

Analysis of Chitin Structure by Nuclear Magnetic Resonance Spectroscopy and Chitinolytic Enzyme Digestion¹

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Received February 11, 1986, and in revised form May 1, 1986

Solid-state ¹³C-NMR analysis of chitin prepared from cuticle of the tobacco hornworm, *Manduca sexta* (L.), and of crab yielded spectra that demonstrate a high degree of chemical homogeneity (>95%) for the preparations. The chemical shifts of the well-resolved carbon signals from both samples matched closely those of the monomeric unit 2-acetamido-2-deoxy-D-glucopyranoside (GlcNAc). Chromatographic analysis of products from the digestion of chitin by the binary chitinase system (endo splitting chitinase and exo splitting β-N-acetylglucosaminidase) isolated from *M. sexta* molting fluid showed that the major product from both chitin preparations is GlcNAc. Also detected was a minor product (product U) that had a chromatographic retention time on the carbohydrate analysis column intermediate between those of chitin penta- and hexasaccharides. Gel filtration chromatography of U indicated that U had an apparent molecular weight intermediate between that of GlcNAc and of N,N'-diacetylchitobiose. Cation-exchange chromatography of U after acid hydrolysis revealed the presence of glucosamine only. Derivatization with trinitrobenzenesulfonate showed the presence of a free amino group in U. Solution proton and carbon NMR spectroscopy were used to identify U as a N-monoacetylchitobiose [O-β-D-2-amino-2-deoxyglucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranose] with the residue at the nonreducing end deacetylated. These studies showed that chitin prepared from alkali- and heat-treated insect or crab cuticle contains trace levels of deacetylated residues that are released as a dead-end product, N-monoacetylchitobiose, after digestion by the binary enzyme system. © 1986

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¹Contribution 86-211-J, Department of Biochemistry, Kansas Agricultural Experiment Station, Manhattan, Kan. Cooperative investigation between ARS, USDA, the Kansas Agricultural Experiment Station, and Monsanto Company. Mention of a proprietary product in this paper does not imply approval of this product by the USDA to the exclusion of other products that may also be suitable. Supported in part by USDA competitive research grant 85-CRCR-1-1667.

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Insect integument is composed of a thin outer epicuticle, a thick inner procuticle, and an epidermis. Although its structural components have been the subject of many investigations (1-3), the supramolecular structure of cuticle is not well understood. Two approaches to the study of cuticle structure are the use of solid state nuclear magnetic resonance spectroscopy and the analysis of enzyme-catalyzed hydrolytic products. The former method (cross-polarization magic-angle spinning NMR or

CPMAS-NMR)⁴ is applicable to investigations of intractable material (4). Enzymatic digestion at neutral pH and relatively low temperature is one of the mildest methods for breaking down cuticle into its components with the chances of generating artifacts being minimized.

One of the main components in the procuticle is chitin, a cellulose-like polysaccharide thought to be a linear chain of β -1,4-linked *N*-acetylglucosamine residues. During the ecdysial period, chitin is digested by chitinolytic enzymes including an endo-bond-splitting chitinase (EC 3.2.1.14) and an exo-bond-splitting β -*N*-acetylglucosaminidase (EC 3.2.1.30). Those two enzymes, which are present in the molting fluid secreted by the epidermis, have been purified from the tobacco hornworm, *Manduca sexta* (L.), and characterized (5-7), and their mechanisms of hydrolytic reaction with chitin and chitin oligosaccharides have been investigated (8, 9). Chitin hydrolysis is initiated by a randomly attacking chitinase producing relatively low molecular weight soluble intermediates which are subsequently hydrolyzed to *N*-acetylglucosamine (GlcNAc) by the β -*N*-acetylglucosaminidase. When mixed together, the two enzymes are referred to as the binary chitinase system.

In a previous study (9), we detected a low molecular weight unknown product in the enzymatic hydrolysate of insect chitin generated by the binary chitinase system. The unknown compound was not GlcNAc or a homooligosaccharide thereof. The present study deals with structural analyses of the unknown product as well as chitin itself by spectroscopic and chemical methods. The unknown compound was identified as a monodeacetylated dimeric derivative of *N*-acetylglucosamine.

MATERIALS AND METHODS

Insects and materials. *M. sexta* eggs were a gift from the Metabolism and Radiation Research Laboratory,

⁴ Abbreviations used: CPMAS, cross-polarization magic-angle spinning; GlcNAc, *N*-acetylglucosamine; TOSS, spinning sideband suppression conditions; TMS, tetramethylsilane.

Agricultural Research Service, U.S. Department of Agriculture, Fargo, North Dakota. Larvae were reared according to Bell and Joachim (10). Intact chitin was prepared by boiling finely ground freeze-dried fifth-instar larval cuticle of *M. sexta* (5 mg ml⁻¹) five times in 1 M sodium hydroxide, washing the ground cuticle with water, and, in some cases, acetylating it with acetic anhydride (10%) in methanol. Crab chitin was prepared in a similar manner from practical grade chitin obtained from Sigma Chemical (St. Louis, Mo.). Colloidal chitin was prepared from intact chitin according to Bade and Stinson (11). β -*N*-Acetylglucosaminidase and chitinase were purified from *M. sexta* molting fluid according to Dziadik-Turner *et al.* (5) and Koga *et al.* (6), respectively. Other reagents were of highest purity commercially available.

