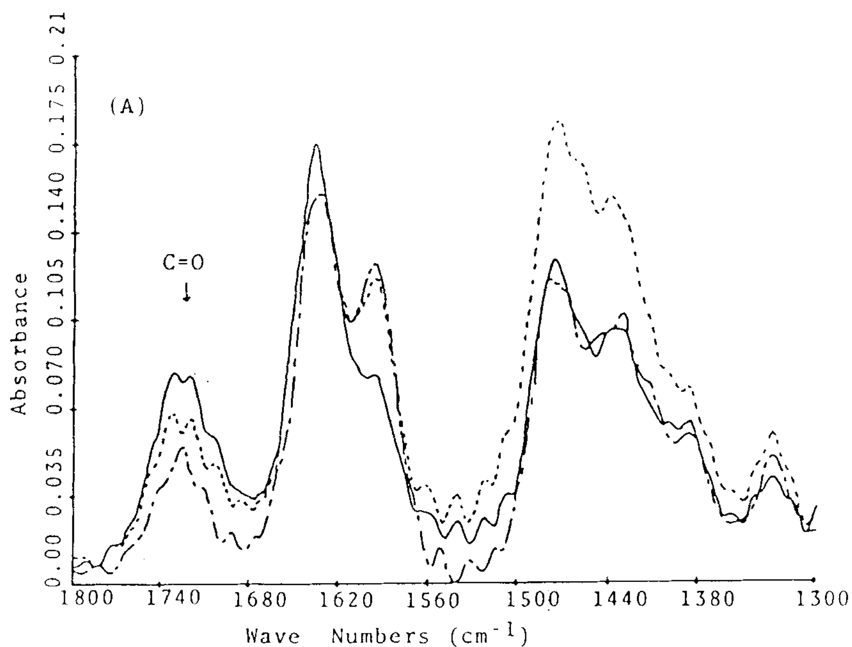
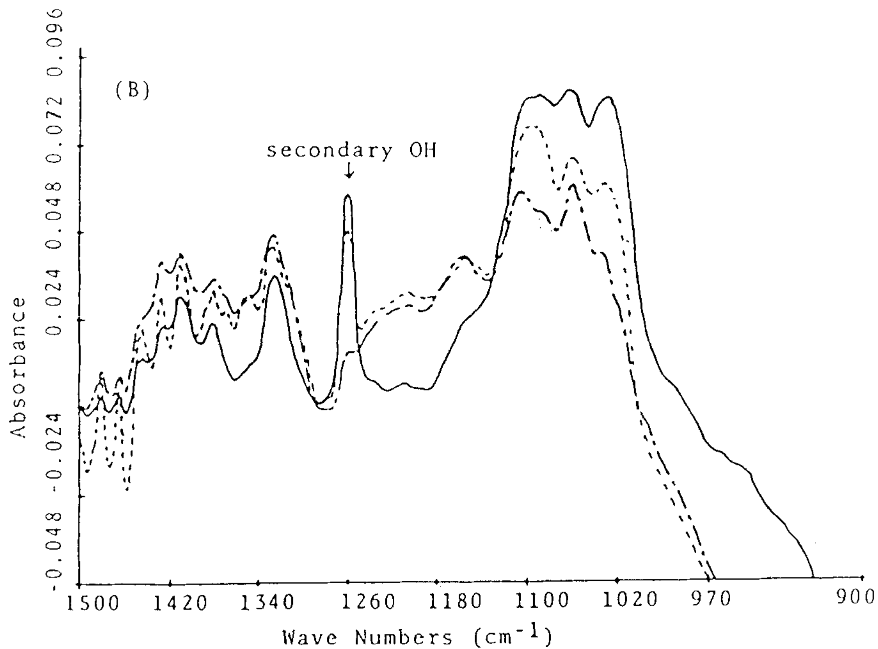


FIG. 2. Relationship between ionic radius and capacity of highly substituted CM-chitin (DS = 0.88) for metal ions.





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FT-IR spectra of Ca-CM-chitin and Ni-CM-chitin complexes (Fig. 3). Though the doublet peak due to stretching of the carbonyl group is simply decreased by the adsorption of nickel ion, the doublet peak is converted to a singlet and decreased more by the adsorption of calcium ion than by that of nickel. The peak at 1280 cm^{-1} due to secondary hydroxyl groups almost disappears in the presence of calcium ion but not in that of nickel ion. Several peaks in the region of 1000 to 1100 cm^{-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. The participation of amide group in the specific adsorption of calcium ion was suggested by differences in the IR spectrum of dried Ca-CM-chitin complex [11]. The Ca-CM-chitin complex might specifically consist of several functional groups as mentioned above.

Adsorption of Amino Acids on CM-Chitin-Metal Ion Complex

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Since CM-chitin enhances the interaction with fibrinogen, one of the blood proteins in the presence of calcium ion, the Ca-CM-chitin complex is expected to interact tightly with the hydrophobic domain of fibrinogen [6, 13]. Thus *D,L*-amino acids having different p*I* values were used to estimate the adsorption capacity on Ca-CM-chitin complex (water insoluble), as listed in Table 1. The adsorption of *D,L*-Phe was enhanced about 50 times in the case of calcium chelation compared to CM-chitin itself.

