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Research Note

N-[^{13}C=O]Acetylchitosan and its digestibility by silkworms

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N-[^{13}C=O]Acetylchitosan was prepared by treating chitosan with a mixture of acetic anhydride and [^{13}C=O]acetic anhydride in an aqueous acetic acid–methanol solution. On feeding silkworms (*Bombyx mori*) with this at the fifth instar, the apparent digestibility of the *N*-[^{13}C=O]acetylchitosan was 21%. The major proportion of the fed ^{13}C was secreted in the faeces, a little was detected in the exuviae and cocoons.

INTRODUCTION

Chitin [(1→4)*N*-acetyl-β-D-glucosaminan] is the main component of exo- and endocuticles of insects (Locke, 1974). Orally administrated chitin and chitosan are almost completely digested by microbial chitinase (EC3.2.1.14) in the intestinal organs of chickens and hens (Hirano *et al.*, 1990), with an enhancement of blood lysozyme activity (Hirano *et al.*, 1992). Chitin and chitosan have been evaluated as feed and food additives (Austin *et al.*, 1981; Knorr, 1984; McCurdy, 1992), and were approved as food additives in Japan (Ministry of Health and Welfare Food Chemistry of Japan, 1983). Chitinase plays an important role at the moulting stage of insects allowing digestion of their cuticle chitin (Kramer *et al.*, 1985; Koga, 1988). However, the fate of orally administered chitin in insects, and its biological significance is not known.

Here we wish to report a simple method for the preparation of *N*-[^{13}C=O]acetylchitosan, and its digestibility in silkworms (*Bombyx mori*).

EXPERIMENTAL

Methods

^{13}C-CP/MAS NMR spectra were recorded on a Chemagnetics CMX 360 NMR spectrometer (Chemagnetics Co., Ltd, Ford Collins), ^{1}H-NMR spectra on a Joel JNM-Gx270 FT-NMR spectrometer (Jeol Co., Ltd, Tokyo), and IR spectra on a Hitachi 251 IR spectrometer (Hitachi Co., Ltd, Tokyo). ^{13}C Atom percentage was measured on a Jasco Ec-130 $^{13}CO_2$ analyser (Jasco Co., Ltd, Tokyo), and specific rotations on a Jasco Dip-181 polarimeter (Jasco Co., Ltd, Tokyo).

Materials

[^{13}C=O]Acetic anhydride was produced by the Commissariat à l'Energie Atomique (Shoko Co., Ltd, Tokyo). Crab shell chitin ($[\alpha]_D^{20}$ − 14° (*c*.1·0, methanesulphonic acid)) and chitosan ($[\alpha]_D^{32}$ − 6·7° (*c*.1·0, aqueous 2% acetic acid); degree of substitution (d.s.) 0·21 for NAc) were products from the Katakurachikarin Co., Ltd (Tokyo). *N*-Acetylchitosan (a regenerated chitin, d.s. 1·0 for NAc) was prepared from chitosan as described by Hirano *et al.* (1976).

N-[^{13}C=O]Acetylchitosan

Chitosan (12 g) was dissolved in aqueous 2% acetic acid (759 ml), and the solution was diluted with methanol (2250 ml). A mixture of acetic anhydride (10 ml) and [^{13}C=O]acetic anhydride (1·0 ml) was added dropwise to the solution whilst stirring continuously, and the mixture was kept at room temperature overnight to produce a slightly viscous solution. Another 15 ml acetic anhydride was added dropwise to the solution

