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Quantitative analysis of carboxymethyl chitin adsorbed on a liposome surface

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ABSTRACT

A convenient and precise method for the quantitative determination of carboxymethyl chitin (CM-chitin) on a liposome surface has been developed. CM-chitin adsorbed on the liposome surface was separated from the phospholipid by extraction with $CHCl_3$. The separated CM-chitin was completely hydrolyzed under acidic conditions, and the hydrolysis product, an *N*-acetyl-D-glucosamine derivative, was colorimetrically determined using the principle that ferricyanide is reduced by hemiacetals or hemiketals to ferrocyanide.

INTRODUCTION

Chitin, a poly-(1-4)-β-2-acetamido-2-deoxy-D-glucopyranose and its derivatives are expected to be usable as biomaterials because of their nontoxic and enzymatically biodegradable properties. Carboxymethyl chitin (CM-chitin) has been used to stabilize liposomes composed of phosphatidylcholine[1–3], however, no quantitative, direct method for the determination of CM-chitin on the surface of a liposome has yet been established. For the complete hydrolysis of chitin α-*N*-acetylglucosaminidase (EC 3.2.1.50) or β-*N*-acetylhexosaminidase (EC 3.2.1.52) is required, in addition to chitinase (EC 3.2.1.14)[4]. The activity of these enzymes toward CM-chitin is significantly lower than that toward chitin because of the presence of bulky carboxymethyl groups.

Ferricyanide is reduced by hemiacetals or hemiketals to ferrocyanide, and a colorimetric determination at 420 nm is an index of the amount of reducing sugar present in the sample[5]. CM-chitin can be completely hydrolyzed under acidic conditions[6], and the amount of the hydrolysis product, a 2-acetamido-2-deoxy-D-

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glucose derivative, can be precisely determined by the ferricyanide procedure in the range of 2–200 μg.

In this study, a convenient, quantitative determination of CM-chitin separated from the liposome surface was developed by means of the chemical hydrolysis of CM-chitin and the quantitative determination of the hydrolysis product using the ferricyanide procedure.

EXPERIMENTAL

Materials and methods.—CM-chitin was kindly provided by Dr. K. Kifune in Unitika Ltd., R & D Center, Kyoto, Japan. Dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), chitinase (from *Streptomyces griseus*), and lysozyme (from chicken egg white) were purchased from Sigma Chemical Co. All other chemicals were of analytical grade or better. Electronic spectra were measured with a Perkin–Elmer Lambda 4B UV/VIS spectrophotometer.

Preparation of liposomes.—A mixture of DMPC (30.6 mg) and DMPG (3.4 mg) was dissolved in 2 mL of $CHCl_3$ and 4 mL of diethyl ether. Two mL of 0.1 M phosphate buffer solution (PBS, pH 7.4) was added to the organic solution, and the resulting two-phase system was sonicated for 1 min in a bath-type sonicator (Laboratory Supplies Co., Hicksville, NY). The mixture was then placed on the rotary evaporator, and the organic solvent was evaporated under reduced pressure (water aspirator) for 10 min at 25°. Two mL of PBS was added, and the suspension was re-evaporated for an additional 30 min at 45° to remove traces of solvent. Two mL of CM-chitin solution (9.8 mg/mL, 19.3 mg/mL or 29.2 mg/mL) was added to 2 mL of liposome suspension with stirring, and the mixture was kept at 4° overnight. In order to separate CM-chitin-coated liposomes from free CM-chitin, the mixture ($\sim$ 6 mL) was centrifuged at 15 000*g* for 30 min, and the pellet of CM-chitin-coated liposomes was resuspended in PBS and washed four times with PBS on the centrifuge. Then, the total volume of CM-chitin-coated liposome suspension was adjusted to 3 mL with PBS. The aliquots of supernatants and pellets suspended in PBS were analyzed for phospholipid and CM-chitin. The concentration of phospholipid was determined by choline assay[7–9]. As the control liposomes, CM-chitin free liposomes were prepared by the same method as described above except that 2 mL of PBS, instead of 2 mL of CM-chitin solution, was added to liposome suspension.

