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Mode of Action on Deacetylation of Acetylated Methyl Glycoside by Cellulose Acetate Esterase from *Neisseria sicca* SB

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The regioselective deacetylation of purified cellulose acetate esterase from *Neisseria sicca* SB was investigated on methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside and 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside. The substrates were used as model compounds of cellulose acetate in order to estimate the mechanism for deacetylation of cellulose acetate by the enzyme. The enzyme rapidly deacetylated at position C-3 of methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside to accumulate 2,4,6-triacetate as the main initial reaction product in about 70% yield. Deacetylation was followed at position C-2, and generated 4,6-diacetate in 50% yield. The enzyme deacetylated the product at positions C-4 and C-6 at slower rates, and generated 4- and 6-monoacetates at a later reaction stage. Finally, it gave a completely deacetylated product. For 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside, CA esterase deacetylated at positions C-3 and C-6 to give 2,4,6- and 2,3,4-triacetate. Deacetylation proceeded sequentially at positions C-3 and C-6 to accumulate 2,4-diacetate in 55% yield. The enzyme exhibited regioselectivity for the deacetylation of the acetylglycoside.

Key words: cellulose acetate; esterase; regioselective deacetylation; *Neisseria sicca*

Cellulose acetate (CA) is one of the most important synthetic organic esters because of its broad applications, such as in fibers, plastics, films, and membranes,[1] and because it is made from cellulose, the most abundant biopolymer on earth. CA is biodegradable by certain unidentified bacteria,[2] *Pseudomonas paucimobilis*,[3] and *Bacillus* sp. strain S2055,[4] but the mechanism of biodegradation of CA on the enzymatic level has not been elucidated. Research on the mechanisms of enzymatic degradation of biodegradable polymers, including CA, is needed to understand the assimilability and safety of degradation products in the environment. In nature, the complete degradation of plant cell-wall polymers calls for a set of enzymes with different activities acting in concert, because the polysaccharides in the plant cell-wall contain many side-chain substituents. In xylan degradation, enzymes act synergistically on the 1,4-β-D-xylan backbone (endo-1,4-β-xylanases) and side-chain-cleaving enzymes (α-L-arabinofuranosidase, acetylxylan esterase, and α-glucuronidase).[5–8] The synergistic action between endo-1,4-β-xylanases and acetylxylan esterase (EC 3.1.1.72) results in efficient degradation of acetylated xylan. The deacetylation reaction by acetylxylan esterase increases the accessibility of endo-1,4-β-xylanase to the polysaccharide backbone.[9]

A bacterium capable of assimilating CA, *Neisseria sicca* SB, was isolated from soil.[10] We purified and characterized a CA esterase from *N. sicca* SB that catalyzes the deacetylation of CA,[11] and endo-1,4-β-glucanases I (EG I)[12] and II (EG II),[13] which hydrolyze the β-1,4 linkages in CA molecules. We have shown synergistic degradation of CA particles by CA esterase with EG I,[12] and by CA esterase with EG II.[13] CA esterase facilitates hydrolysis of the CA backbone by the glucanases. CA esterase is important for complete degradation of CA particles. The degradation of CA by a mixture of CA esterase and endo-1,4-β-glucanases from *N. sicca* SB resembled heteroxylan degradation in its synergistic action. The N-terminal amino acid sequence of CA esterase was found to have 45% similarity with that of an acetylxylan esterase from *Aspergillus niger*.[11] CA esterase catalyzed the hydrolysis of *p*-nitrophenyl esters of short-chain fatty acids.[11] The enzyme showed only slight activity toward aliphatic and aromatic acetyl esters. It showed significant activity on acetyl saccharides, though the compounds are low solubility. To know the mode of action of CA esterase in detail is important for elucidating the mechanism of CA degradation by the enzymes. This paper describes regioselective deacetylation by CA esterase for methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (2,3,4,6-Ac-

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Abbreviations: CA, cellulose acetate; EG I, endo-1,4-β-glucanase I; EG II, endo-1,4-β-glucanase II; 2,3,4,6-Ac-MeβGlc, methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside; 2,3,4,6-Ac-MeβGal, methyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside; MeβGlc, methyl-β-D-glucopyranoside; MeβGal, methyl-β-D-galactopyranoside; TLC, thin-layer chromatography; TMS, trimethylsilyl; GLC, gas-liquid chromatography; GLC–MS, gas-liquid chromatography mass spectrometer; COSY, correlation spectroscopy

MeβGlc) and methyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (2,3,4,6-Ac-MeβGal) as model compounds for CA. Especially, 2,3,4,6-Ac-MeβGlc resembles triacetyl 1,4-linked glucopyranosyl units in the CA molecule, and this provided insight concerning its function in CA degradation.

Materials and Methods

Materials. Lipase from *Candida rugosa* L-8525 (EC 3.1.1.3) was purchased from Sigma Chemical (St. Louis, MO), and was dialyzed before use. CA esterase was produced by *N. sicca* SB in CA medium, and was purified as described previously.[11] CA esterase activity was assayed in a reaction mixture containing 50 mM Tris–HCl buffer, pH 8.0, 1.0% CA (degree of substitution, 0.88) as the substrate, at 30 °C, and the acetic acid released was measured as described previously.[11] One unit of the esterase was defined as the amount of enzyme releasing 1.0 µmol of acetic acid per min.

