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Non-Catalytic Dehydration of *N,N'*-diacetylchitobiose in High-temperature Water

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Abstract

Non-catalytic synthesis of 4-*O*-β-2-acetamido-2-deoxy-D-glucopyranosyl 2-acetamido-2,3-dideoxydidehydro-glucopyranose (GND) from chitin disaccharide, *N,N'*-diacetylchitobiose $(GlcNAc)_2$, was achieved, with a maximum yield of 24.7% in high-temperature water at 120–220ºC and 25 MPa with a reaction time of 8–39 sec.

Introduction

N,N'-diacetylchitobiose, $(GlcNAc)_2$, is a dimer of *N*-acetyl-D-glucosamine (GlcNAc), and a chitin oligosaccharide. $(GlcNAc)_2$ can be obtained from chitin by acid hydrolysis or enzymatic degradation.[1-4] After cellulose, chitin is the second most abundant biomass on earth, and is a major component in the cell walls of fungi and the exoskeletons of insects and crustaceans.[1,2] Chitin has a remarkable potential for the production of functional value-added chemicals, especially N-containing compounds.[1,2] The biological activities of chitin oligosaccharides such as $(GlcNAc)_2$ have been well documented and these have been used in foods, cosmetics, pharmaceuticals, and functional materials.[5-7] In addition, transformation of chitin and its oligosaccharides into derivatives has recently been studied to obtain renewable N-containing compounds.[8-11] $(GlcNAc)_2$ derivatives such as 4-*O*-β-2-acetamido-2-deoxy-D-glucopyranosyl 2-acetamido-2,3-dideoxydidehydro-glucopyranose (GND) and 4-*O*-β-2-acetamido-2-deoxy-D-glucopyranosyl 2-acetamido-2,3-dideoxydidehydro-gluconolactone (GNL), have attracted recent attention for a wide range of applications from plant protection to clinical administration in human medicine.[8] For example, GND is expected as an antioxidant and GNL has been found to exhibit antimutagenic activity.[8] So far, these $(GlcNAc)_2$ derivatives are not commercially available from chemical reagent companies because their syntheses generally involve

multi-step pathways, but, their market could be increased if a simple synthesis of $(GlcNAc)_2$ derivatives was developed.

Ogata et al. have reported the conversion of $(GlcNAc)_2$ into its dehydration products by incubation in sodium borate solution at 100ºC for 2 hours, obtaining GND in 13% yield.[8] They also reported that GND was easily oxidized to GNL over charcoal catalyst at 60ºC for 120 hours. This previous study found that the dehydration of $(GlcNAc)_2$ requires a catalyst (sodium borate) at temperatures below 100ºC. However, to utilize $(GlcNAc)_2$ derivatives as functional food additives or medicines requires the complete elimination of the catalyst, resulting in an increase in the cost and energy consumption of the production process. In addition, the reaction time with sodium borate catalyst below 100ºC is a number of hours. A shorter reaction time is thus required for practical production.

On the other hand, high-temperature water is recognized as a green chemical medium for saccharide conversion, promoting such reactions without any catalyst.[12] We have reported that non-catalytic dehydration of GlcNAc in water at 120–220ºC and 25 MPa with a reaction time of 7–39 sec affords 2-acetamido-2,3-dideoxy-D-erythro-hex-2-enofuranose (Chromogen I) and 3-acetamido-5-(1',2'-dihydroxyethyl)furan (Chromogen III).[13] We found that the presence of the *N*-acetyl group in GlcNAc leads to a different dehydration mechanism from that of glucose. There are reports on the reaction of oligosaccharides containing only hydroxy groups in high-temperature water.[14-19] Bobleter and Bonn

hydrolyzed cellobiose, in water at 180-249ºC for 1-14 min by using an autoclave reactor to give glucose in 60% yield.[11] Both the hydrolysis and the retro-aldol condensation of cellobiose have been reported in water at temperatures of 100-400ºC.[15-19] To date, however, there have been no studies on the reaction of amino oligosaccharides containing an *N*-acetyl group at temperatures above 100ºC. The probable reason is that the products from amino oligosaccharides (for example, the products of this study: GND, GNL, Chromogen I, and III) are generally not commercially available. As a result, researchers would have to synthesize these compounds in an initial step and confirm their chemical structures by NMR and electrospray ionization mass spectrometry (ESI-MS). Although the abundance of amino oligosaccharides from chitin is similar to oligosaccharides from cellulose, there is little knowledge of the reactions of amino oligosaccharides in high-temperature water. If environmentally-friendly and effective production methods for the synthesis of amino oligosaccharide derivatives are developed, the research of new biological functions of amino oligosaccharide derivatives is also likely to blossom.

