*Carbohydrate Research*, 146 (1986) 251–258
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

# BIOACTIVE CHITIN DERIVATIVES. ACTIVATION OF MOUSE-PERITONEAL MACROPHAGES BY *O*-(CARBOXYMETHYL)CHITINS

SHIN-ICHIRO NISHIMURA, NORIO NISHI, SEIICHI TOKURA,
*Department of Polymer Science, Faculty of Science, Hokkaido University, Sapporo 060 (Japan)*

KEIKO NISHIMURA, AND ICHIRO AZUMA
*Institute of Immunological Science, Hokkaido University, Sapporo 060 (Japan)*

(Received March 14th, 1985; accepted for publication in revised form, August 15th, 1985)

ABSTRACT

The effect of *O*-(carboxymethyl)chitins (CM-chitins) on the activation of mouse-peritoneal macrophages *in vivo* and their mitogenic activity on mouse spleen-cells were investigated. The induction of cytotoxic macrophages is enhanced by an increase of negative charge at O-6 and decreased by further modification at O-3 of the GlcNAc residue. CM-Chitins had a minor effect on mitogenic activity that was independent of the site of modification; partially *N*-deacetylated chitins had little activity. Although there was remarkable enhancement of accessibility to lysozyme upon modification at O-6 of the GlcNAc residue, the accessibility was decreased by further substitution at O-3.

## INTRODUCTION

Chitin, a neutral, (1→4)-linked polysaccharide composed of 2-acetamido-2-deoxy-β-D-glucopyranose residues, is widely distributed in Nature as the skeletal material of crustaceans, insects, mushrooms, and the cell walls of bacteria.

This series of studies on chitin derivatives, their preparation, and their physicochemical and biochemical characterization, have been performed for the development of new biomedical materials[1].

It has been shown recently that partially deacetylated chitins have potent immunological activities, such as activation of peritoneal macrophages *in vivo*, suppression of growth of Meth-A tumor cells in syngeneic mice, and stimulation of non-specific host resistance against *Escherichia coli* infections; these were not observed for chitin itself[2]. As 70% deacetylated chitin was the most effective for the foregoing phenomena in comparison with other partially deacetylated chitins (0%, 30%, and 90% of deacetylation), an optimal charge-distribution along the polysaccharide chain was suggested, together with high retentivity. During immunological studies, CM-chitin of d.s. 0.80 was found to show macrophage activation of a similar level as that of 70%, deacetylated chitin, despite a poor retentive ability. These results suggest that the type, amounts, and distribution of

0008-6215/86/$ 03.50 

ionizable substituent groups in the sugar skeleton contribute to the activation of the immune system by chitin derivatives, in addition to the general polysaccharide-chain structure.

In the present work, immunological and biological properties of CM-chitins were investigated to seek a fundamental understanding of the charge effect of these compounds, because negatively charged chitin derivatives induce a potent activation of the immune system, as does deacetylated chitin. The biodegradability of CM-chitins was also studied in relation to the substitution site on the GlcNAc residue, in connection with the retention of immune activity.

## EXPERIMENTAL

*Materials.* — Chitin was prepared from Queen crab shells according to the method of Hackman[3] 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. Egg-white lysozyme [EC 3.2.1.17] was purchased from Seikagaku Kogyo Ltd. and used without further purification. Maleic anhydride–divinyl ether of mol. wt. 15,500 was kindly supplied by Dr. Michael Chirigos, Immunopharmacology section, NCI-FCRF, Frederic, MD 21701, U.S.A. The amino content in chitin was <8% from potentiometric titration.

*Preparation of CM-chitin.* — CM-Chitins were prepared as described previously and their degrees of substitution (d.s.) were 0.25, 0.56, and 0.80, respectively[1]. Only the d.s. 0.80 material was completely soluble in water. Its molecular weight was estimated as 63,000 from its intrinsic viscosity[4] in 0.1M NaCl solution at 30°. CM-Chitin (d.s. 0.80, 1.0 g) was dissolved in 50% (w/w) NaOH solution containing 0.2% (w/w) of sodium dodecyl sulfate (25 mL) at 4° for further carboxymethylation. 2-Propanol (25 mL) was added to the foregoing, viscous solution, and then monochloroacetic acid was added in portions with mechanical stirring at room temperature until the mixture became neutral. The product was filtered off, washed with methanol, and then dissolved in water. The solution of CM-chitin was dialyzed against deionized water to remove any traces of metal ions. The product was precipitated with acetone, collected by centrifugation, and dried *in vacuo*; yield; 1.2 g, $[\alpha]_D$ −18.6° (*c* 0.5, $H_2O$).