2,3,4,6-Ac-MeβGlc and 2,3,4,6-Ac-MeβGal were obtained by acetylation with acetic anhydride in pyridine from commercially available methyl-β-D-glucopyranoside (MeβGlc, Nacalai Tesque, Kyoto, Japan) and methyl-β-D-galactopyranoside (MeβGal, Nacalai Tesque) respectively. A solution of 2 g of MeβGlc or MeβGal in 12 ml of pyridine was treated at 0 °C with 7 ml of acetic anhydride. After 16 h, the products were crystallized from water and recrystallized from ethanol. The structures of the products were confirmed by NMR spectroscopy by way of comparison of the obtained and reported NMR spectra.[14] Methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (2,3,4-Ac-MeβGlc) and methyl 2,3,4-tri-*O*-acetyl-β-D-galactopyranoside (2,3,4-Ac-MeβGal) were enzymatically prepared from 2,3,4,6-Ac-MeβGlc and 2,3,4,6-Ac-MeβGal *via* C-6 selective hydrolysis catalyzed by *Candida rugosa* lipase respectively.[15]

Deacetylation of 2,3,4,6-Ac-MeβGlc and 2,3,4,6-Ac-MeβGal by CA esterase. A saturated solution of 2,3,4,6-Ac-MeβGlc or 2,3,4,6-Ac-MeβGal in 50 mM potassium phosphate buffer, pH 7.0, at 20 °C was filtered and mixed with a solution of CA esterase to give a final CA esterase concentration of 0.15 U/ml in a total volume of 30 ml. The reaction conditions were neutral pH and relatively low temperature, because the products were not very stable. Control mixtures without the enzyme were run in parallel. Initial substrate concentrations were 6.4 mM for 2,3,4,6-Ac-MeβGlc and 6.4 mM for 2,3,4,6-Ac-MeβGal, measured by the phenol-sulfuric acid method.[16] After incubation with CA esterase, aliquots of the mixture were taken periodically and subjected to thin-layer chromatography (TLC), or were frozen immediately in liquid nitrogen and lyophilized.

GLC and GLC mass spectrometry of trimethylsilyl (TMS) derivatives of partially acetylated methyl β-glycosides. Standard compounds and freeze-dried reaction mixtures were subjected to trimethylsilylation using Silblender-HTP (Nacalai Tesque). After incubation at 25 °C for 1 h, samples were analyzed by gas-liquid chromatography (GLC) with a Shimadzu GC-1700 equipped with a flame ionization detector, and a Supelco SPB-170l fused-silica capillary column (30 m × 0.32 mm, 0.25 µm film). The peak areas were integrated with a Shimadzu C-R8A Chromatopack integrator. Helium was used as a carrier gas at a flow rate 1.4 ml/min. The derivatives of MeβGlc and MeβGal were chromatographed at an initial temperature of 120 °C for 6 min, which was then increased at a rate of 20 °C/min to 180 °C, then increased at 2 °C/min to 240 °C. The injector and detector temperatures were both 260 °C. Concentrations of the reaction components were determined by electronic integration. Differences in detector responses to derivatives having different contents of acetyl and TMS groups were taken into consideration. Acetylated and trimethylsilylated derivatives were analyzed with a gas-liquid chromatography mass spectrometer (GLC–MS) for estimation of number of acetyl groups. The analysis was done with a Varian Saturn 2000 mass spectrometer at an ionization voltage of 70 eV. The mass spectra were obtained by electron-impact ionization at 70 eV, using a Supelco MDN-5S capillary column (30 m × 0.25 mm, 0.25 µm film).

TLC and NMR spectroscopy. Enzymatic deacetylation products of 2,3,4,6-Ac-MeβGlc and 2,3,4,6-Ac-MeβGal were analyzed on TLC plates of silica gel 60 (Merck, Darmstadt, Germany). The solvent system was ethyl acetate/benzene/2-propanol (2:1:0.1, v/v). The separated saccharides were detected by spraying H_2SO_4–methanol (1:1, w/w), and by heating the plate at 150 °C for a few minutes. Large-scale isolation of partially deacetylated compounds for NMR spectroscopy was accomplished by TLC. The compounds were eluted from the silica gel with ethyl acetate (tri- and di-acetates) or methanol (mono-acetates).

^{1}H- and ^{13}C-NMR spectra of partially acetylated MeβGlc and MeβGal derivatives were recorded with a JEOL JNM-AL300 spectrometer at 25 °C in $CDC1_3$ or CD_3OD, operated at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts were given as the δ-value (ppm) against tetramethylsilane as an internal reference. The sites of the acetyl group were confirmed by 2D ^{1}H–^{1}H and ^{1}H–^{13}C correlation spectroscopy (COSY) NMR spectroscopy.

Results

Enzymatic deacetylation of 2,3,4,6-Ac-MeβGlc and 2,3,4,6-Ac-MeβGal

2,3,4,6-Ac-MeβGlc, 2,3,4-Ac-MeβGlc, and MeβGlc were available as standard compounds for GLC. 2,3,4,6-Ac-MeβGlc was deacetylated by CA esterase, and the products were analyzed by GLC (Fig. 1). CA esterase hydrolyzed 2,3,4,6-Ac-MeβGlc rapidly and generated

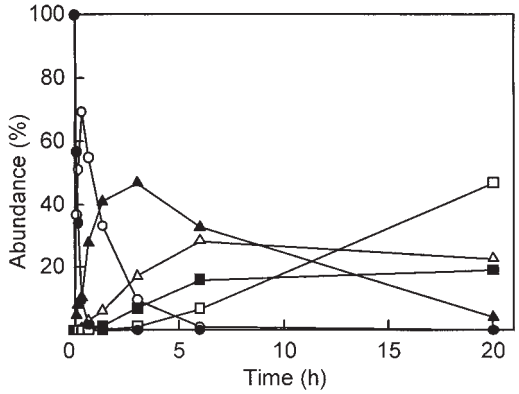

Fig. 1. Time Course of 2,3,4,6-Ac-MeβGlc Deacetylation by CA Esterase from *N. sicca* SB.