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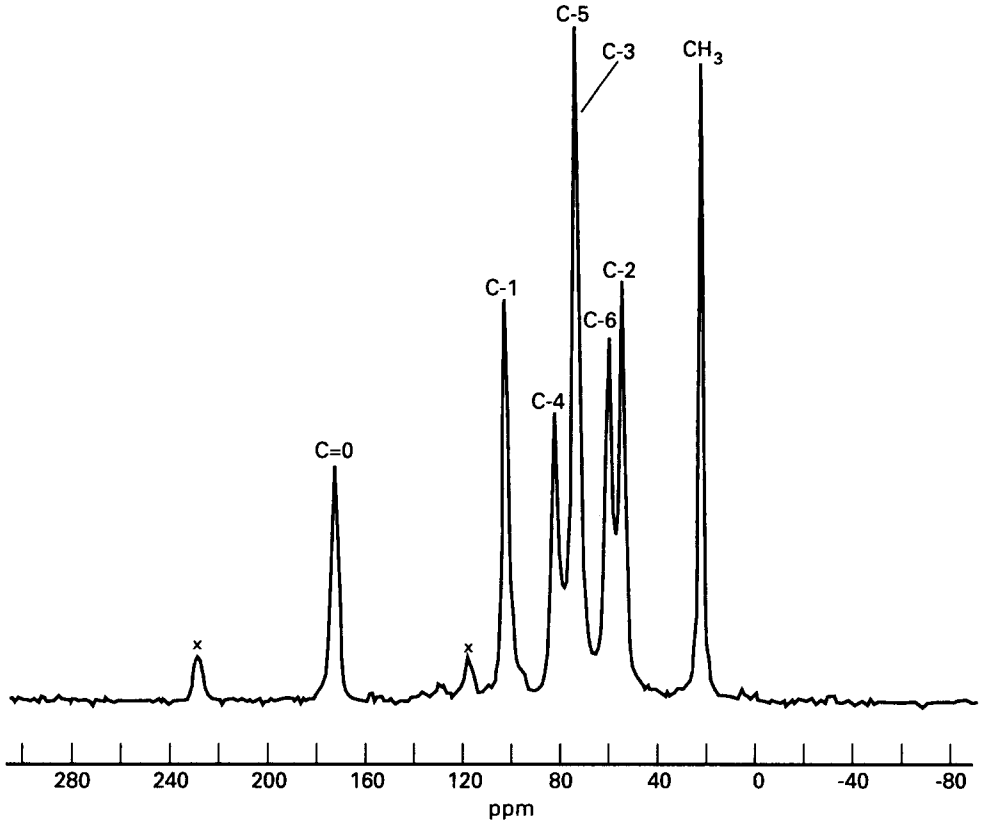
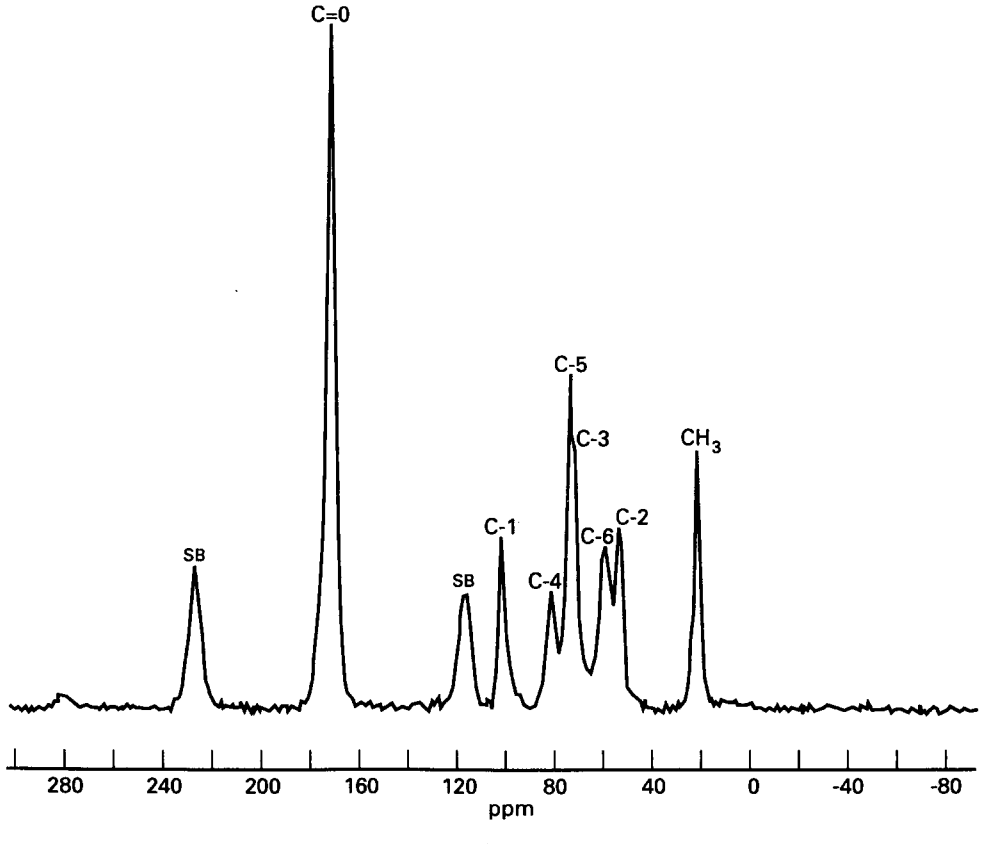


