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THE ADSORPTION OF BOVINE BLOOD PROTEINS ONTO THE SURFACE OF *O*-(CARBOXYMETHYL)CHITIN

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ABSTRACT

Chitin was found to interact with bovine blood proteins and the affinities of these proteins for chitin tended to be decreased by the introduction of *O*-carboxymethyl (CM) groups onto the chitin surface, especially with fibrinogen. As the adsorption of blood proteins to the CM-chitin (d.s. 0.35) was assumed to follow an isothermal adsorption-curve, the adsorption coefficients were estimated by applying the Langmuir equation. Bovine serum albumin showed the highest affinity among the proteins applied in this experiment [K_{BSA} (bovine serum albumin); 20.0, $K_{B\gamma G}$ (bovine gamma globulin); 1.96, K_{BF} (bovine fibrinogen); 1.20]. The binding site of BSA for CM-chitin was assumed to be regulated not only by the cationic groups of BSA but also by other factors such as the recognition capacity of BSA to bind to GlcNAc residues in CM-chitin.

INTRODUCTION

Chitin, a widely distributed supporting polysaccharide of crustacea, is known to be accessible to lysozyme in the human body despite its similar chemical and crystalline structure to that of cellulose[1]. Chitin is insoluble in general organic solvents as a result of its rigid, crystalline structure supported by hydrogen bonds between hydroxyl groups and acetamido groups of GlcNAc residues[2]. Chitin does not contain any other functional groups except hydroxyl groups, although there are a few free amino groups in chitin that may bind with native proteins. As the acetamido group of the GlcNAc residue is unstable under alkaline conditions and the glycoside linkage of chitin is labile to acid, the chemical modification of chitin has to be performed under conditions milder than for cellulose.

We have chemically functionalized chitin under mild conditions[3–11]. A 6-*O*-(carboxymethyl)chitin was prepared from alkali-chitin and monochloroacetic acid in 2-propanol at room temperature. The degree of carboxymethylation of chitin, contributing to the water solubility, was found to be regulated by the concentration of sodium hydroxide during the freezing process in preparation of the alkali-chitin. The adsorption properties of *O*-(carboxymethyl)-(CM)-chitin flake (d.s. 0.35)

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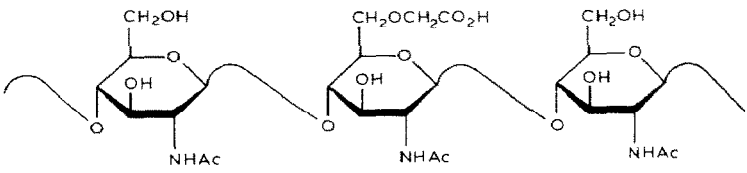

Partially carboxymethylated chitin (D.s 0.33)

toward alkaline earth metals were reported previously[12]. It was found that calcium ion was adsorbed to CM-chitin specifically from among other alkaline earth metals, and magnesium ion was replaced by calcium ion, even in the presence of a monovalent cation such as Na^+ or K^+. The cation-exchange property of CM-chitin is also expected to participate with some specificity in the adsorption of a protein.

In this study, the interactions of CM-chitin with blood proteins were investigated by adsorption chromatography in order to determine the relationship between carboxyl groups and GlcNAc residues on the adsorption of blood proteins. The effect of Ca^{2+} on the interaction of CM-chitin with proteins is also described in preliminary form.

EXPERIMENTAL

Materials. — Chitin was prepared from Queen Crab shells according to the method of Hackman[13] and powdered to 45–60 mesh before use. Monochloroacetic acid and other reagents of reagent grade were obtained from Wako Pure Chemical Industries Ltd. and used without further purification. Bovine serum albumin, bovine fibrinogen, and bovine gamma globulin were purchased from Nakarai Chemical Ltd. and used without further purification.

Preparation of alkali-chitin. — Alkali-chitin was prepared as described previously[10]. Aqueous solutions (25–40%, w/w) of sodium hydroxide were used to prepare the variously substituted CM-chitins.

Preparation of CM-chitin. — CM-chitins were prepared as described previously[10]. The excess of sodium hydroxide was not removed to prevent deacetylation during the isolation procedure. As CM-chitins prepared under our experimental conditions were water-insoluble, the products were treated with 0.01M HCl and then washed successively with water, acetone, ethanol, and then dried *in vacuo*.

Estimation of the degree of substitution. — The degree of carboxymethylation was determined by elemental analysis with a Yanagimoto CHN Corder MT-2, by i.r. absorption spectroscopy with a JASCO i.r. spectrophotometer A-302, and also by potentiometric titration with a Radiometer Automatic Titor TTTc-SBR2c under a nitrogen atmosphere, as described previously[10].