Enzymatic reaction. The enzymatic reaction was initiated by addition of 0.57 μ M chitinase and 0.26 μ M β -*N*-acetylglucosaminidase mixture to a suspension of insoluble chitin substrate (10 mg/ml) in 50 mM sodium phosphate buffer (pH 7.0) at 32°C with constant stirring. At an appropriate time, an aliquot was withdrawn and acidified to pH 2.5 with cold 1.42 M phosphoric acid in order to terminate the reaction. After ultrafiltration using an Amicon PM-10 membrane to remove protein and particulate material, the sample was analyzed for oligosaccharide and monosaccharide products by high-performance liquid chromatography (8).

Purification of the unknown product. Colloidal chitin and the binary enzyme mixture were incubated under the same conditions as described in the previous section. After 24 h incubation, the reaction mixture was directly applied to a column of Bio-Gel P-4 (2.3 \times 200 cm) and eluted with distilled water. The unknown product was purified by gel filtration repeated three times. The purity was checked by high-performance liquid chromatography as described in the following section.

High-performance liquid chromatography. For the separation of hydrolytic products from insoluble chitin, two types of column systems were employed: a carbohydrate analysis (aminopropyl) column purchased from Waters Associates, with a mobile phase of acetonitrile:50 mM sodium phosphate buffer, pH 5.6 (7:3) at 1.5 ml min⁻¹, and a gel filtration column of TSK-GEL G2000PW from Toyo Soda Co. Ltd., which was eluted by distilled water at 0.3 ml min⁻¹.

Spectroscopic determination. Ultraviolet absorbance at 220 nm was used for detection of the acetamido group. Free amino groups were derivatized with 2,4,6-trinitrobenzenesulfonic acid and the absorbance was measured at 420 nm (12). All spectroscopic determinations were made with a Cary 118C spectrophotometer.

Hexosamine and amino acid analysis. Samples were hydrolyzed for 20 h *in vacuo* at 110°C in 3 N HCl, and analyzed for hexosamine and amino acid content by

cation-exchange chromatography with postcolumn ninhydrin derivatization.

Nuclear magnetic resonance spectroscopy. Magic-angle spinning ^{13}C -NMR spectra were obtained at 50.3 MHz by using 2-ms cross-polarization transfers from protons and 50-kHz radio frequency fields with the dried samples spinning at 3.2 kHz in a boron nitride double-bearing 700- μl hollow rotor (13). The chemical shifts are in parts per million downfield from external tetramethylsilane (TMS). The CPMAS ^{13}C -NMR spectra shown in Fig. 1 were collected under spinning sideband suppression conditions (TOSS) (14). Quantitation of CPMAS signal intensities (Table I) was performed on non-TOSS spectra. Areas under resonance peaks were determined by triangulating to the base line, cutting, and weighing the paper on an analytical balance.

Solution ^1H - and proton-decoupled ^{13}C -NMR spectra of the unknown product from insect cuticle chitin, *N,N*-diacetylchitobiose from crab chitin (6), and standard compounds (Sigma Chemical) were obtained by using a 5-mm C/H probe on a Bruker WM-400 instrument. Typically, about 3 mg of each of the materials was lyophilized twice from 99.8% $^2\text{H}_2\text{O}$ and once from 99.99% $^2\text{H}_2\text{O}$ before being dissolved in 0.4 ml of 99.99% $^2\text{H}_2\text{O}$. Solvent suppression in the ^1H spectra was accomplished with a pulsed presaturation procedure. A *p*-dioxane in $^2\text{H}_2\text{O}$ reference, taken to be 67.4 ppm relative to internal TMS, was used for the ^{13}C -spectra, whereas the ^1H -spectra were referenced directly against the methyl signals of sodium 2,2-dimethyl-2-silapentanesulfonate in $^2\text{H}_2\text{O}$ taken as 0 ppm.

RESULTS

Solid-State Nuclear Magnetic Resonance Spectroscopy of Chitin

Chitin, by traditional definition, is the cuticular material that remains insoluble in hot alkali (15). It has been routinely prepared from arthropod cuticle by means of hot alkaline and/or cold acidic solvents. Such harsh conditions, however, have led some investigators to question the chemical integrity of some of those preparations (16, 17). To determine the quality of our preparations and especially their degree of acylation following hot alkaline conditions, we obtained ^{13}C -CPMAS NMR spectra of intact *M. sexta* larval cuticle and its extracted chitin, the latter both before and after acetic anhydride treatment (Fig. 1). The eight major resonances in the cuticle spectrum are due primarily to carbon atoms from chitin (see Table I for chemical shift assignments). Except for a minor dif-

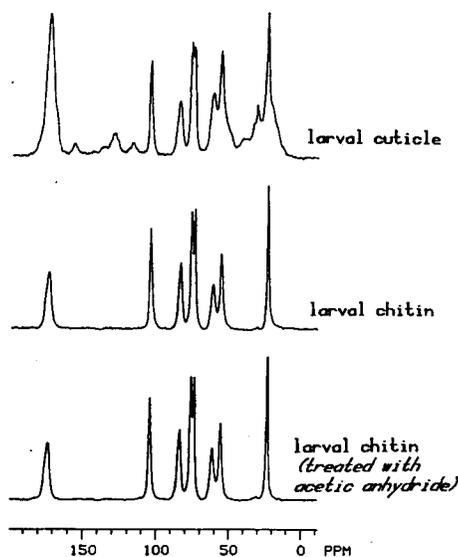


FIG. 1. ^{13}C natural abundance cross-polarization magic-angle sample spinning nuclear magnetic resonance of *M. sexta* larval cuticle (upper spectrum), intact chitin (middle spectrum), and intact chitin treated with 10% acetic anhydride in methanol saturated with sodium acetate (bottom spectrum). Sample size was 100–200 mg and spectra were normalized for direct intensity comparison. Spinning sidebands have been suppressed. The carbohydrate-carbon line assignments are carbonyl, 172 ppm; C-1, 105 ppm; C-4, 85 ppm; C-5, 74 ppm; C-3, 72 ppm; C-6, 62 ppm; C-2, 56 ppm; methyl, 24 ppm.