(a)



(b)

Fig. 1. ^{13}C-CP/MAS NMR spectra of (a) *N*-acetylchitosan and (b) *N*-[^{13}C=O]acetylchitosan (down); SB, Side band.

Table 1. The weight of pupae, exuviae, and cocoons of silkworms fed each of the N-acetylchitosan- and chitosan-supplemental diets at the fifth instar

Supplements for the diet	Weight (g/silkworm)[a]						
	Day			Pupae		Exuviae	Cocoons
	First	Second	Last	Male	Female		
1% *N*-Acetylchitosan	3·86	4·91	5·74	1·76	2·87	0·014	0·60
1% Chitosan	3·91	5·05	5·87	1·64	2·86	0·012	0·61
Basal diet (control)	3·66	4·84	5·75	1·73	2·94	0·014	0·60

[a]An average value is shown, $n = 15$–20.

whilst stirring continuously, and the mixture was again kept at room temperature overnight. The solution then became a transparent gel, which was crushed in a homogenizer. The suspension was adjusted to pH 7–8 by adding an aqueous 10% sodium hydroxide solution, and kept at room temperature overnight. The gel suspension was centrifuged at 1500*g* for 10 min, and the precipitate was re-suspended in distilled water (approx. 1 litre) and the suspension was stirred continuously at room temperature overnight. The precipitate was then freeze-dried (15 g, 89%) ($[\alpha]_D^{21} + 2{\cdot}5°$ (*c*.1·0, methanesulphonic acid). The product was soluble in methanesulphonic acid, but insoluble in water, aqueous hydrochloride, aqueous sodium hydroxide, dimethylformamide, and dimethylsulphoxide. γ_{KBr}^{max} cm^{-1}: 1650 and 1550 (C=O and NH of NAc). ^{13}C CP/MAS NMR data: 173·6 (C=O), 104·3 (C-1), 83·5 (C-3), 75·9 (C-4), 75·0 (C-5), 61·5 (C-6), 55·8 (C-2), 23·2 (CH_3) ppm.

Anal. Calc. for [$^{12}C_{7\cdot61}{}^{13}C_{0\cdot39}H_{13}O_5N \cdot 0{\cdot}25H_2O$]n: C, 46·37; H, 6·49; N, 6.73. Found: C, 46·63; H, 6·70; N, 6·35.

Silkworms and diets

Silkworms, *Bombyx mori* (Shinzan x Reiko, Yunichika Sanshi Co., Ltd, Osaka), were reared on mulberry leaves until the second instar during August and September at Tottori University farm, and were then fed on an autoclaved artificial diet containing powdered mulberry leaves (Yakult, Co., Ltd, Tokyo). For the present feeding experiment, a total of 80 silkworms were reared at 25°C to their pupal stage on an artificial diet that contained 1% each of *N*-[^{13}C=O]acetylchitosan, *N*-acetylchitosan and chitosan, and the results were compared with that of silkworms fed the basal diet. The hexosamine content of diets and faeces was determined as described previously (Hirano *et al.*, 1990).