2,3,4,6-Ac-MeβGlc was incubated with CA esterase. The products were trimethylsilylated and analyzed by gas-liquid chromatography. (●) 2,3,4,6-Ac-MeβGlc; (○) product A; (▲) product B; (△) product C; (■) product D; (□) MeβGlc.

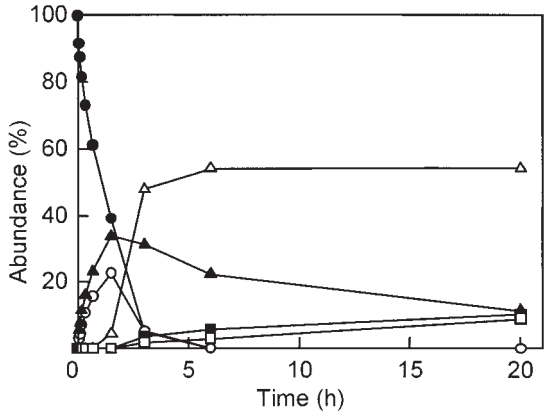

Fig. 2. Time Course of 2,3,4,6-Ac-MeβGal Deacetylation by CA Esterase from *N. sicca* SB.

2,3,4,6-Ac-MeβGal was incubated with CA esterase. The products were trimethylsilylated and analyzed by gas-liquid chromatography. (●) 2,3,4,6-Ac-MeβGal; (○) product E; (▲) product F; (△) product G; (■) product H; (□) MeβGal.

product A as the major initial product in about 70% yield at 20 min. The initial decreasing rate of the substrate was 0.56 mM/min when the initial substrate and enzyme concentrations were 6.4 mM and 0.15 U/ml respectively. The apparent increasing rate of product A was almost the same. As the reaction progressed, product B was accumulated as an intermediate product in about 50% yield at 3 h. A mixture of products C and D was generated at a later reaction stage. The formation of MeβGlc proceeded at a much slower rate. In the case of deacetylation for 2,3,4,6-Ac-MeβGal, CA esterase rapidly generated products E and F in about 25% and 35% yield respectively at 1.5 h (Fig. 2). The initial decreasing rate of the substrate was 0.22 mM/min. The apparent increasing rates of products E and F were 0.077 and 0.14 mM/min respectively. As the reaction progressed, product G was accumulated in about 55% yield at 6 h.

Table 1. Estimation of Number of Acetyl Groups for Various Acetylated MeβGlc by GLC–MS

TMS derivatives	Fragment ion peak (m/z)	Molecular weight	Number of acetyl group
2,3,4,6-Ac-MeβGlc*	331 (M^+-CH_3O)	362	4
2,3,4-Ac-MeβGlc*	377 (M^+-CH_3)	392	3
Product A	377 (M^+-CH_3)	392	3
Product B	407 (M^+-CH_3)	422	2
Product C	437 (M^+-CH_3)	452	1
Product D	437 (M^+-CH_3)	452	1
MeβGlc*	467 (M^+-CH_3)	482	0

*Standard compounds

At a later reaction stage, product H and MeβGal were generated at a much slower rate.

Identification of deacetylated products from 2,3,4,6-Ac-MeβGlc and 2,3,4,6-Ac-MeβGal by GLC–MS

The various molecular weights of the enzymatic products A, B, C, and D were determined by the spectra of GLC–MS, and the number of acetyl groups of the products was estimated (Table 1). The enzymatic products were trimethylsilylated and analyzed by GLC–MS. If the substrate is deacetylated by the enzyme, hydroxyl groups will arise at the deacetylated positions in the products. When the hydroxyl groups of the products are trimethylsilylated, the total molecular weights of the products increase. The mass spectra of trimethylsilyl ethers of MeβGlc by electron ionization mass spectrometry at 70 eV have been reported.[17,18] A more intense peak is present for the loss of $CH_3\cdot$ from one of the trimethylsilyl groups. The molecular weight can therefore be determined from this if no molecular-ion peak is present. That was confirmed by GLC–MS spectra of trimethylsilylated MeβGlc and 2,3,4-Ac-MeβGlc as standard compounds. Trimethylsilylated MeβGlc and 2,3,4-Ac-MeβGlc gave the M^+-CH_3 ion at m/z 467 and at m/z 377 respectively. 2,3,4,6-Ac-MeβGlc gave the M^+-CH_3O ion at m/z 331. Product A gave the M^+-CH_3 ion at m/z 377. The fragment ion suggested that the number of the acetyl group for product A was three. Product B gave the M^+-CH_3 ion at m/z 407, and products C and D gave the M^+-CH_3 ion at m/z 437. The fragment ions suggested that the numbers of the acetyl group were two for product B and one for products C and D. The reaction products E, F, G, and H were estimated from the number of acetyl groups in the same way. Products E, F, G, and H gave the M^+-CH_3 ion at m/z 377, 377, 407, and 437 respectively. These fragment ion peaks suggest that the numbers of the acetyl group were three for products E and F, two for G, and