ference in the absolute signal level on a per milligram basis, the two chitin spectra were very similar to one another and to previously published solid-state NMR spectra of chitin (18, 19). Well-resolved carbon signals that corresponded to resonances of the monomeric unit GlcNAc were exhibited (20–22). There were very minor resonances apparent at approximately 30 ppm and at 130 and 150 ppm that indicated the presence of trace amounts of constituents other than GlcNAc.

Acid hydrolysis of the larval chitin released some residual amino acids (<5 mol % relative to total glucosamine and amino acid content as determined by amino acid analysis after hydrolysis). Since glucosamine is substantially more labile to acid than are amino acids and since the resonances possibly attributable to amino acids in the solid-state spectra are so low, the

TABLE I
¹³C-CPMAS CHEMICAL SHIFTS AND INTENSITIES OF INSECT AND CRAB CHITINS

Sample ^a	Carbon	Chemical shift (ppm) ^b	Peak ht ^c	Area ^c	T _{1(H)} ^d (ms)	Area ^e
I	-CH ₃	24	0.91	0.85	8.7	1.00
C			0.93	0.86	7.8	1.03
I	C-2	56	0.57	0.89	8.3	1.01
C			0.62	0.96	7.7	1.10
I	C-6	62	0.40	0.88	9.3	0.99
C			0.41	0.92	8.0	1.02
I	C-5 or C-3	75	1.00	1.00	8.5	1.13
C			1.00	1.00	7.7	1.15
I	C-4	85	0.54	0.84	8.2	1.00
C			0.56	0.88	7.8	1.00
I	C-1	105	0.76	0.93	8.3	1.06
C			0.77	0.95	7.7	1.07

^a I, insect chitin; C, crab chitin. Standard error for area values, $\pm 5\%$.

^b Assignments of Saito *et al.* (1981) and this study.

^c Value at 1 ms contact time and normalized relative to C-5 or C-3.

^d Spin-lock lifetime of protons.

^e Extrapolated to zero contact time.

actual mole percentage of amino acids in the *M. sexta* larval chitin preparation was estimated to be substantially less than 2%.

CPMAS NMR spectra of insect and crab chitins were used to compare quantitatively the relative abundance of carbon atoms in insect and crab chitin preparations (Table I and Fig. 2). Relative areas (or peak heights) were determined by extrapolation of data to zero transfer time (13). The carbonyl carbon has been omitted from the analysis because of approximately 10% sidebands in non-TOSS spectra which complicate quantitation. The insect and crab chitin samples were remarkably similar in carbon intensities, as shown by the near unity of values for the extrapolated peak areas (Table I). The fact that C-3 and C-5 were slightly higher and C-4 lower than unity indicated a systematic error in the method chosen to separate the overlapping tails of the lines. The observed buildup of carbon polarization for the C-6 and methyl carbons depends on dipolar coupling between carbons and protons (Fig. 2). Therefore, polarization transfer was slightly slower for the methyl carbon since the in-

ternal rotation of the methylene group reduces the net C-H dipolar coupling. Nevertheless, both signals were near their maximum values in a matched spin-lock transfer experiment after 1 ms (Fig. 2). For longer transfer times, the carbon magnetization decreased because of the finite spin-lock lifetime of the protons, $T_{1(H)}$. Overall, these results demonstrated that the products from alkali-heat treatment of insect and crab cuticles are indeed polymers of *N*-acetylglucosamine and thus are suitable substrates for chitinolytic enzymes. There appeared to be little or no glucosamine in the chitin preparations since the degree of *N*-acylation, as inferred from the zero contact time intensities of the methyl signal, was comparable in acetic anhydride-treated and untreated samples, and those relative carbon intensities were near theoretical values for GlcNAc (Table I).

Hydrolytic Products from Insect and Crab Chitins

The products from the incubation of insect and crab colloidal chitins with the bi-

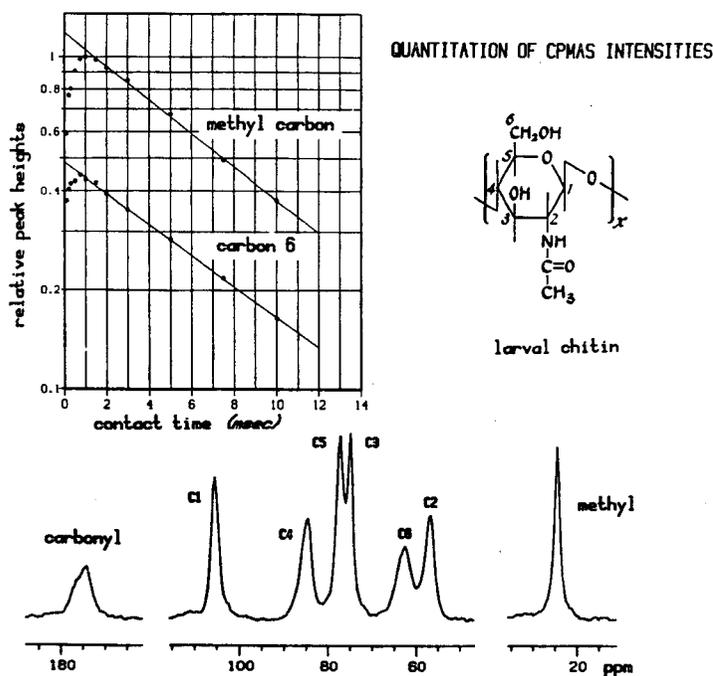


FIG. 2. Quantitation of CPMAS intensities of insect chitin. Extrapolation of relative peak heights of methyl carbon and C-6 to zero contact time. See Table I for relative areas.