Adsorption chromatography. — The water suspension of CM-chitin was loaded onto a short column (1.1 × 10.0 cm) which was then equilibrated with buffer solutions (0.05M Tris-HCl, 0.05M acetate, and 0.05M formate buffers; the ionic

strength was adjusted to 0.15 by NaCl). Phosphate and borate buffers could not be used owing to precipitation with calcium ion. The protein, equilibrated with a buffer solution, was applied to the CM-chitin column. The amount of applied protein was adjusted to 1.0 mg in each case. After the adsorption isotherm had been determined, the column was rinsed with the buffer solution until no absorption at 280 nm was observed in the effluent. The bound protein was eluted by 0.1M HCl at a flow rate of 0.5–0.6 m.min^{-1}, and the amount of adsorbed protein was estimated, using calibration curves, from the absorption at 280 nm. The adsorption coefficients of proteins were determined by applying the Langmuir isothermal-adsorption equation.

$$\text{CM-chitin} + \text{protein} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{CM-chitin–protein complex}$$

The adsorption coefficients ($b = k_1/k_{-1}$) of the CM-chitin–protein complex were calculated from the following equation:

$$A = \frac{\alpha b[P]}{1 + b[P]} \tag{1}$$

where $[P]$ is the initial protein concentration applied (mg.mL^{-1}), A is the amount of adsorbed protein (mg) obtained from the absorption at 280 nm per g of CM-chitin, and α denotes the maximum amount of adsorption for each protein (mg of protein/g of CM-chitin). In this study, the constants were obtained separately by using a rearranged form of Eq. *1*:

$$\frac{1}{A} = \frac{1}{\alpha} + \frac{1}{\alpha b[P]} \tag{2}$$

When chromatography was performed at various protein concentrations, b was obtained from the slope and α was also determined from the intercept on the ordinate of double-reciprocal plots of A and $[P]$.

RESULTS AND DISCUSSION

Estimation of the degree of substitution. — The carboxymethylation of chitin was successfully performed by varying the alkali concentration, although the excess of alkali was not removed to prevent deacetylation during the squeezing process. The concentrations of sodium hydroxide used, the degree of carboxymethylation, and the elemental analyses are shown in Table I. The degree of carboxymethylation estimated from the elemental analysis agreed very well with that from the potentiometric titration of CM-chitin in 0.1M NaCl solution, as reported previously[10,12].

TABLE I

RELATIONSHIP BETWEEN DEGREE OF SUBSTITUTION AND THE CONCENTRATION OF NaOH ADDED

[NaOH] % (w/w)	*Degree of substitution*[a]	*Found (%)*			*Calc. values (%)*		
		C	*H*	*N*	*C*	*H*	*N*
0	0	44.9	6.50	6.57	45.3	6.60	6.60
25	0.20	44.9	6.51	6.39	45.1	6.44	6.26
30	0.35	44.3	6.51	6.15	44.9	6.33	6.18
40	0.58	44.5	6.36	5.88	44.7	6.17	5.70

[a]Carboxymethylated groups/GlcNAc residue.

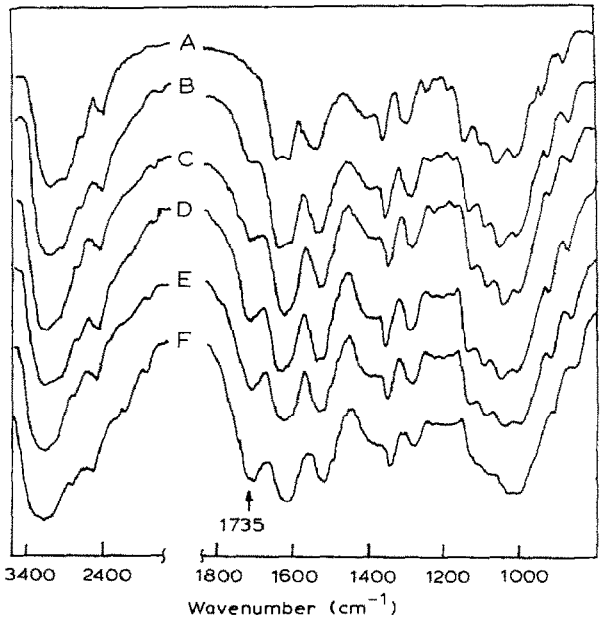

Fig. 1. The i.r. spectra of variously carboxymethylated chitins: A, chitin; B, d.s. 0.15; C, d.s. 0.20; D, d.s. 0.35; E, d.s. 0.58; and F, d.s. 0.78. D.s. 1.0 corresponds to chitin fully carboxymethylated at O-6.