Table 2. ^{1}H-NMR Data for Deacetylated Products of 2,3,4,6-Ac-MeβGlc by CA Esterase from *N. sicca* SB

Sample	Chemical shifts (ppm)							
	H1	H2	H3	H4	H5	H6	H6′	OCH_3
2,3,4,6-Ac-MeβGlc	4.43	4.99	5.21	5.09	3.70	4.28	4.15	3.51
Product A (2,4,6-Ac)	4.37	4.84	3.72	4.95	3.63	4.29	4.17	3.51
Product B (4,6-Ac)	4.23	3.46	3.68	4.93	3.64	4.29	4.14	3.57
Product C (4-Ac)	4.20	3.40	3.60	4.88	3.52	3.92	3.71	3.56
Product D (6-Ac)	4.21	3.39	3.55	3.55	3.45	4.45	4.32	3.55
MeβGlc	4.17	3.16	3.35	3.28	3.26	3.87	3.67	3.53

Table 3. ^{1}H-NMR Data for Deacetylated Products of 2,3,4,6-Ac-MeβGal by CA Esterase from *N. sicca* SB

Sample	Chemical shifts (ppm)							
	H1	H2	H3	H4	H5	H6	H6′	OCH_3
2,3,4,6-Ac-MeβGal	4.40	5.20	5.02	5.39	3.91	4.18	4.16	3.53
Product E (2,3,4-Ac)	4.43	5.22	5.05	5.38	3.77	3.77	3.58	3.53
Product F (2,4.6-Ac)	4.36	4.96	3.84	5.33	3.85	4.18	4.16	3.52
Product G (2,4-Ac)	4.38	4.98	3.83	5.25	3.66	3.76	3.76	3.51
MeβGal	4.12	3.49	3.47	3.83	3.50	3.75	3.73	3.52

one for H. The numbers of acetyl groups of the enzymatic products were also confirmed by comparing relative mobility on TLC (data not shown).

Structure determinations of partially deacetylated products by NMR

The deacetylated positions in the enzymatic products were established by ^{1}H- and ^{13}C-NMR. The ^{1}H-NMR spectra of products A, B, C, and D were measured after purification by TLC (Table 2). In the chemical shifts of the ^{1}H-NMR spectra, the corresponding positions of the products were confirmed by ^{1}H–^{1}H COSY. Deacetylation at positions C-2, C-3, C-4, and C-6 of acetylated methyl glucosides results in an upfield shift (1–3 ppm) of the peak corresponding to the proton of O-acetylated carbon.[19] The shift values are virtually independent of the nature of the acyl moiety and the solvent. The NMR spectrum of the triacetate was compared to that of authentic tetraacetate, and the spectrum of the diacetate was compared to that of the previously identified triacetate. The spectra of the monoacetates were compared to that of the previously identified diacetate and that of unmodified, authentic methyl glucoside. When the chemical shifts of product A were compared with that of 2,3,4,6-Ac-MeβGlc, the chemical shift at position C-3 for product A was an upfield shift of about 1.5 ppm, and the values at the other positions were almost equal. This result suggests that product A was a deacetylated compound of 2,3,4,6-Ac-MeβGlc at position C-3, namely 2,4,6-Ac-MeβGlc. When product B was compared with product A, the chemical shift at position C-2 was an upfield shift of about 1.4 ppm. This result suggests that product B was 4,6-Ac-MeβGlc. When product C was compared with product B, the chemical shift at position C-6 was an upfield shift, suggesting that product C was 4-Ac-MeβGlc. When product D was compared with product B, the chemical shift at position C-4 was an upfield shift, suggesting that product D was 6-Ac-MeβGlc. These results were confirmed by comparison products C and D with MeβGlc.

In order to identify the position of deacetylation, the enzymatic products were measured by ^{13}C-NMR also (data not shown). In the chemical shifts of ^{13}C-NMR spectra, the corresponding positions of glucoside derivatives were confirmed by ^{1}H–^{13}C COSY. For acetylated saccharides, deacetylation of acetylated hydroxyl groups at positions C-2, C-3, C-4, and C-6 results in a downfield shift (1–3 ppm) of the peak(s) corresponding to the neighboring carbon(s).[20] When the chemical shifts of product A were compared with those of 2,3,4,6-Ac-MeβGlc, chemical shifts at positions C-2 and C-4 for product A were downfield shifts of about 3 ppm each, and the values at the others positions were almost equal. This result suggests that product A was a deacetylated compound of 2,3,4,6-Ac-MeβGlc at position C-3, namely 2,4,6-Ac-MeβGlc. When product B was compared with product A, chemical shifts at positions C-1 and C-3 were downfield shifts. This suggests that product B was 4,6-Ac-MeβGlc. When product C was compared with product B, the chemical shift at position C-5 was a downfield shift, suggesting that product C was 4-Ac-MeβGlc. When product D was compared with product B, chemical shifts at positions C-3 and C-5 were downfield shifts, suggesting that product D was 6-Ac-MeβGlc. These results were confirmed by comparing products C and D with MeβGlc. These results from ^{13}C-NMR corresponded with the results from ^{1}H-NMR.