nary chitinase system or chitinase alone were separated by HPLC as shown in Fig. 3. The binary chitinase system released one major product, *N*-acetylglucosamine, I (38% yield), and a minor unknown product, U (retention time \sim 32 min, 2.4% yield), which appears between the pentamer and hexamer in both cases (Figs. 3B and D). Chitinase alone (Figs. 3C and E) also released the unknown product (2.9% yield) together with the chitin oligosaccharides, *N,N'*-diacetylchitobiose, II (29% yield), and *N,N',N''*-triacetylchitotriose, III (0.1% yield). From these results, the dimer and trimer were again demonstrated to be real intermediates of the chitin degradation process, as described initially in previous reports (8, 9). The possibility of glucosamine, which would not have been detected by the HPLC procedure, being a minor product of the enzymatic digestion was considered. However, when the reaction mixture was analyzed directly for hexosamine content by cation-exchange chromatography, no glucosamine was found. Therefore it seemed important to characterize the structure of the unknown product

in order to obtain additional information in regard to chitin structure and the specificity of the chitinolytic enzymes.

Purification of the Unknown Product

The enzymatic reaction was performed on a scale large enough (chitinase, $0.6 \mu\text{M}$; β -*N*-acetylglucosaminidase, $0.3 \mu\text{M}$; insect chitin, 10 mg ml^{-1}) to obtain milligram quantities of the unknown product. After 24 h incubation the reaction mixture was applied directly to a gel filtration column of Bio-Gel P-4. The unknown product was eluted just before the elution of *N*-acetylglucosamine on Bio-Gel P-4. After rechromatography two times a single peak of the unknown product was obtained, which was pooled and lyophilized. Purity of this sample was confirmed by high-performance liquid chromatography both on a carbohydrate analysis column and on a TSK-GEL G2000PW gel filtration column. A single peak was observed in both cases. Figure 4 shows the gel filtration profile of authentic chitin oligosaccharides and the purified unknown product. The unknown

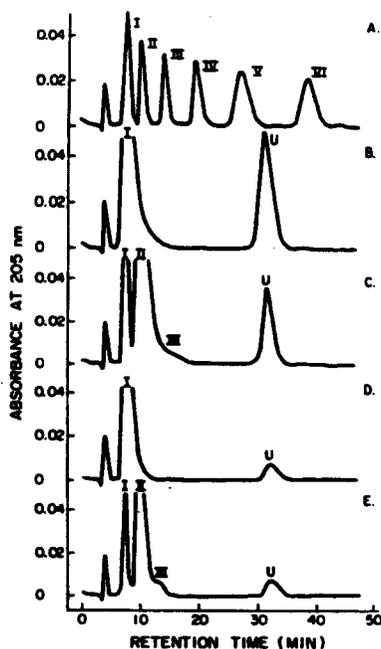


FIG. 3. Typical high-performance chromatographic separation of chitin oligosaccharide standard compounds and hydrolytic products from insoluble insect and crab chitin digested by chitinolytic enzymes from *M. sexta*. (A) Chitin oligosaccharide mixture:GlcNAc, I through β GlcNAc, VI (hexamer); (B) products from incubation of colloidal crab chitin with enzyme mixture for 6 h; (C) products from incubation of colloidal crab chitin with chitinase for 6 h; (D) products from incubation of colloidal *M. sexta* chitin with enzyme mixture for 4.5 h; (E) products from incubation of colloidal *M. sexta* chitin with chitinase alone for 4.5 h. The peak at 4.1 min is due to buffer components.

product was eluted just after *N,N'*-diacetylchitobiose, indicating that the unknown product has an apparent molecular weight intermediate between those of *N*-acetylglucosamine and a dimeric chitin oligosaccharide. When the purified unknown compound was incubated with the binary chitinase mixture, no further degradation was observed, confirming that the unknown compound is a dead-end product of chitin hydrolysis by chitinolytic enzymes.

Cation-Exchange Chromatography of the Unknown Product

Cation-exchange chromatography coupled with postcolumn ninhydrin derivatization, the standard method for amino acid

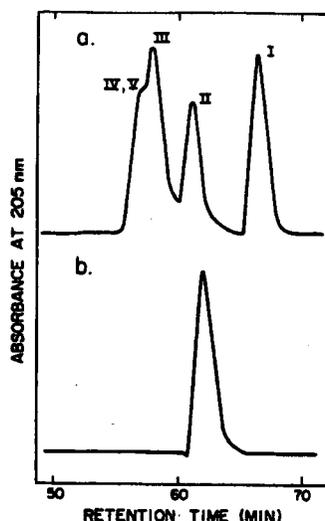


FIG. 4. High-performance gel filtration of (a) chitin oligosaccharide standard compounds and (b) *M. sexta* larval chitin unknown product on TSK-Gel G2000 PW. Roman numerals indicate the degree of polymerization of oligosaccharides.