RESULTS AND DISCUSSION

A partially [^{13}C=O]-labelled derivative of *N*-acetylchitosan was prepared by treating chitosan with a mixture of [^{13}C=O]acetic anhydride and acetic acid. The d.s. for combined *N*-acetyl and *N*-[^{13}C=O]acetyl was 1·0, and the product was isolated in an 89% yield. In the ^{13}C CP/MAS NMR spectrum of *N*-[^{13}C=O]acetylchitosan (Fig. 1), the strong ^{13}C=O signal of NAc appeared at 173·6 ppm, and it was stronger than that in *N*-acetylchitosan (^{13}C CP/MAS NMR data: 174·3 (C=O), 104·3 (C-1), 83·7 (C-3), 75·5 (C-4), 74·0 (C-5), 61·4 (C-6), 55·7 (C-2), 23·4 (CH_3) ppm]. No *O*-acetyl group was detected in the IR and ^{13}C CP/MAS NMR spectra of the product. However, the distribution of *N*-[^{13}C=O]acetyl group in the chain is unknown.

In silkworms fed each of the 1% chitosan- and 1% *N*-acetylchitosan-supplemental diets, their body weight slightly increased on the second day of the fifth instar (Table 1). In silkworms fed the chitosan-supplemental diet at the fourth instar, their molting stage was one day earlier than that of the control. However, no significant increase appeared in the body weight on the last day of the fifth instar, and in the weights of pupae, exuviae, and cocoons. A chitin-like substance was isolated from faeces of the silkworms fed the *N*-acetylchitosan-supplemental diet (data not shown). This demonstrates the safety of chitin and chitosan for use as a novel feeding additive for silkworms (Arai, *et al.*, 1968).

At the fifth instar, each silkworm fed daily on an average 635 ± 103 mg (dry wt) of the 1% *N*-[^{13}C=O]acetylchitosan-supplemental diet, daily secreted

Table 2. ^{13}C atom % in faeces, exuviae, and cocoons in silkworms fed the 1% *N*-[C=O]acetylchitosan-supplemental diet[a]

Supplements for the diet	^{13}C Atom/(^{12}C and ^{13} atoms) (%)		
	Faeces	Exuviae	Cocoons
1% *N*-[^{13}C=O]acetylchitosan	1·155 (1·018)[b]	1·127 (0·994)	1·122 (1·002)
1% *N*-Acetylchitosan	1·135 (1·000)	1·139 (1·004)	1·123 (1·003)
1% Chitosan	1148 (1·011)	1136 (1·002)	1124 (1·004)
Basal diet (control)	1·135 (1·000)	1·134 (1·000)	1·120 (1·000)

[a]Each (1·0–1·3 mg) of the samples was analysed.
[b]Numbers in parentheses indicate the net increase of the ^{13}C atom percentage relative to that of the control.

an average 399 ± 77 mg (dry wt) of faeces, resulting in 37% of the apparent digestibility of the diets. The hexosamine content as D-glucosamine was 6·99 mg in 635 mg of the 1% *N*-[^{13}C=O]acetylchitosan-supplemental diet, and 5·51 mg in 399 mg of the faeces, resulting in 21% of its apparent digestibility. At the fifth instar of silkworms (Table 2), about 80% of the fed ^{13}C was excreted in the faeces, and little accumulated in the exuviae and cocoons. These data conclude that the apparent digestibility of *N*-[^{13}C=O]acetylchitosan is 21% in silkworms, and the limited digestion is probably due to dietary chitinase as well as intestinal microbial chitinase in silkworms. In fact, chitinase activity (52 mU/g by dry weight) was detected even in the autoclaved commercial diet, and the enzyme activity probably originated from mulberry leaves (Hirano *et al.*, 1988).

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