In order to identify the position of deacetylation, ^{1}H-NMR spectra of products E, F, and G were measured (Table 3). The results suggest that products E, F, and G were 2,3,4-Ac-MeβGal, 2,4,6-Ac-MeβGal, and 2,4-Ac-MeβGal respectively. These results were confirmed by the ^{13}C-NMR spectra (data not shown). We could not analyze product H due to an insufficient amount of the pure product.

Discussion

CA esterase from *N. sicca* SB had been reported on as to purification and characterization.[11] The enzyme showed significant activity on acetyl saccharides. Investigation of the mode of action of CA esterase is

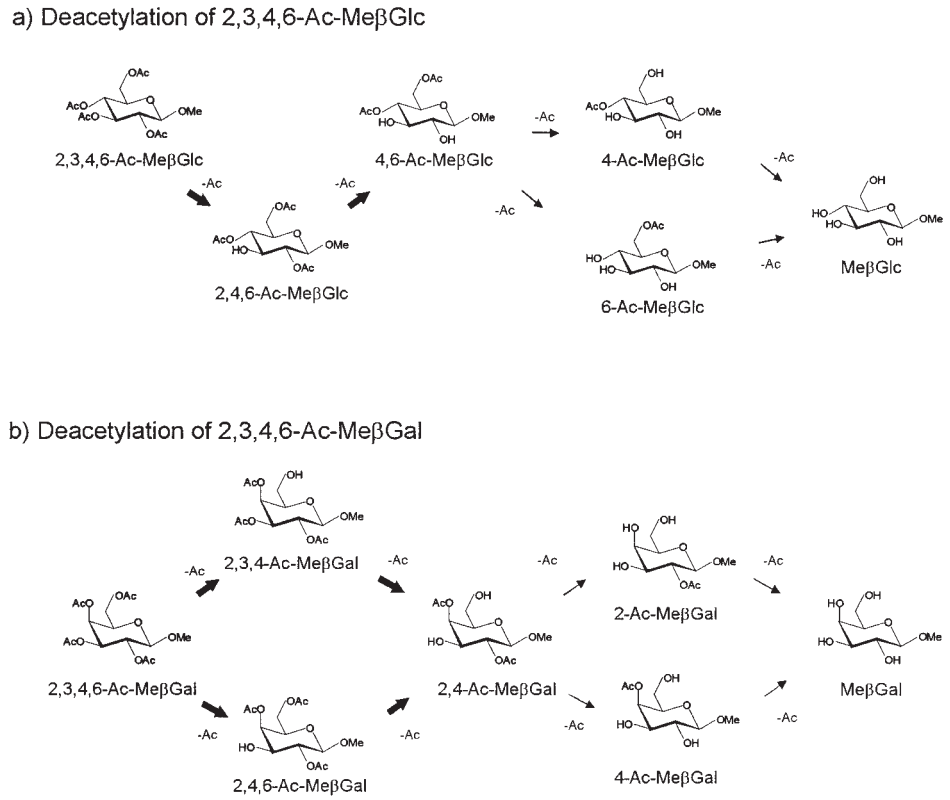

Fig. 3. Main Deacetylation Reactions Catalyzed by CA Esterase from *N. sicca* SB for 2,3,4,6-Ac-MeβGlc and for 2,3,4,6-Ac-MeβGal.

necessary in order to understand the deacetylation mechanism of CA degradation by CA esterase in detail. But it is difficult to analyze deacetylation products of CA polymer directly because partially deacetylated CA is a heterogeneous molecule with a high molecular weight and changed solubility. Acetylated methyl glucoside was chosen as a model compound of CA. The compound mimics acetylated glucoside residues in the CA molecule. Since CA has the β-1,4 glycosidic linkage, we used a methylated saccharide at anomeric carbon by β-linkage. In this study, we investigated the substrate specificity of CA esterase as to whether the enzyme hydrolyzes 2,3,4,6-Ac-MeβGlc and 2,3,4,6-Ac-MeβGal regioselectively or randomly.

When CA esterase hydrolyzed 2,3,4,6-Ac-MeGlc, we observed several partially deacetylated products by GLC. The reaction products were mainly four compounds, products A, B, C, and D. Each product was estimated as to the number of acetyl residues by GLC–MS. The positions of deacetylation were identified by NMR spectroscopy. The deacetylation reactions of 2,3,4,6-Ac-MeβGlc catalyzed by CA esterase from *N. sicca* SB are summarized in Fig. 3. There are four positions of the acetyl group in the 2,3,4,6-Ac-MeβGlc molecule that CA esterase can attack initially. 2,3,4,6-Ac-MeβGlc was most rapidly deacetylated at position C-3 to give 2,4,6-Ac-MeβGlc as the main initial product. Deacetylation was followed at position C-2, and generated 4,6-Ac-MeβGlc. Deacetylation continued at position C-4 or C-6 to produce a mixture of 6-Ac-MeβGlc and 4-Ac-MeβGlc transiently. 4-Ac-MeβGlc and 6-Ac-MeβGlc were subjected to further slow deacetylation and were converted to MeβGlc. CA esterase deacetylated 2,3,4,6-Ac-MeβGlc in preferential order. These results suggest that CA esterase most rapidly deacetylated at position C-3 of 2,3,4,6-Ac-MeβGlc, and that the deacetylation rate at position C-2 was slower than at position C-3, and further that deacetylation at positions C-4 and C-6 was yet slower.