Analysis of CM-chitin in aqueous solution.—Analysis of CM-chitin in PBS was carried out by means of the chemical hydrolysis of CM-chitin and the determination of the hydrolysis product using the modified method of Robyt and coworkers[5]. Thus, to 0.1 mL of CM-chitin solution in PBS, 0.5 mL of concd. HCl was added, and the mixture was heated for 2.5 h at 85° in a test tube with a glass stopper. After cooling the mixture, 0.6 mL of 0.1 N NaOH and 0.5 mL of 0.02 M $K_3Fe(CN)_6$–0.5 M Na_2CO_3 were added to the mixture, and it was heated for 15

min at 85°. Then, the total volume of the mixture was adjusted to 5 mL by the addition of distilled water, and the absorbance at 420 nm was measured. For the control experiments, 0.1 mL of PBS instead of CM-chitin solution was used (PBS control). The decrease in absorbance at 420 nm due to the 2-acetamido-2-deoxy-D-glucose derivative liberated from CM-chitin in the reaction (ΔA) was obtained by subtraction of the absorbance at 420 nm of the samples from that of PBS control. The product analysis was carried out by paper chromatography [6:4:3 (v/v) butanol–pyridine–H_2O][6].

Analysis of CM-chitin on the liposome surface.—To a CM-chitin-coated liposome suspension (0.3 mL PBS) was added 1 mL of $CHCl_3$, and the mixture was shaken vigorously for 1 min in a separating funnel. After the mixture separated into two phases, the $CHCl_3$ phase was removed from the separating funnel. This procedure was repeated four times to extract the phospholipid from the CM-chitin-coated liposomes. The aliquots of the aqueous phase were analyzed for CM-chitin by the same method as described above, for the analysis of CM-chitin in aqueous solution. For the control experiments, the same experiments were carried out using CM-chitin-free liposomes, and the absorbance at 420 nm obtained for the CM-chitin-coated liposomes was subtracted from that measured for the CM-chitin-free liposomes. Each $CHCl_3$ solution containing phospholipid was placed on the vacuum pump to remove $CHCl_3$, and the remaining phospholipid was suspended in 1 mL of PBS and analyzed for phospholipid.

RESULTS AND DISCUSSION

Analysis of CM-chitin in aqueous solution.—Fig. 1 shows the decrease in absorbance at 420 nm (ΔA) for the reduction of ferricyanide as a function of the concentration of CM-chitin. The reducing power of the hydrolyzed CM-chitin increased linearly with an increase in the CM-chitin concentration, and it was shown that the amount of CM-chitin can be determined precisely in the order of a few μg (see Fig. 1b).

Fig. 2 shows the time-dependent hydrolysis of 200 μg of CM-chitin under acidic conditions. ΔA at 420 nm became constant after heating for 2 h at 85°. Fig. 3 shows ΔA for the reduction of ferricyanide as a function of the concentration of 2-acetamido-2-deoxy-D-glucose. Taking account of the degree of carboxymethylation of our CM-chitin of 0.7 (ref. 10), the average molecular weight of the 2-acetamido-2-deoxy-D-glucose derivative in CM-chitin is estimated to be 277. Using this value, the amount of 2-acetamido-2-deoxy-D-glucose derivative in 20 μg of CM-chitin is estimated to be 7.2×10^{-2} μmoles. As shown in Figs. 1 and 3, ΔA for the reduction of ferricyanide by 20 μg of CM-chitin (0.112) is consistent with that by 7.2×10^{-2} μmoles of 2-acetamido-2-deoxy-D-glucose (0.110). These results suggest that the complete chemical hydrolysis of CM-chitin takes place by heating for 2.5 h at 85° under acidic conditions. On the other hand, the hydrolytic activity of the enzymes, chitinase or lysozyme, toward CM-chitin was very low (see Fig. 2).