and hexosamine analyses, was employed for further study of the unknown product. The chromatographic behavior of glucosamine, *N,N'*-diacetylchitobiose, and the unknown product is reproduced in Fig. 5. Glucosamine exhibited two peaks (Fig. 5a)

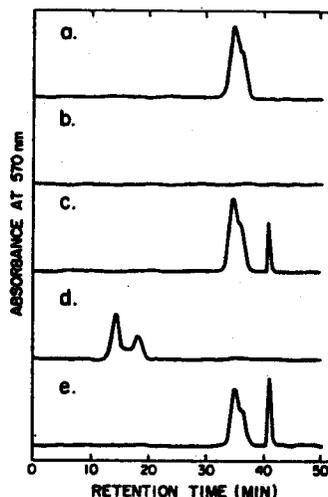


FIG. 5. Cation-exchange chromatography of (a) glucosamine, (b) *N,N'*-diacetylchitobiose, (c) acid hydrolysate of (b), (d) *M. sexta* larval chitin unknown product, and (e) acid hydrolysate of (d).

that were partially superimposed on each other, a result attributed to the anomeric nature of the carbohydrate. Although *N,N'*-diacetylchitobiose showed no peaks (Fig. 5b) because of the absence of a free amino group, this compound when hydrolyzed by 3 N HCl for 20 h exhibited one major peak (Fig. 5c) corresponding to glucosamine and a minor peak that was probably ammonia. The unknown compound exhibited two partially fused peaks (Fig. 5d) that eluted relatively early during chromatography and probably represented separation of anomers. When hydrolyzed under the same conditions as for *N,N'*-diacetylchitobiose hydrolysis, the unknown compound hydrolysate exhibited a chromatographic profile (Fig. 5e) similar to that of the *N,N'*-diacetylchitobiose hydrolysate, which contained glucosamine and ammonia. These results indicated that the unknown compound has a structure similar to that of *N,N'*-diacetylchitobiose but that it also has a free amino group.

Spectroscopic Properties

Spectroscopic analyses of the unknown compound were made with a focus on both the acetamido group and free amino group. The molar extinction coefficients of the unknown material and its trinitrophenylated derivative were estimated on the assumption of a parent compound molecular weight of *N*-monoacetylchitobiose hydrochloride salt (Table II). The extinction coefficient of the unknown compound at 220 nm

due to the acetamido group was approximately one-half that of *N,N'*-diacetylchitobiose. The molar extinction coefficient of U at 420 nm caused by modification of the free amino group(s) with trinitrobenzenesulfonate was apparently one-half that of glucosamine-HCl. From these results, the unknown product appeared to have the structure of a monoacetylated chitobiose derivative.

Solution Nuclear Magnetic Resonance Spectroscopy of Unknown Compound

A more definitive analysis of the structure of the unknown product was made with proton and carbon nuclear magnetic resonance spectroscopy. A total of 22 peaks were resolved in the ¹H-decoupled 100.6-MHz ¹³C-NMR spectrum of the unknown product with none of the lines showing abnormal widths or intensities indicative of overlapping resonances. Comparisons of chemical shifts and peak heights to those of authentic *N,N'*-diacetylchitobiose revealed several differences. First, the *N,N'*-diacetylchitobiose gave 24 peaks in the 100.6-MHz ¹³C-NMR spectrum, most of which matched the 21 resolved shifts reported for that compound (20) after a constant +1.0 ppm correction of the literature values for differences between their external and our internal TMS references. One of the additional peaks resolved was at 23.32 ppm in the methyl region and appeared just downfield from the larger peak at 23.27 ppm (Fig. 6A). Its intensity relative

TABLE II

SPECTROSCOPIC PROPERTIES OF *N*-ACETYLGLUCOSAMINE, *N,N'*-DIACETYLCHITOBIOSE, GLUCOSAMINE, AND UNKNOWN CHITIN DIGESTION PRODUCT^a

Compound	Molecular weight	ϵ_{220} ($M^{-1} \text{ cm}^{-1}$)	ϵ_{420} ($M^{-1} \text{ cm}^{-1}$) of TNBS derivative ^b
GlcNAc	221.2	309	<100
GlcNAc- β -(1 \rightarrow 4)-GlcNAc	424.4	506	<100
GlcNH ₂ -HCl	215.6	<10	12,085
Unknown metabolite	(418.9) ^c	358	6,578

^a All determinations performed in 50 mM sodium phosphate, pH 5.0.

^b TNBS, 2,4,6-trinitrobenzenesulfonate.

^c For GlcNH₂-HCl- β -(1 \rightarrow 4)-GlcNAc.