*Anal.* Calc. for ($C_{10.4}H_{17.4}N_{1.0}O_{8.4}$): C, 42.9; H, 5.30; N, 4.82. Found: C, 42.2; H, 5.72; N, 4.99.

The degree of carboxymethylation was 1.20 from the elemental analysis. The molecular weight was estimated as 26,000 from viscosity measurements.

*Infrared absorption spectra.* — I.r. spectra of chitin and its derivatives were measured as KBr pellets with a Jasco A-302 i.r. spectrophotometer.

*Enzymic degradation of water-soluble chitin derivatives.* — The lysozyme susceptibility of CM-chitins was determined with an Ubbelhode type of viscometer at 30°. One mL of lysozyme solution (final concentration, 0.63$\mu$M) was applied to

10 mL of CM-chitin solution (1.0 mg/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[5].

*Assay for macrophage activation.* — Cytolysis of EL-4 lymphoma cells and Lewis lung carcinoma (3LL) was quantitated as previously described[2]. Tumor cells ($5 \times 10^5$) in RPMI (Roswell Park Memorial Institute, NY, U.S.A.) 1640 medium supplemented with 10% heat-inactivated fetal-calf serum (Lot. R 781615; GIBCO Laboratories, Grand Island, NY, U.S.A.) were incubated for 1 h at 37° with $Na^{51}CrO_4$ (Japan Radio Isotope Association, Tokyo, Japan) and washed three times with Hanks balanced salt solution. The $^{51}Cr$-labelled tumor cells ($5 \times 10^3$) were cultured with peritoneal macrophages ($2.5 \times 10^5$) for 18–20 h at 37°. The radioactivity in the culture supernatant was determined by $\gamma$-counting to estimate target cytolysis by the formula:

$$\% \text{ Cytolysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

Maximum release of $^{51}Cr$ was determined by freezing and thawing labelled target cells three times. The spontaneous release was measured as the radioactivity release from labelled cells in the absence of macrophages.

*Mitogenic activity on normal mouse-spleen cells.* — Spleen cells ($5 \times 10^5$) of C57BL/6 or BALB/c mice suspended in 100 $\mu$L of RPMI–fetal-calf serum were cultured with or without chitin derivatives or mitogens in 96-well micro tissue-culture plates for 72 h at 37°. At 24 h, before the end of the culture, 0.5 $\mu$Ci of $^3H$-thymidine (The Radiochemical Centre, Amersham, Buckinghamshire, UK) was added, and the spleen cells were harvested on a glass filter by using a multiple-cell harvester. The incorporation of $^3H$-thymidine was measured by a liquid-scintillation method.

## RESULTS AND DISCUSSION

*I.r. spectra.* — Fig. 1a shows the spectrum of CM-chitin (d.s. 0.56) and the baseline for the measurement of peak intensity. The amide I band at 1655 $cm^{-1}$ and C=O stretching at 1735 $cm^{-1}$ were used for the calibration curve, because the absorption at 1655 $cm^{-1}$ was not changed by the introduction of *O*-carboxymethyl groups on the GlcNAc residues.

Fig. 1b shows plots of the ratio of absorption at 1735 $cm^{-1}$ to that at 1655 $cm^{-1}$ against the degree of substitution determined from the elemental analyses. From this relationship between the value of A(1735 $cm^{-1}$)/A(1655 $cm^{-1}$) and d.s. from elemental analyses, it is suggested that the degree of carboxymethylation may be estimated by this i.r. method for the region of d.s. 1.0.