Regioselective deacetylation for acetylsaccharides has been reported as to a few acetylxylan esterases. Acetylxylan esterases from *Streptomyces lividans*,[21] *Trichoderma reesei*,[22] and *Schizophyllum commune*[23] were investigated as to deacetylation of 2,3,4,6-Ac-MeβGlc. These enzymes deacetylated 2,3,4,6-Ac-MeβGlc at positions C-2 and C-3, yielding 4,6-Ac-MeβGlc as an final product or an intermediate. But there were some differences in the action of the acetylxylan esterases from that of CA esterase. Acetylxylan esterase from *S. lividans* removed two acetyl groups simultaneously from positions C-2 and C-3, to give 4,6-Ac-MeβGlc as an final product in 90–95% yield.[21] Acetylxylan esterase from *T. reesei*[22] exhibited similar regioselectivity to acetylxylan esterase from *S. lividans*. The enzyme initially removed two acetyl groups at positions C-2 and C-3, producing 4,6-Ac-MeβGlc in 30% yield. The intermediates of this conversion, 2,4,6- and 3,4,6-Ac-MeβGlc, were not detected in the reaction

mixture. 4,6-Ac-MeβGlc was further converted, mainly to 4-Ac-MeβGlc as an final product in 60% yield. Acetylxylan esterase from *S. commune* most rapidly deacetylated at position C-3 of 2,3,4,6-Ac-MeβGlc, and generated 2,4,6-Ac-MeβGlc as the major initial product in 50–60% yield.[23] 2,4,6-Ac-MeβGlc was further deacetylated, mainly at position C-2, to give 4,6-Ac-MeβGlc in 55% yield. 4,6-Ac-MeβGlc was not further converted to 4-Ac-MeβGlc or 6-Ac-MeβGlc. CA esterase from *N. sicca* SB produced 2,4,6-Ac-MeβGlc in about 70% yield, the highest yield among these enzymes. These facts suggest that the deacetylation rate by CA esterase at position C-3 was faster and/or that those at other positions were slower than those enzymes. CA esterase from *N. sicca* SB gave 4,6-Ac-MeβGlc, 4-Ac-MeβGlc, and 6-Ac-MeβGlc in 50%, 30%, and 15% yield respectively as the maximum yield from 2,3,4,6-Ac-MeβGlc. This indicates that CA esterase deacetylated at positions C-4 and C-6, though the reaction rates are very slow, as well as at positions C-2 and C-3.

In nature, deacetylation of acetylxylan residues in heteroxylans by acetylxylan esterase leads to an efficient degradation of heteroxylans by xylanases.[9] The property of rapid deacetylation at positions C-2 and C-3 of acetylxylose[21–23] is important for deacetylation of acetylxylan by acetylxylan esterase, but the deacetylation at positions C-4 and C-6 is not important because there are no acetyl groups at position C-4 and no C-6 carbon in the acetylxylan molecule. On the other hand, the property of deacetylation of 2,3,4,6-Ac-MeβGlc at position C-6 as well as positions C-2 and C-3 is important for complete degradation of CA. Kinetic studies of the hydrolysis of carboxymethyl cellulose by crude cellulase from *Trichoderma viride* have been reported.[24] It was concluded that hydrolysis occurred easily when three adjacent unsubstituted glucosyl residues were present, and very slowly when only two adjacent unsubstituted residues were present. Endo-1,4-β-glucanases from *N. sicca* SB did not hydrolyze the β-1,4 linkage backbone in the CA molecule with a degree of substitution of 1.77.[12,13] When the degree of substitution for CA was low, for example less than 1.0, endo-1,4-β-glucanases hydrolyzed the CA backbone. When CA with a degree of substitution of 0.88 was further deacetylated by CA esterase, the glucanases hydrolyzed the products at a faster rate.[12,13] This suggests that the substituents in the cellulose molecule influenced hydrolysis by cellulase and that removing the substituents led to effective hydrolysis of the cellulose molecule by cellulase. CA esterase deacetylated 2,3,4,6-Ac-MeβGlc at position C-6 as well as at positions C-2 and C-3. This suggests that CA esterase can deacetylate the CA molecule at position C-6, and that the properties of CA esterase lead to effective degradation of CA by synergistic action with endo-1,4-β-glucanases, and further that CA esterase is superior for degradation of CA to acetylxylan esterase, which cannot deacetylate at position C-6 of acetylated glucosyl residues in CA molecules.

There have been several reports of regioselective deacetylation of acetylated glycosides by lipases in order to search for preparation of useful intermediates for synthesis of saccharide derivatives. Lipase from *Candida rugosa* hydrolyzed the acetyl group esterifing at position C-6 in glucoside, and did not hydrolyze at any other position of the acetyl group.[15] Lipase from *Pseudomonas cepacia* hydrolyzed 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside at position C-4.[25] Lipase from *Aspergillus niger* hydrolyzed 2,3,4,6-Ac-MeβGlc at position C-3 to give 2,4,6-Ac-MeβGlc only.[26] Crude porcine pancreatic lipase hydrolyzed β-D-glucose pentaacetate at position C-1.[27] These lipases displayed high regioselectivity for deacetylation of acetylsaccharides. Deacetylation of 2,3,4,6-Ac-MeβGlc by CA esterase from *N. sicca* SB proceeded easily at position C-3, slowly at position C-2, and at positions C-4 and C-6 at a much slower rate. CA esterase had a different regioselectivity from these lipases.