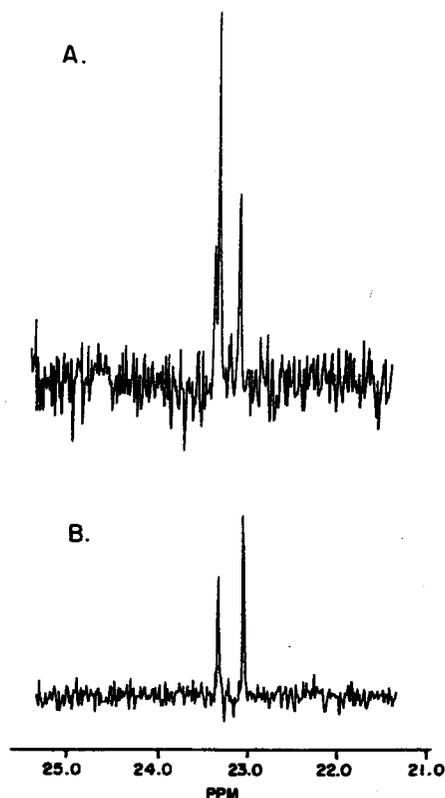


FIG. 6. Methyl-carbon regions of ^1H -decoupled 100.6-MHz ^{13}C -NMR spectra of N,N' -diacetylchitobiose and N -monoacetylchitobiose. (A) N,N' -Diacetylchitobiose. Spectrum acquired on a 3-mg sample in 0.4 ml of $^2\text{H}_2\text{O}$ using 4096 ca. 60° pulses over a 20,000-Hz spectral width, a 3-s period, 1,221-Hz/point resolution and processed with 0.2-Hz line broadening. (B) N -Monoacetylchitobiose from *M. sexta* cuticle chitin. Spectrum acquired identically to that of (A) on a 3 mg/0.4 ml sample except for the use of 10,240 pulses.

to the other two methyl carbon peaks corresponded to that of a β conformer and, thus, has been assigned to $\text{C}8\beta$ (Fig. 7) since carbon intensities of hexoses generally reflect the proportions of α and β anomers. Importantly, the large central methyl peak (23.27 ppm) in the N,N' -diacetylchitobiose spectrum previously assigned to $\text{C}8'$ (20) is missing in the unknown product (Fig. 6B). A 400-MHz ^1H -NMR spectrum of U (Fig. 8B) also showed only a single methyl proton resonance (2.019 ppm), whereas that for N,N' -diacetylchitobiose (Fig. 8A) showed a second methyl proton peak of comparable intensity slightly downfield

(2.050 ppm) from that of the unknown compound. Other features of the ^1H -NMR spectrum (not shown) of the unknown compound were in general agreement with the 300-MHz spectrum of the β -methyl glycoside of N,N' -diacetylchitobiose (23) except for some overlapping resonances from the α -anomeric form of the reducing ring. For example, the β -H1 peak at 4.70 ppm for the unknown product (4.68 ppm in N,N' -diacetylchitobiose) looked like an unresolved pair of doublets (apparent $J_{1,2} = 7.0$ Hz) at 23°C , most likely due to virtual coupling with H2 and H3 as shown by Brisson and Carver (23), and persisted even in the 500-MHz ^1H -NMR spectra of that residue in an oligosaccharide (24, 25). The α -H1 resonance (5.178 ppm for the unknown product and 5.175 ppm for N,N' -diacetylchitobiose), on the other hand, appeared as a sharp singlet ($J_{1,2} < 0.5$ Hz) for both compounds in contrast to that of the α form of N -acetylglucosamine which had a $J_{1,2}$ of 3.6 Hz (22). No additional detailed analysis of the ^1H -NMR spectrum of the unknown product has been attempted at this time primarily because of the complexity introduced by the anomeric mixture.

The other two newly resolved resonances in the ^{13}C -NMR spectrum of N,N' -diacetylchitobiose were in the carbonyl region and showed three well-resolved peaks (Fig. 9A). The middle resonance, which is missing in the spectrum of the unknown product (Fig. 9B), had an intensity essentially equal to the sum of the other two and was assigned to $\text{C}7'$. Of the remaining two, the most downfield resonance had the least intensity and was assigned to $\text{C}7\beta$. By difference the most upfield of the three signals was taken to correspond to $\text{C}7\alpha$. Collec-

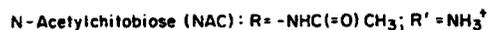
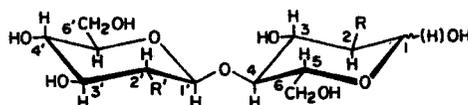


FIG. 7. Cyclic structures of N,N' -diacetylchitobiose and N -monoacetylchitobiose.

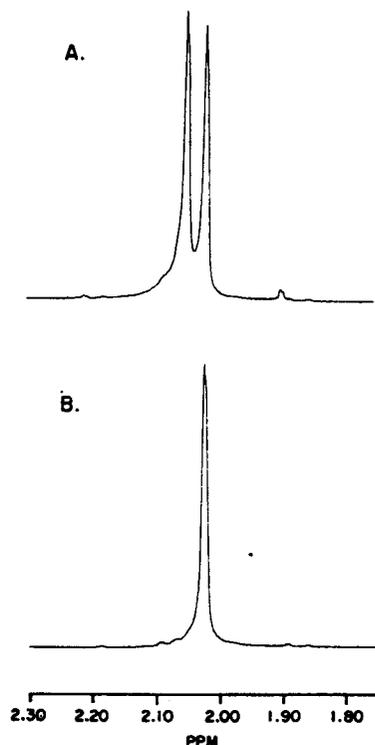


FIG. 8. Methyl proton regions of 400-MHz ^1H -NMR spectra of N,N' -diacetylchitobiose and N -monoacetylchitobiose. (A) N,N' -Diacetylchitobiose. Spectrum acquired on the same solution used for Fig. 6A by collecting 256 ca. 60° pulses over a 4000-Hz spectral width with a 4.048-s period, 0.488-Hz resolution, and processing with 0.25-Hz line broadening. The ^2HOH signal was suppressed by a pulsed homonuclear pre-saturation technique. (B) N -Monoacetylchitobiose from *M. sexta* cuticle chitin. Spectrum acquired on the same solution used for Fig. 6B utilizing the same conditions as in (A).

tively, the NMR spectra strongly suggested that the unknown product is N -monoacetylchitobiose [O - β -D-2-amino-2-deoxyglucopyranosyl-(1 \rightarrow 4)-2-acetamido-2-deoxy- β -D-glucopyranose]; that is, a monoacetylated chitobiose with the acetyl group missing from the nitrogen of the nonreducing ring.