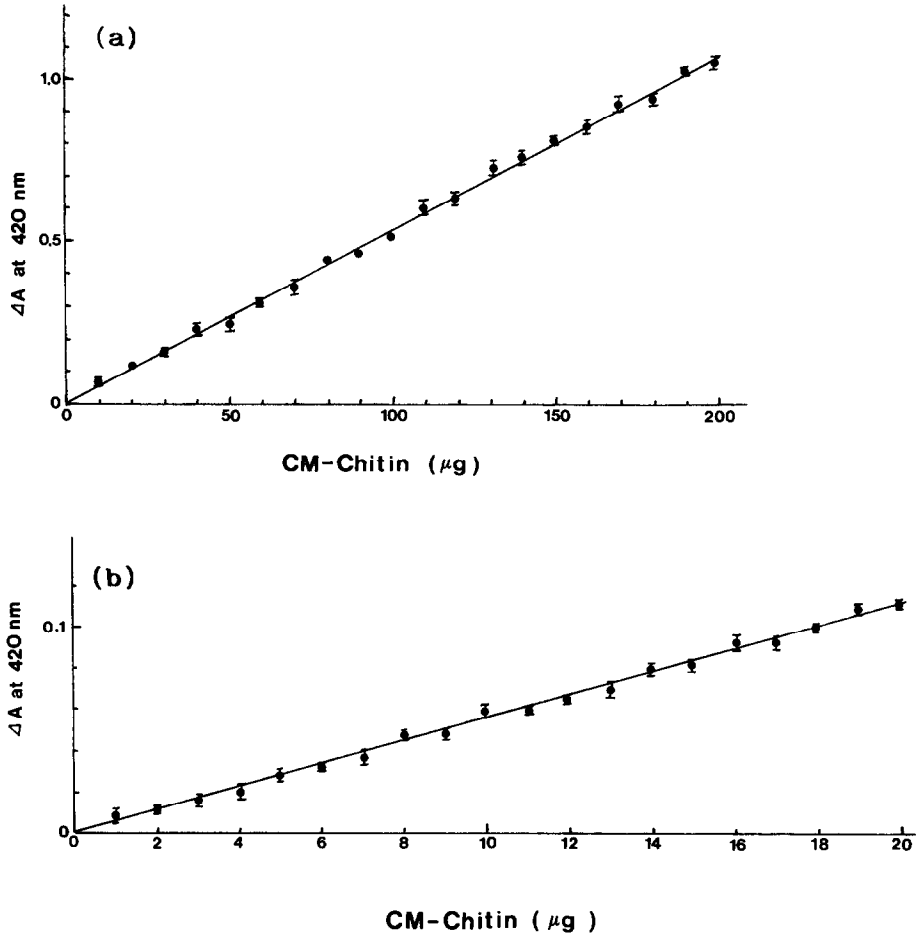

Fig. 1. (a and b) ΔA at 420 nm as a function of CM-chitin concentration. Mean ± SEM ($n = 3$) are shown.

Incubation of 200 μg of CM-chitin with 30 units of chitinase or lysozyme for 13 h at 37°, pH 7.4, showed only 50% hydrolysis of CM-chitin.

Analysis of CM-chitin on the liposome surface.—The amount of free CM-chitin in the supernatants after washing the liposomes in the centrifuge is shown in Table I. After the 1st washing, 96% of the free CM-chitin was separated from the liposomes, and 2% of free CM-chitin was removed by the 2nd washing, and even a trace of free CM-chitin was not detected in the 3rd and 4th supernatants, indicating that the free CM-chitin was not present in the liposome suspension after washing four times in the centrifuge.