As the effective number of calcium ion complex 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-GlcNAc residue would be the driving force for 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, respectively, by calcium chelation. The adsorbed phenylalanine was eluted only by EDTA, while the other adsorbed amino acids were eluted by a shift in the ionic strength. Since there was hardly any calcium effect on the adsorption of amino acids to fibrous 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, and there is also little selectivity for the optical isomer of phenylalanine.

FIG. 3. FT-IR absorption spectra of water-soluble CM-chitin (*DS* = 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.

TABLE 1. 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.

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The adsorption profiles of *D,L*-amino acids for water-soluble CM-chitin-Ca or -Mn complexes were studied by using equilibrium dialysis, as shown in Table 2. The adsorption capacities of CM-chitin toward *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 neutral amino acids in the presence of calcium ion. *D,L*-Ala adsorption was decreased by calcium ion. This 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 with *D,L*-Phe was increased about threefold and hardly any effect was observed on the adsorption of other amino acids in the presence of manganese ion.

As the affinity of neutral amino acids toward the Ca-CM-chitin complex was suggested to depend on the hydrophobicity of the side chain, the adsorption capacity was plotted according to Nozaki and Tanford [14] as in Fig. 4. This shows that the adsorption capacities of the neutral amino acids other than Phe were found to be simply related to the hydrophobicity of the side chain. The abnormally high adsorption of Phe seems to be due to an additive factor, probably the phenyl side chain.

TABLE 2. Adsorption of Amino Acids on Water-Soluble CM-Chitin in the Presence of Ca^{2+} or Mn^{2+} [a]

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

[a]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.

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A preliminary ^{1}H-NMR study also suggested some influence upon the aromatic region. Since the amount of adsorbed *D,L*-Phe was three times as much as that of *L*-Phe and little *D*-Phe was adsorbed to the Ca-CM-chitin complex (as estimated by UV analysis of adsorbed Phe after equilibrium dialysis, as shown in Table 3), *L*-Phe was suggested to be adsorbed rather selectively to the Ca-CM-chitin complex without optical resolution. It was assumed that there was site-specific adsorption for the *D,L*-Phe conjugate in addition to the site for *L*-Phe, because only a small amount of *D*-Phe was adsorbed.

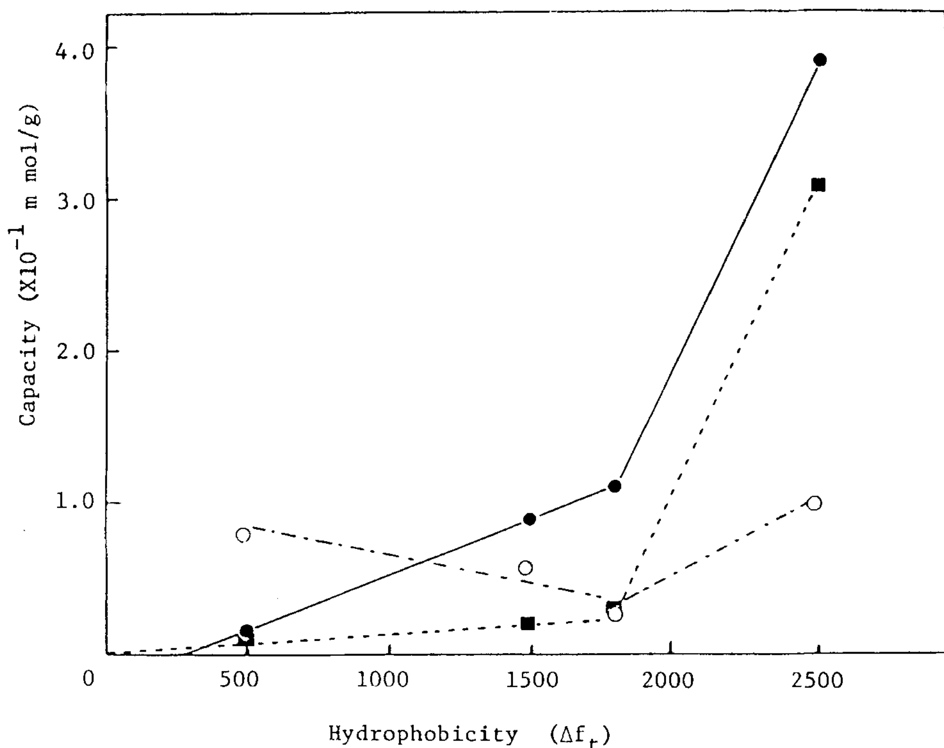

FIG. 4. Relationship between adsorption capacities of amino acids for water-soluble CM-chitin and hydrophobicity of the side chain. (●) In the presence of Ca^{2+}, (■) in the presence of Mn^{2+}, (○) in the absence of metal ion.