The i.r. absorption spectra of chitin and variously *O*-(carboxymethylated)-chitins are shown in Fig. 1. The absorption at 1735 cm^{-1}, attributable to carbonyl stretching of carboxyl group, was increased by the carboxymethylation procedure. As the hydroxyl-group absorptions, which were expected to decrease through substitution, were not observed clearly, it was assumed from n.m.r. studies that the substitution site of the GlcNAc residue would be O-6, the primary hydroxyl group[10]. The intensity of the C-6 peak of GlcNAc (63.0 p.p.m.) was decreased, and a new peak could be observed at 69.0 p.p.m. attributable to the substituted carbon atom. There was no significant indication of a shift of C-3 (76.8 p.p.m.) resulting from 3-substitution. Miyazaki and Matsushima[14] reported also that 2-amino-3-*O*-

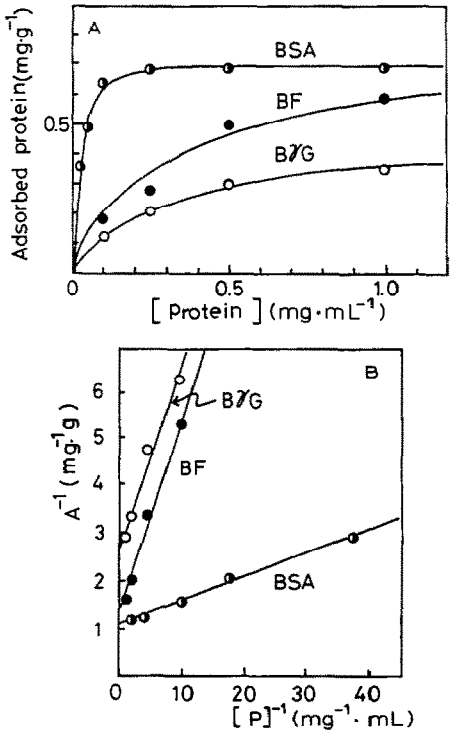

Fig. 2A. Isothermal adsorption-curves of blood proteins. Conditions: pH 7.4, ionic strength 0.15, 25°, in 50mM of Tris-HCl buffer; B. Double-reciprocal plot of isothermal adsorption-curves.

TABLE II

AFFINITY CONSTANTS OF BLOOD PROTEINS

	BSA	*BF*	*BγG*
b[a]	20.0	1.20	1.96
α[b]	0.87	0.77	0.43

[a]Adsorption coefficient (k_1/k_{-1}). [b]The maximum amount of adsorbed proteins (mg/g of CM-chitin).

(carboxymethyl)-2-deoxy-D-glucose hydrochloride is barely detectable by t.l.c. of the acid hydrolyzate of CM-chitin when the degree of carboxymethylation was <1.0. Attack at O-6 seems to be particularly favored because HO-3 may be engaged by hydrogen bonds, as proposed by Blackwell *et al.*[2]

Adsorption chromatograpy. — The adsorption isotherms of BSA, BF, and BγG on the CM-chitin flake (d.s. 0.35) are shown in Fig. 2A for various concentrations of protein. The ordinate is the amount of adsorbed protein (mg) per g of CM-chitin flake and the abscissa is the concentration of protein applied ($mg.mL^{-1}$). The double-reciprocal plots of Fig. 2A give straight lines, suggesting that the

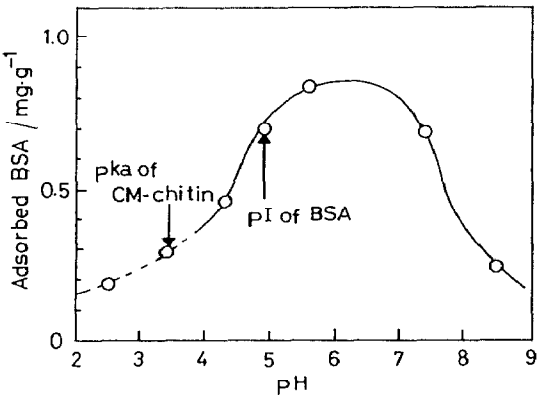

Fig. 3. Dependence of pH on the adsorption of BSA onto CM-chitin at 25°; pH 2.5, 50mM formate buffer; pH 3.5, 4.5, and 4.8, 50mM acetate buffer; pH 5.8, 7.4, and 8.5 50mM Tris-HCl buffer. The ionic strength was adjusted to 0.15 by NaCl in each case.

adsorption proceeds under Langmuir's conditions as shown in Fig. 2B. The α and b values are obtained respectively from the slope and the intercept, as described in the experimental section. The results are summarized in Table II. The affinity of BSA toward CM-chitin (d.s. 0.35) is the highest of the proteins studied (K_{BSA} 20.0, $K_{B\gamma G}$ 1.96, and K_{BF} 1.20). As there is no noteworthy difference in the maximum amounts of adsorbed proteins (α), monomolecular layer-adsorption on the surface of the CM-chitin molecule is indicated. The highest affinity of BSA is also clearly shown at lower protein concentrations, as seen in Fig. 2A.