After extraction of phospholipid with $CHCl_3$ three times, almost 100% of phospholipid was removed from CM-chitin-coated liposomes. Thus, the 1st, 2nd, and 3rd $CHCl_3$ extract contained 91.3, 8.4, and 0.1% of the total phospholipid,

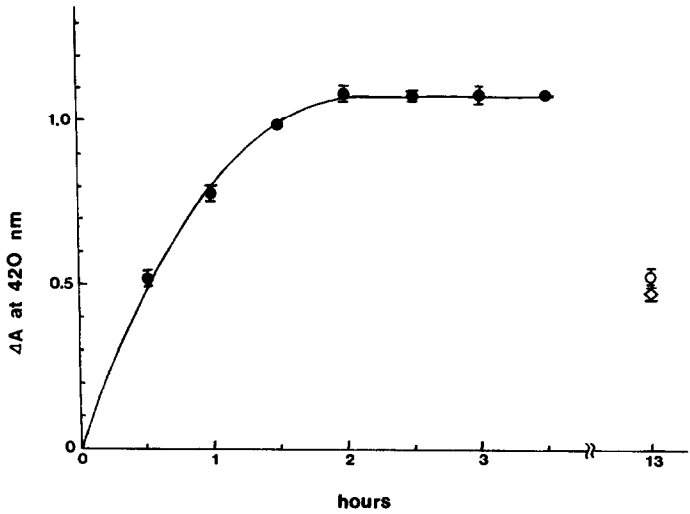

Fig. 2. Time dependent hydrolysis of 200 μg of CM-chitin. Mean $\pm$ SEM ($n = 3$) are shown: (•) ΔA for hydrolysis under acidic conditions; (○) ΔA for hydrolysis with chitinase; (◇) ΔA for hydrolysis with lysozyme.

respectively. The amount of phospholipid in the CM-chitin-coated liposome suspension, both before and after the extraction of phospholipid with $CHCl_3$ four times, was determined to be 9.7 mg/mL and 3 μg/mL, respectively, and these values were independent of the initial CM-chitin concentration. The amount of

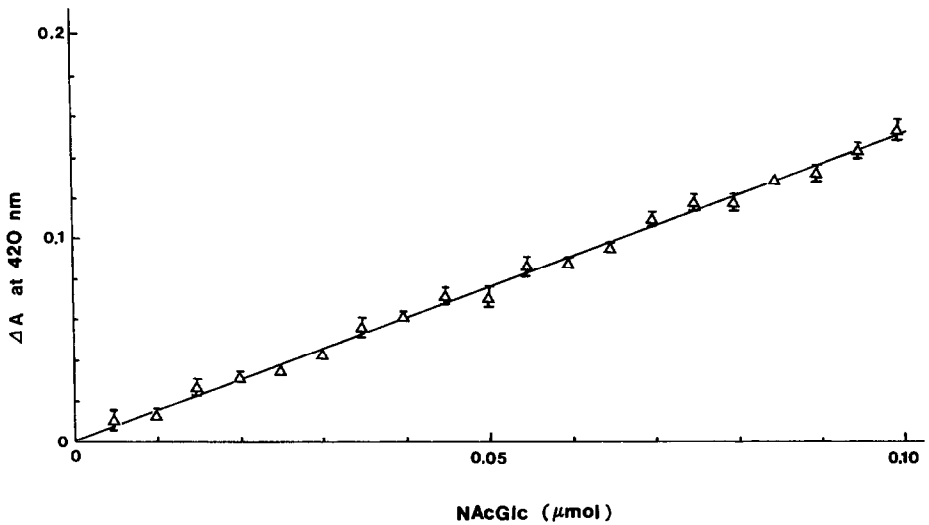

Fig. 3. ΔA at 420 nm as a function of 2-acetamido-2-deoxy-D-glucose concentration. Mean $\pm$ SEM ($n = 3$) are shown.

TABLE I

Amount of free CM-chitin in the supernatants after washing of liposomes in the centrifuge

Total amount of CM-chitin (mg)	Amount (mg) after washing No.			
	1st	2nd	3rd	4th
19.6	18.6	0.6	ND [a]	ND [a]
38.6	37.1	0.7	ND [a]	ND [a]
58.4	56.1	1.2	ND [a]	ND [a]

[a] ND, not detectable.