*A highly substituted CM-chitin.* — In order to obtain a highly substituted

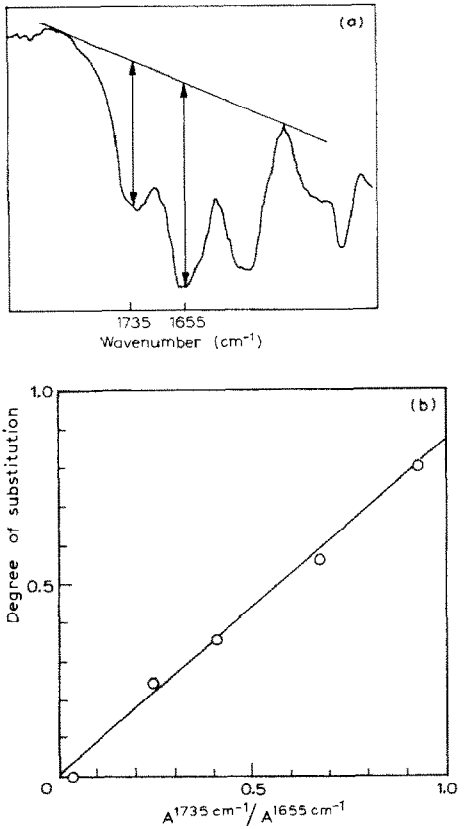


Fig. 1(a) I.r. absorption spectrum of CM-chitin (d.s. 0.56) and the baseline for facile determination of the degree of *O*-carboxymethylation. (b) Calibration curve for the determination of degree of substitution by i.r. spectrometry.

derivative containing 3-*O*-(carboxymethyl) groups in the sugar skeleton, further carboxymethylation of CM-chitin (d.s. 0.80) was successively performed in a homogeneous system. From the elemental analyses of this product, its d.s. was estimated to be 1.20; its molecular weight (Mw) was estimated to be 26,000 by use of the viscosity equation $[\eta] = 7.92 \times 10^{-5}$ $Mw^{1.0}$, 0.1M NaCl, 30°. Some decomposition of the polymer chain occurred during the recarboxymethylation procedure. In the i.r. spectrum of the product, the C=O stretching-band at 1735 $cm^{-1}$ and C-O stretching at 1240 $cm^{-1}$ showed apparent increase compared with the spectrum of the starting CM-chitin, but the absorptions at 1655 $cm^{-1}$ (Amide I band) and at 1550 $cm^{-1}$ (Amide II band) were scarcely changed. It is concluded that no significant *N*-deacetylation occurred during the reaction.

*Biological activities of CM-chitins.* — The effect of chitin and CM-chitins on the activation of mouse-peritoneal macrophages *in vivo* was examined as shown in Table I. Chitin and CM-chitin (d.s. 0.25) did not activate macrophages at all. CM-Chitins of d.s. 0.56 and 0.80 were effective for the activation of macrophages and

that of d.s. 0.80 was superior to CM-chitin of d.s. 0.56. A highly substituted derivative (d.s. 1.20) showed no macrophage activation. These results suggest that CM-chitin of d.s. 0.56–0.80 is suitable for macrophage activation, and that the charge distribution in the sugar skeleton is also important, in addition to the degree of *O*-carboxymethylation.

Normal spleen cells of BALB/c or C57BL/6 mice were cultured with CM-chitins dissolved or suspended in saline in order to measure the mitogenic activity. CM-Chitins showed weak mitogenic activity, independent of the substitution site, as shown in Table II, whereas partially deacetylated chitins demonstrated no mitogenic activity[6]. A different type of pathway for the adjuvant activity appears indicated for the CM-chitins as compared with deacetylated chitins.

*Enzymic degradation of CM-chitins.* — Fig. 2 showed the time course of the reduced viscosity of CM-chitin upon the treatment with egg-white lysozyme. The reduced viscosity reflects the apparent molecular weight of polysaccharide.

CM-Chitins of d.s. 0.80 was readily hydrolyzed by lysozyme and its reduced viscosity decreased rapidly from 6.0 to 0.4 within 16 h. On the other hand, a highly carboxymethylated derivative (d.s. 1.20) was scarcely digested. Chemical modification at O-3 is evidently not effective for conversion of chitin into bioactive and biocompatible materials, because CM-chitin (d.s. 1.20) having 6-*O*- and 3-*O*-(carboxymethyl) groups depressed both the macrophage activation and biocompatibility observed with CM-chitin of d.s. 0.80.