Deacetylation of cellulose triacetate in dimethylsulfoxide was achieved with dimethylamine.[28] The contents of acetyl groups in the products at positions C-2, C-3, and C-6 were 17%, 25%, and 58% respectively when the average of degree of substitution for the mixture of the products was 1.45. The rate of deacetylation was fast in the order of positions C-2, C-3, and C-6. This suggests that deacetylation of acetylated saccharides by the basic catalysts showed much lower regioselectivity than that by CA esterase, and that the way of deacetylation by CA esterase was different from that by the basic catalysts. Regioselective deacetylation by CA esterase is probably derived from the affinity of acetylated saccharides and CA esterase. The affinity of CA esterase to acetylated saccharides probably leads to an effective deacetylation of CA in CA degradation under neutral conditions.

We also studied the regioselectivity of CA esterase for 2,3,4,6-Ac-MeβGal in order to estimate the effect of stereoisomers. Deacetylation reactions are summarized in Fig. 3. For 2,3,4,6-Ac-MeβGal, CA esterase rapidly deacetylated at position C-3 or position C-6 to give 2,4,6-Ac-MeβGal or 2,3,4-Ac-MeβGal as the intermediate. Deacetylation was followed at position C-6 for 2,4,6-Ac-MeβGal and at position C-3 for 2,3,4-Ac-MeβGal, and generated 2,4-Ac-MeβGal in 55% yield. 2,4-Ac-MeβGal was subjected to further deacetylation to generate product H and MeβGal at a much slower rate. The structure of product H is assumed to be 2-Ac-MeβGal or 4-Ac-MeβGal generated by hydrolysis of 2,4-Ac-MeβGal. The amount of product H was much smaller, and we could not analyze it further. These results suggest that CA esterase rapidly deacetylated at positions C-3 and C-6 of 2,3,4,6-Ac-MeβGal, and that the deacetylation rate at position C-3 was slightly faster than at position C-6, and further that deacetylation at positions C-2 and C-4 proceeded at a much slower rate. A different regioselectivity for 2,3,4,6-Ac-MeβGal from 2,3,4,6-Ac-MeβGlc was observed on deacetylation by

CA esterase. Deacetylation by CA esterase at position C-3 at a much faster rate for acetylgalactoside was similar to that for acetylglucoside. The rate of deacetylation at position C-6 in acetylgalactoside was much faster than that in acetylglucoside. On the other hand, the rate of deacetylation at position C-2 in acetylgalactoside was much slower than that in acetylglucoside. The acetyl group at position C-4 in 2,3,4,6-Ac-MeβGlc is equatorial, whereas that in 2,3,4,6-Ac-MeβGal is axial. These results suggest that the configuration at position C-4 in the acetylglycoside molecule affected the rate of deacetylation at positions C-2 and C-6, and that CA esterase recognizes not only the acetyl moiety in the acetylglycoside molecule but also part of the saccharide structure in the acetylglycoside molecule. The configuration of acetylglycoside is important for the deacetylation reaction by CA esterase. There are few detailed reports on the regioselectivity of lipases and acetylxylan esterases for 2,3,4,6-Ac-MeβGal. Lipase from *C. rugosa* has been shown to deacetylate 2,3,4,6-Ac-MeβGal only at position C-6, not at any other position.[15] When we deacetylated 2,3,4,6-Ac-MeβGlc and 2,3,4,6-Ac-MeβGal by lipase from *C. rugosa*, the rate of deacetylation at position C-6 was very slow. This indicates that regioselective deacetylation by lipase from *C. rugosa* was not affected by the configuration of the acetylsaccharides, and that the enzyme did not bind to the whole molecule of acetylsaccharides but recognized only the acetyl group at the primary hydroxy group. Other acetylated carbohydrates must be examined in order to understand the recognition of substrate by CA esterase in detail.