CM-chitin in the CM-chitin-coated liposome suspension after the extraction of phospholipid with $CHCl_3$ was determined using the calibration curve shown in Fig. 1. The amount of CM-chitin adsorbed on the liposome surface increased linearly with initial CM-chitin concentration (Fig. 4). By an independent experiment it was shown that the solubility of CM-chitin in $CHCl_3$ at 25° was negligible (i.e., less than 1 μg/100 mL), indicating that not even a trace of CM-chitin was present in the $CHCl_3$ extract.

The quantitative determination of CM-chitin in the liposome system would be difficult without the separation of CM-chitin from the liposome system because of the interfering effects of the perturbation from the phospholipids. A turbidity method for the quantitative determination of CM-chitin in coated liposomes has been reported[11]. However, the property of CM-chitin depends on the degree of carboxymethylation and the average molecular weight of CM-chitin, which affects the solubility and the turbidity of CM-chitin in solution and the conformation of

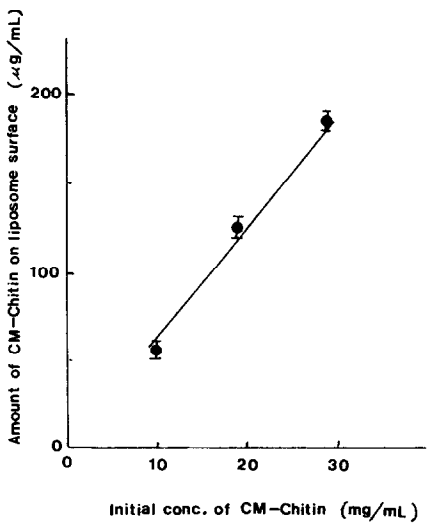

Fig. 4. Amount of CM-chitin on the liposome surface as a function of the initial CM-chitin concentration. Mean ± SEM ($n = 3$) are shown.

CM-chitin on the liposome surface. The chemical hydrolysis of CM-chitin separated from the liposome system and the colorimetric determination of the hydrolysis product as described herein is a convenient and precise means for the quantitative determination of CM-chitin. This method could be applied to CM-chitin with different degrees of carboxymethylation and average molecular weight, and the extraction of phospholipid with $CHCl_3$ should also be applicable to almost every kind of phospholipid.

The effects of CM-chitin on the membrane dynamics as a function of CM-chitin concentration and the property of phospholipid will be discussed in a forthcoming article.

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REFERENCES

1 T. Kondo, in G. Gregoriadis (Ed.), *Liposomes as Drug Carriers*, Wiley, New York, 1988, pp 585–595, and references therein.
2 C.Dong and J.A. Rogers, *Proc. Intl. Symp. Control., Rel. Bioact. Materials* 16 (1989) 358–359.
3 T. Nishiya, and S. Ahmed, *J. Biochem.*, 107 (1990) 217–221.
4 A. Ohtakara, *Methods Enzymol.*, 161 (1988) 462–470.
5 J.F. Robyt, R.J. Ackerman, and J.G. Keng, *Anal. Biochem.*, 45 (1972) 517–521.
6 R.T. Mayer, A.C. Chen, and J.R. DeLoach, *Insect Biochem.*, 10 (1980) 549–556.
7 H.-O. Beutler and G. Henninger, *Swiss Food*, 3 (1981) 27–29.
8 K. Diedrich, S. Hepp, H. Welker, D. Krebs, and H.-O. Beutler, *Geburtshilfe Frauenheikunde*, 39 (1979) 849–856.
9 S. Schiefer and H.-O. Beutler, in H.U. Bergmeyer (Ed.), *Methods Enzym. Anal.*, Vol. 8, 3rd edn., Verlag, Deefield Beach, FL, 1985, pp 87–104.
10 K. Kifune, personal communication.
11 C. Dong and J.A. Rogers. *J. Microencapsulation*, 8 (1991) 153–160.