*Crystalline structure and $D_2O$ adsorption.* — In our previous report, the i.r. spectra of $D_2O$-treated chitin and its cyanoethylated derivatives were investigated to seek fundamental information on their affinity for water, which has been proposed to be incorporated in the crystalline structure of native chitin[7].

Spectrum (A) of Fig. 3 shows absorption at ~2550 $cm^{-1}$, attributable to the crystalline part of chitin and absorption at ~2450 $cm^{-1}$ attributable to the

TABLE I

MACROPHAGE ACTIVATION BY CM-CHITINS: EFFECT OF DEGREE OF SUBSTITUTION BY CM GROUPS

| *Treatment*[a] | *D.s.*[b] | *Dose* | *Timing* | *% Cytolysis*[c] | |
|---|---|---|---|---|---|
| | | *(μg)* | *(days)* | *EL-4* | *3LL* |
| Chitin | 0 | 500 | −3 | 3.1 ±3.1 | 10.8 ±2.7 |
| CM-chitin | 0.25 | 500 | −3 | 4.4 ±6.5 | 11.5 ±1.3 |
| CM-chitin | 0.56 | 500 | −3 | 19.4 ±2.8[d] | 19.0 ±3.8[e] |
| CM-chitin | 0.80 | 500 | −3 | 33.4 ±3.2[e] | 21.1 ±4.3[d] |
| CM-chitin | 1.20 | 500 | −3 | −1.7 ±7.9 | 3.5 ±3.3 |
| MVE-2[f] | | 500 | −3 | 46.6 ±5.1[e] | 43.6 ±5.4[e] |
| control | | | | 2.6 ±3.1 | 4.5 ±2.8 |

[a]Mice were injected intraperitoneally with each polysaccharide at indicated days before harvest of macrophages. [b]Degree of substitution. [c]Each value is the mean ±standard error of six wells in each groups. [d]Significant difference from the control by student's t-test($p<0.05$). [e]Significant difference from the control ($p<0.005$). [f]A maleic anhydride–divinyl ether preparation.

TABLE II

MITOGENIC ACTIVITY OF CM-CHITINS ON NORMAL MOUSE-SPLEEN CELLS

| *Mitogen* | *Dose* ($\mu g/mL$) | $^3H$-*TdR incorporation* (*mean c.p.m.* + *S.E.*) |
|---|---|---|
| *Expt. 1*[a] | | |
| CM-chitin (0.25)[b] | 10 | 2713 ±62 (0 9) |
| | 50 | 3766 ±121 (1.2) |
| | 250 | 3888 ±604 (1.3) |
| CM-chitin (0.56) | 10 | 2725 ±392 (0.9) |
| | 50 | 3609 ±304 (1.2) |
| | 250 | 5292 ±645 (1.7) |
| CM-chitin (0.80) | 10 | 2389 ±201 (0.8) |
| | 50 | 3332 ±267 (1.1) |
| | 250 | 4664 ±323 (1.5) |
| | 1000 | 8210 ±781 (2.7) |
| CM-chitin (1.20) | 10 | 2860 ±303 (0.9) |
| | 50 | 3575 ±282 (1.2) |
| | 250 | 3819 ±274 (1 3) |
| | 1000 | 7742 ±86 (2.5) |
| Concanavalin A | 1 | 56520 ±1614 (18.6) |
| | 5 | 59900 ±1574 (19.7) |
| Lipopolysaccharide (*Salmonella typhimarium*) | 10 | 20209 ±138 (6.6) |
| | 50 | 17019 ±1456 (5.6) |
| Medium control | | 3044 ±135 (1.0) |
| *Expt. 2*[b] | | |
| Chitin | 10 | 2665 ±147 (1.5) |
| | 50 | 3293 ±344 (1.9) |
| | 250 | 2747 ±501 (1.6) |
| CM-chitin (0.80) | 10 | 3478 ±296 (2.0) |
| | 50 | 3822 ±280 (2.2) |
| | 250 | 6065 ±208 (3.4) |
| Lipopolysaccharide (*E coli*) | 10 | 10466 ±190 (5.9) |
| control (medium) | | 1770 ±169 (1.0) |