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Various peptides were examined to investigate the adsorption sites of amino acids for the Ca-CM-chitin complex (water insoluble), as listed in Table 4. Since the adsorption capacity for H-Gly-Phe-Gly-OH is maintained at almost the same level as that of H-Phe-Gly-OH, the α-amino and the α-carboxyl groups of Phe do not seem to affect the adsorption of Phe very much. The increase of the adsorption capacity for the peptides containing tyrosine suggest a contribution of the 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 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 great decrease in ionic linkages.

TABLE 3. Adsorption of Phenylalanine Optical Isomers on Ca-CM-Chitin Complex (in μmol/g)[a]

D,L-Phe	390
L-Phe	130
D-Phe	40

[a]Mixtures of *L*, *D*, or *D,L*-Phe and water-soluble CM-chitin were dialyzed against deionized water for 48 h at room temperature. The optical densities of the inner solutions were measured at 257 nm.

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These data are treated stoichiometrically to explain the results more clearly and summarized in Table 5. It is clear from Table 5 that only a part of the bound calcium contributes to the adsorption of these neutral amino acids. For example, the effective number for the *D,L*-Phe adsorption is 13% of bound calcium ions on low substituted CM-chitin and 31% on highly substituted CM-chitin. However, the effective number of calcium ion for *D,L*-Phe adsorption on the fibrous CM-chitin is much less in spite of the threefold increase of calcium ion adsorption. This result suggests the contribution of the geometrical arrangement of the functional groups for the specific adsorption of phenylalanine, but the adsorption of lysine seems not to depend on the bound calcium ions but on the ionic binding through carboxyl groups. The speculative adsorption mechanism for phenylalanine shown in Scheme 2 assumes that the adsorption of calcium ion and amino acid is a local phenomenon because little conformational change was suggested by a CD study of the adsorption of small molecules. Further studies of the adsorption mechanism are now under way by NMR and EXAFS.

However, CM-chitin might become very useful in the near future for peptide drug carriers with controlled release because CM-chitin is highly biodegradable, has a poor antigenicity, and calcium ion is very abundant in the animal body.

TABLE 4. Adsorption of Peptides to Low-Substituted CM-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]1 g CM-chitin was packed into a column (1.0 X 10.4 cm), and 4.0 m*M* peptide solutions were passed through the column at a rate of about 0.2 mL/min.

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

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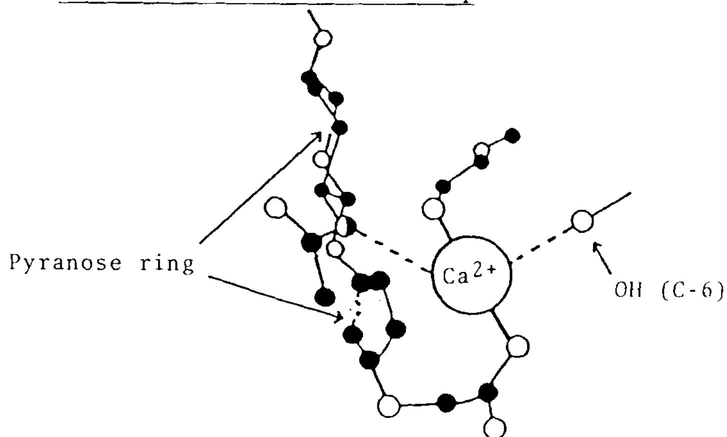

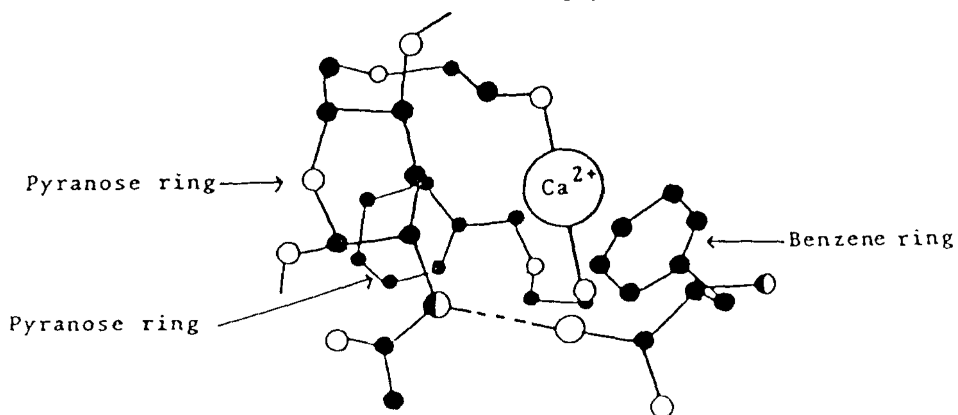

● ; Carbon atom

○ ; Oxygen atom

◐ ; Nitrogen atom

SCHEME 2. Speculative model for Ca-CM-chitin complex and Ca-CM-chitin-Phe complex.

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TABLE 5. Summary of Unit Capacities of CM-Chitin

Sample	Low 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

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ACKNOWLEDGMENTS

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