The pH dependence of the maximal adsorption of BSA to CM-chitin is shown in Fig. 3. A specific adsorption-site is suggested to be present on the surface of the native BSA molecule, because the adsorption of BSA is considerably decreased upon loss of the native conformation by a shift in pH.

Binding is maximal in the pH range 5–7. Both surfaces should be charged negatively in the pH region 5–7, as the pK_a of the carboxyl group of CM-chitin was reported to be 3.4 by potentiometric titration[10], and the isoelectric point of BSA is known to be 4.8. The pH dependence of the adsorption of protein would also suggest the presence of a type of driving force to overcome such an electrostatic repulsion between BSA and CM-chitin.

In the previous report[12], CM-chitin was shown to adsorb Ca^{2+} specifically from among the alkaline earth metals, and to be equilibrated with 0.3 mmol of Ca^{2+} per g of CM-chitin. The Ca^{2+} was assumed to chelate onto the CM-chitin surface, with the tetrahedral profile being assisted by the acetamido and hydroxyl groups as well as the carboxyl groups[12]. In order to reveal the effect of Ca^{2+} on the adsorption of proteins, blood proteins were applied to the CM-chitin in the presence of Ca^{2+}, as summarized in Table III. The adsorption of BSA is depressed to 0.34 mg/g from 0.79 mg/g of CM-chitin and that of bovine fibrinogen is enhanced to 0.81 from 0.59 mg/g. There is little effect on the adsorption of BγG. As these tendencies on the adsorption of blood proteins have also been observed for Ca^{2+}-

TABLE III

THE EFFECTS OF Ca^{2+} ON THE ADSORPTION OF PROTEINS[a] (mg/g)

	BSA	*BF*	*BγG*
$-Ca^{2+}$	0.79	0.59	0.36
$+Ca^{2+}$	0.34	0.81	0.39

[a]The concentration of proteins are all 1.0 mg/mL.

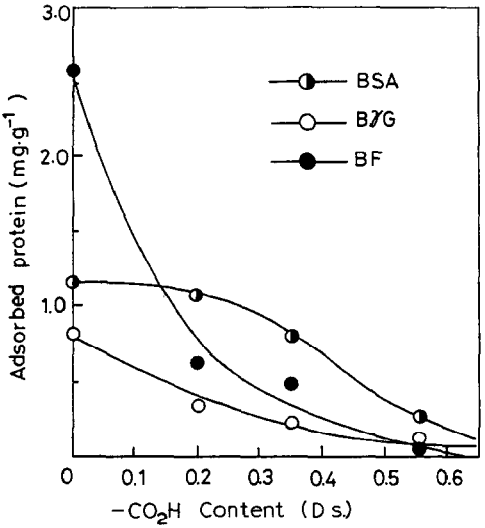

Fig. 4. Relationship between the degree of carboxymethylation of chitin and the amount of adsorbed proteins. Proteins were adsorbed onto resin at pH 7.4 (50mM Tris-HCl buffer and ionic strength 0.15) and 25°.

pretreated CM-chitin, bovine fibrinogen seems to have an enhanced affinity to the Ca^{2+}-chelated CM-chitin surface, and the adsorption of BSA is somewhat inhibited.

As the participation of carboxyl groups has been involved in the adsorption of blood proteins, the dependence of carboxyl content on the adsorption of proteins was investigated, as shown in Fig. 4. There is a noteworthy influence on the adsorption of bovine fibrinogen, but BSA and BγG are not so sensitive to changes of carboxyl content. The increased electrostatic repulsion between charged groups disturbs the interaction of protein with CM-chitin.

In conclusion, the chitin surface that has high initial affinity toward BF may be converted into a BSA-selective surface by the introduction of carboxyl group, preferentially at low protein concentration (0.1 mg/mL), and a 0.58-substituted CM-chitin has little affinity toward BF. As the adsorption property is suggested to be influenced by pretreatment with Ca^{2+}, the interaction between blood-proteins and a non-charged chitin surface would be controlled by the amount of carboxyl group and by the participation of calcium ion.

The further introduction of SO_3H groups into the CM-chitin molecule will be required for fundamental understanding of the interaction of blood proteins with heparinoids[15-17]. The results of further investigations will be reported in elsewhere.

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