[a]C57BL/6 mouse. [b]Numbers in parentheses give d.s. values [c]BALB/c mouse

amorphous part[7]. The absorption at ~2450 $cm^{-1}$ was enhanced relatively by *O*-carboxymethylation at O-6, and the total amount of adsorbed $D_2O$ tended to increase for CM-chitin of d.s. 0.80. On the other hand, the amount of adsorbed $D_2O$ was depressed markedly at higher d.s., as shown in spectrum (C), suggesting disruption of the crystalline structure by chemical modification.

From the present study, activation of mouse peritoneal macrophages by CM-chitins is indicated to depend highly not only on the amount of carboxymethyl groups introduced but also on the site of substitution on the GlcNAc residues in chitin. There might also be a significant relation between the interaction with lysozyme and the activation of macrophages by CM-chitins, because the highly carboxymethylated derivative (d.s. 1.20) having poor lysozyme susceptibility

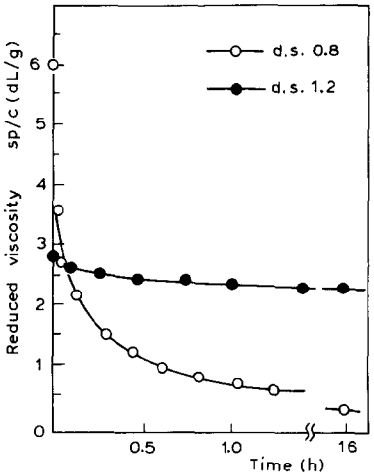


Fig. 2. Enzymic degradation of water-soluble CM-chitins by a viscometric method (pH 7.4, 30°).

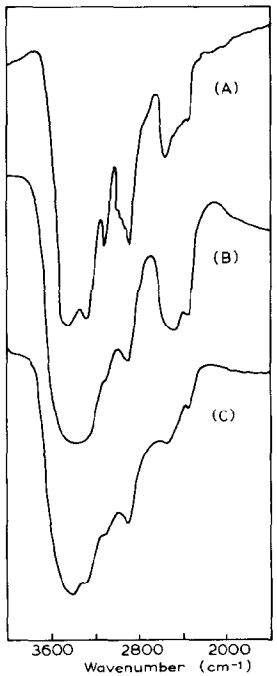


Fig. 3. I.r. absorption spectra of $D_2O$-treated chitin and CM-chitins. (A) chitin powder, (B) CM-chitin (d.s. 0.80), and (C) CM-chitin (d.s. 1.20). Samples were stirred in $D_2O$ for 12 h at 25° and precipitated by acetone. The powder was dried *in vacuo* after washing with EtOH.

showed a little activity in comparison with 6-*O*-CM-chitins. Low retentive capacity for the induction of cytotoxic macrophages with CM-chitin seems to be related directly to its rapid degradation under physiological conditions.

REFERENCES

1 S. NISHIMURA, Y. IKEUCHI, AND S. TOKURA, *Carbohydr Res.*, 134 (1984) 305–312.
2 K. NISHIMURA, S. NISHIMURA, N. NISHI, I SAIKI, S. TOKURA, AND I. AZUMA, *Vaccine*, 2 (1984) 93–99.
3 R. H. HACKMAN, *Aust. J. Biol. Sci.*, 7 (1954) 168–178.
4 Y. INOUE, S. TOKURA, AND M. KANEKO, *Rep. Proc. Polym. Phys. Jpn.*, (1982) 759.
5 K. HAMAGUCHI AND M. FUNATSU, *J. Biochem. (Tokyo)*, 46 (1959) 1659–1660.
6 K. NISHIMURA, S. NISHIMURA, N. NISHI, S. TOKURA, AND I. AZUMA, *Vaccine*, 3 (1985) in press.
7 S. TOKURA, N. NISHI, S. NISHIMURA, AND Y. IKEUCHI, *Polym. J.*, 15 (1983) 553–556