This structural determination was substantiated by comparing the chemical shifts of N,N' -diacetylchitobiose and the unknown product (Table III) based on the assignments described above and those of Saito *et al.* (21). Differences in chemical shifts of more than 1 ppm were noted only

for $\text{C}1'$, $\text{C}2'$, $\text{C}3'$, $\text{C}4\alpha$, and $\text{C}4\beta$. Downfield shifts of 1.09 ppm at $\text{C}2'$ and 2.1 ppm at $\text{C}3'$ in the unknown product were larger, but in the same direction as the ca. 0.3 and 1.5 ppm differences at $\text{C}2$ and $\text{C}3$, respectively, between glucosamine (26) and N -acetylglucosamine (27). Similar comparisons for $\text{C}1'$, on the other hand, revealed a smaller shift difference between the two disaccharides (1.26 ppm) than between the two monosaccharides (ca. 2.4 ppm). Thus, the difference between the unknown product and N,N' -diacetylchitobiose appears to reflect more than just the greater electron withdrawing capacity of NH_3^+ relative to $-\text{NHC}(=\text{O})\text{CH}_3$ and probably indicates that there are conformational differences between the two disaccharides. Indeed, the

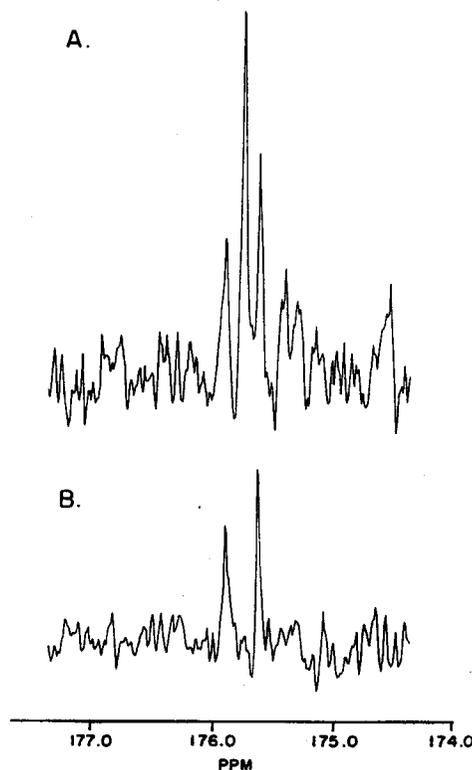


FIG. 9. Carbonyl-carbon regions of ^1H -decoupled 100.6-MHz ^{13}C -NMR spectra of N,N' -diacetylchitobiose and N -monoacetylchitobiose. From the same spectra as shown in Fig. 6 except that these were processed with a 2.4-Hz line broadening. (A) N,N' -Diacetylchitobiose; (B) N -monoacetylchitobiose from *M. sexta* cuticle chitin.

TABLE III
¹³C CHEMICAL SHIFTS OF *N*-ACETYLCHITOBIOSE AND
N,N'-DIACETYLCHITOBIOSE

Carbon ^b	Chemical shift, δ ^a (ppm)		δ ^d
	<i>N</i> -Acetyl- chitobiose	<i>N,N'</i> -Diacetyl- chitobiose ^c	
C _{1a}	91.65	91.58	0.07
C _{1β}	95.97	95.97	0
C _{1'}	103.88	101.60	1.26
C _{2a}	54.97	54.77	0.20
C _{2β}	57.25	57.25	0.27
C _{2'}	57.85	56.76	1.09
C _{3a}	70.31	70.45	-0.14
C _{3β}	73.51	73.68	-0.17
C _{3'}	76.73	74.63	2.10
C _{4a}	79.92	81.03	-1.11
C _{4β}	79.54	80.60	-1.06
C _{4'}	70.73	70.89	-0.16
C _{5a}	71.27	71.11	0.16
C _{5β}	75.86	75.71	0.15
C _{5'}	77.32	77.05	0.27
C _{6a}	61.34	61.21	0.13
C _{6β}	61.44	61.33	0.11
C _{6'}	61.78	61.70	0.08
C _{7a}	175.61	175.61	0
C _{7β}	175.87	175.88	-0.01
C _{7'}	None	175.73	N/A
C _{8a}	23.04	23.03	0.01
C _{8β}	23.33	23.32	0.01
C _{8'}	None	23.27	N/A

^a Relative to internal TMS.

^b See Fig. 7.

^c Assignments of Saito *et al.* (21) and this study.

^d δ(*N*-Monoacetylchitobiose)-δ(*N,N'*-Diacetylchitobiose).

relatively large changes at C4 supported the possibility of a conformational change around the β-1,4 linkage. Furthermore, lesser, but probably real, shift differences were observed for the other carbons nearest the glycosidic bond in both rings (Table III).