References

1) Bogan, R. T., and Brewer, R. J., "Encyclopedia of Polymer Science and Engineering" 2nd ed. Vol. 3, eds. Mark, H. F., Bikales, N. M., Overberger, C. G., and Menges, G., John Wiley, New York, pp. 174–178 (1985).
2) Cantor, P. A., and Mechalas, B. J., Biological degradation of cellulose acetate reverse-osmosis membranes. *J. Polym. Sci. C*, **28**, 225–241 (1969).
3) Nelson, M., McCarthy, S. P., and Gross, R. A., Isolation of a *Pseudomonas paucimobilis* capable of using insoluble cellulose acetate as a sole carbon source. *Proc. ACS Div. Polym. Mater. Sci. Eng.*, **67**, 139–140 (1992).
4) Ishigaki, T., Sugano, W., Ike, M., and Fujita, M., Enzymatic degradation of cellulose acetate plastic by novel degrading bacterium *Bacillus* sp. S2055. *J. Biosci. Bioeng.*, **90**, 400–405 (2000).
5) Christov, L. P., and Prior, B. A., Esterases of xylan-degrading microorganisms: production, properties, and significance. *Enzyme Microb. Technol.*, **15**, 460–475 (1993).
6) Nanmori, T., Watanabe, T., Shinke, R., Kohno, A., and Kawamura, Y., Purification and properties of thermostable xylanase and β-xylosidase produced by a newly isolated *Bacillus stearothermophilus* strain. *J. Bacteriol.*, **172**, 6669–6672 (1990).
7) Tuohy, M. G., Puls, J., Claeyssens, M., Vrsanska, M., and Coughlan, M. P., The xylan-degrading enzyme system of *Talaromyces emersonii*: novel enzymes with activity against aryl β-D-xylosides and unsubstituted xylans. *Biochem. J.*, **290**, 515–523 (1993).
8) Shao, W., Obi, S. K. C., Puls, J., and Wiegel, J., Purification and characterization of the α-glucuronidase from *Thermoanaerobacterium* sp. strain JW/SL-YS485, an important enzyme for the utilization of substituted xylans. *Appl. Environ. Microbiol.*, **61**, 1077–1081 (1995).
9) Biely, P., MacKenzie, C. R., and Schneider, H., Production of acetyl xylan esterase by *Trichoderma reesei* and *Schizophyllum commune*. *Can. J. Microbiol.*, **32**, 767–772 (1988).
10) Sakai, K., Yamauchi, T., Nakasu, F., and Ohe, T., Biodegradation of cellulose acetate by *Neisseria sicca*. *Biosci. Biotechnol. Biochem.*, **60**, 1617–1622 (1996).
11) Moriyoshi, K., Ohmoto, T., Ohe, T., and Sakai, K., Purification and characterization of an esterase involved in cellulose acetate degradation by *Neisseria sicca* SB. *Biosci. Biotechnol. Biochem.*, **63**, 1708–1713 (1999).
12) Moriyoshi, K., Ohmoto, T., Ohe, T., and Sakai, K., Purification and characterization of an endo-1,4-β-glucanase from *Neisseria sicca* SB that hydrolyzes β-1,4 linkages in cellulose acetate. *Biosci. Biotechnol. Biochem.*, **66**, 508–515 (2002).
13) Moriyoshi, K., Ohmoto, T., Ohe, T., and Sakai, K., Role of endo-1,4-β-glucanases from *Neisseria sicca* SB in synergistic degradation of cellulose acetate. *Biosci. Biotechnol. Biochem.*, **67**, 250–257 (2003).
14) Bock, K., and Pedersen, C., Carbon-13 nuclear magnetic resonance spectroscopy of monosaccharides. *Adv. Carbohydr. Chem. Biochem.*, **41**, 27–66 (1983).
15) Sweers, H. M., and Wong, C.-H., Enzyme catalyzed regioselective deacylation of protected sugars in carbohydrate synthesis. *J. Am. Chem. Soc.*, **108**, 6421–6422 (1986).
16) Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F., Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **28**, 350–356 (1956).
17) DeJongh, D. C., Radford, T., Hribar, J. D., Hanessian, S., Bieber, M., Dawson, G., and Sweeley, C. C., Analysis of trimethylsilyl derivatives of carbohydrates by gas chromatography and mass spectrometry. *J. Am. Chem. Soc.*, **91**, 1728–1740 (1969).
18) Biemann, K., DeJongh, D. C., and Schnoes, H. K., Application of mass spectrometry to structure problems. XIII. Acetates of pentoses and hexoses. *J. Am. Chem. Soc.*, **85**, 1763–1771 (1963).
19) Iwashita, T., In "Jikken Kagaku Koza Vol. 5, NMR" 4th ed. (in Japanese), Maruzen, Tokyo, pp. 99–178 (1991).
20) Yoshimoto, K., Itatani, Y., Shibata, K., and Tsuda, Y., Utilization of sugars in organic synthesis. II. Syntheses and proton- and carbon-13-nuclear magnetic resonance spectra of all positional isomers of methyl mono-*O*-tetradecanoyl-α- and β-D-glucopyranosides. *Chem. Pharm. Bull.*, **28**, 208–219 (1980).
21) Biely, P., Cote, G. L., Kremnicky, L., Greene, R. V., Dupont, C., and Kluepfel, D., Substrate specificity and mode of action of acetylxylan esterase from *Streptomyces lividans*. *FEBS Lett.*, **396**, 257–260 (1996).

22) Biely, P., Cote, G. L., Kremnicky, L., Greene, R. V., and Tenkanen, M., Action of acetylxylan esterase from *Trichoderma reesei* on acetylated methyl glycosides. *FEBS Lett.*, **420**, 121–124 (1997).
23) Biely, P., Cote, G. L., Kremnicky, L., Weisleder, D., and Greene, R. V., Substrate specificity of acetylxylan esterase from *Schizophyllum commune*: mode of action on acetylated carbohydrates. *Biochim. Biophys. Acta*, **1298**, 209–222 (1996).
24) Eriksson, K.-E., and Hollmark, B. H., Kinetic studies of the action of cellulase upon sodium carboxymethyl cellulose. *Arch. Biochem. Biophys.*, **133**, 233–237 (1969).
25) Lopez, R., Perez, C., Fernandez-Mayoralas, A., and Conde, S., Enzymic transesterification of alkyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside. *J. Carbohydr. Chem.*, **12**, 165–171 (1993).
26) Chen, H.-P., Hsiao, K.-F., Wu, S.-H., and Wang, K.-T., Regioselectivity enhancement by partial purification of lipase from *Aspergillus niger*. *Biotechnol. Lett.*, **17**, 305–308 (1995).
27) Hennen, W. J., Sweers, H. M., Wang, Y.-F., and Wong, C.-H., Enzymes in carbohydrate synthesis: lipase-catalyzed selective acylation and deacylation of furanose and pyranose derivatives. *J. Org. Chem.*, **53**, 4939–4945 (1988).
28) Heinze, T., and Liebert, T., Chemical characteristics of cellulose acetate. In "Cellulose Acetates: Properties and Applications", ed. Rustemeyer, P., Wiley-VCH, Freiburg, pp. 167–237 (2004).