Hydrolysis of Intact Cuticle

The cuticle from fifth-instar larvae of *Manduca sexta* was incubated with diluted molting fluid from pharate pupae to deter-

mine whether *N*-monoacetylchitobiose is produced from intact cuticle *in vitro*. The reaction conditions were the same as used for chitin hydrolysis except for the addition of 10⁻⁴ M 1-phenyl-2-thiourea to prevent darkening of the reaction mixture which is caused by trace amounts of tyrosinase associated with the larval cuticle. Although a considerable amount of *N*-acetylglucosamine was generated in the 24-h incubation, *N*-monoacetylchitobiose was undetected by HPLC analysis (data not shown). Apparently, glucosamine residues are either not present or are present at only trace levels in larval chitin. Consequently, the presence of *N*-monoacetylchitobiose in the enzymatic digestion mixture would seem to be a result of incomplete reacetylation during chitin preparation. The binary chitinase mixture alone did not produce *N*-acetylglucosamine, chitin oligosaccharides, or *N*-monoacetylchitobiose from intact larval cuticle. Apparently proteases that are also present in molting fluid are required to digest protein in the chitin-protein matrices of the cuticle so that chitin becomes susceptible (exposed) to chitinolytic enzymes (28, 29).

DISCUSSION

In previous studies (8, 9), we reported on the mechanism of chitin hydrolysis by the binary chitinase system from *M. sexta*. *N*-Acetylglucosamine is the major end product of the catalysis while the dimer and trimer of *N*-acetylglucosamine are intermediates in chitin degradation. In the present study, the structure of another product was elucidated to be *N*-monoacetylchitobiose which is a dead-end product of enzymatic chitin degradation. Treatment of our chitin preparations with red flour beetle chitinolytic enzymes also yielded *N*-monoacetylchitobiose as a dead end product of those enzymes (H. Aoki and K. J. Kramer, unpublished data). We estimate that the degree of deacylation of chitin prepared from insect and crab cuticle is very low (<3% of the residues). It is possible that some deacylation of chitin occurred during the rather harsh alkaline heat treatment of the cuticle. If that is the

case, the liberated amino groups are not reacylated by the usual acetic anhydride procedure.

Solid-state NMR has been used herein to demonstrate a relatively high degree of chemical homogeneity for chitin that had been extracted from tobacco hornworm or crab cuticle by the traditional method of hot alkali digestion (15). Chitins with variable chemical composition have been noted previously and chemical heterogeneity depends on both the source of species and the method of preparation (16, 17). Our chitin preparation contained trace levels of amino acids and glucosamine (<2 mol %). Since the occurrence of glucosamine in most arthropod chitins is rare, and not even detectable as the *N*-monoacetylchitobiose product from intact cuticle digested with molting fluid, that residue probably does not play a major role in determining the physical properties of chitin or in the kinetic mechanism of chitin degradation by chitinolytic enzymes. Thus, the mechanism of chitin degradation by the binary chitinase system previously proposed (8, 9) can be considered a valid one.

Deacetylated residues in chitin or peptidoglycan components of arthropod cuticles or fungal and algal cell walls have been studied by several investigators (17, 30-38). In some of those studies, chitin or peptidoglycan was obtained by harsh acid and/or alkaline treatments. Thus, the natural occurrence of deacetylated residues in intact invertebrate cuticle or microbial cell wall remains uncertain. The chitin in the cuticle of *Macrotermes estherea* was reported to undergo deacetylation *in vivo* in order to facilitate enlargement of the cuticle during the physogastric phase (39). In the present study of hornworm and crab chitins, a deacetylated degradation product was purified by high-performance liquid chromatography and identified as *N*-monoacetylchitobiose. Direct hydrolysis of larval cuticle by molting fluid generated no *N*-monoacetylchitobiose, although a considerable amount of *N*-acetylglucosamine was found in the enzymatic hydrolysate. That result showed that there are at most only trace amounts of deacetylated residues in *M. sexta* and crab chitins *in situ*,

assuming molting fluid chitinolytic enzymes have the same specificities as those of the binary enzyme mixture and that complete digestion occurs.

The absence of any detectable glucosamine combined with the presence of *N*-monoacetylchitobiose in the binary chitinase digestion mixture indicated that neither enzyme is able to cleave β -1,4-glycosidic bonds when the amino function on the glycon or nonreducing side (see Fig. 7) of the bond is deacetylated. On the other hand, the apparent absence of any detectable trimer containing glucosamine as the middle residue, which would be expected to elute between peak III (Fig. 3A) and peak U (Figs. 3B-E), shows that a glucosamine residue on the aglycon or reducing side of the β -1,4 bond does not prevent bond cleavage by the chitinolytic enzymes. The apparent inability of the enzymes to form a productive complex when glucosamine is on the nonreducing side of the β -1,4 glycosidic bond may be a direct consequence of deacetylation or an indirect result of the change in conformation about the β -1,4 glycosidic bond inferred from the NMR chemical shift data.

The presence of glucosamine in chitin prepared from arthropod cuticle was a somewhat surprising result since the solid-state NMR data do not reveal any difference between the spectra of native and acetic anhydride-treated samples. In the insoluble state, those residues appear to be shielded from solvent and inaccessible to acylating agents. CPMAS ^{15}N -NMR is a method that could be used to measure directly amino and amide nitrogen content in chitin (4), but the low natural abundance of ^{15}N makes that experiment difficult at the present time. Instead, enzymatic product analysis can be used to reveal whether chitin samples contain residues of glucosamine. *M. sexta* or crab cuticular chitin may not be a strict homopolymer of β -(1 \rightarrow 4)-linked GlcNAc residues, but if it is not, then glucosamine or amino acids are present in only trace amounts. In chitin samples prepared by alkali-heat treatment of cuticle, the frequency of glucosamine residues in the polysaccharide preparations may be increased.

ACKNOWLEDGMENT

We are grateful to Roy Speirs, USGMRL, for assistance with tissue preparation.

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