From the previous studies of cellobiose, it is expected that the hydrolysis of $(GlcNAc)_2$ to GlcNAc would be a major reaction pathway. However, the market price of $(GlcNAc)_2$ is approximately 2,000 US$/g, which is significantly higher than the 0.2 US$/g price of GlcNAc. Therefore, the hydrolysis of $(GlcNAc)_2$ is not desired in view of the market price and the preservation of the disaccharide structure is important. The aim of the study was

84 to produce GND from $(GlcNAc)_2$ in water at temperatures ranging from 120 to 220ºC In
85 addition, we developed a kinetic model for estimating the optimum reaction conditions for
86 the formation of GND from $(GlcNAc)_2$.
87
88 **Experimental**
89 $(GlcNAc)_2$ was kindly provided by Yaizu Suisan Kagaku Industry Co. (Shizuoka,
90 Japan). Chemicals were used without further purification. Distilled water was obtained from a
91 water distillation apparatus (Yamato Co., model WG-220). GND, GNL, Chromogen I, and
92 Chromogen III were synthesized by methods reported previously[8, 20] and used as standard
93 samples for HPLC analysis.
94 The experimental flow apparatus was reported previously.[13] The concentration of
95 $(GlcNAc)_2$ aqueous solution was 1.0 wt%. The reaction temperatures were varied from 120 to
96 220ºC. Residence time in the reactor was from 8 to 39 sec.
97 HPLC analysis was carried out using an Anidius column (4.6 × 250 mm, Develosil,
98 Japan) with a Shimadzu Intelligent liquid chromatography system and detection at 210 nm.
99 The bound material was eluted with 75% CH_3CN at a flow rate of 1.0 mL min^{-1} at 40ºC. The
100 ESI-MS spectra were measured on a JMS-T100LC mass spectrometer. ^1H and ^{13}C NMR
101 spectra were recorded on a JEOL JNM-LA 500 spectrometer at 25ºC. Chemical shifts are
102 expressed in δ relative to the external standard, sodium 3-(trimethylsilyl) propionate. A

representative HPLC chromatograph and results of ESI-MS and NMR are shown in supplementary information.

The product yields, Y_{i2}, of disaccharides such as $(GlcNAc)_2$, GND and GNL were defined as follows:

$$Y_{i2}\ (\%) = C_{i2} / C_{o,(GlcNAc)2} \times 100. \quad (1)$$

The product yields, Y_{i1}, of monosaccharides such as GlcNAc, Chromogen I and III were defined as follows:

$$Y_{i1}\ (\%) = C_{i1} / (2 \times C_{o,(GlcNAc)2}) \times 100. \quad (2)$$

where $C_{o,(GlcNAc)2}$ is the concentration at the reactor inlet [$mol \cdot L^{-1}$] and C_{i2} and C_{i1} are the concentrations of product i at the reactor outlet [$mol \cdot L^{-1}$]. A number of experiments were repeated three times to confirm reproducibility.

Results and discussion

Scheme 1 shows the products obtained from the reaction of $(GlcNAc)_2$. The products were GND, GNL, GlcNAc, Chromogen I, and Chromogen III. The formation of 2-acetamido-3,6-anhydro-2-deoxy-D-glucofuranose and 2-acetamido-3,6-anhydro-2-deoxy-D-mannofuranose, which we reported in the previous work,[10] was observed and their yields were less than 1.0%; therefore, we did not show these yields in Figures 1 and 2. The compounds were separated and identified by comparison of

122 their ^{1}H and ^{13}C NMR and ESI-MS data, and HPLC retention times with standard samples.[8, 13, 20]

124 Fig. 1 shows the temperature dependence of the product yields from $(GlcNAc)_2$ at
125 reaction times from 32 sec (at 200ºC) to 39 sec (at 120ºC). The results indicate that
126 $(GlcNAc)_2$ is stable at temperatures up to 130ºC for up to 40 sec. $(GlcNAc)_2$ was gradually
127 consumed at 140ºC and above, yielding GND as the major product, which reached a
128 maximum at 180ºC. Above 180ºC, the yield of GND decreased as this compound was
129 oxidized to GNL. $(GlcNAc)_2$, GND and GNL were also hydrolyzed to GlcNAc, which was
130 subsequently dehydrated resulting in Chromogen I, and III. The yield of GlcNAc and
131 Chromogen I increased to a maximum at 190ºC, and then decreased as these compounds were
132 further dehydrated to give Chromogen III. The yields of Chromogen III increased to a
133 maximum at 200ºC. At temperatures above 150ºC, the total yields of identified compounds
134 were below 80%, indicating that other decomposition products were formed, (not shown in
135 Scheme 1). We observed some minor peaks on the HPLC chromatograms of samples heated
136 to temperatures above 150ºC; but, these products were not identified.

137 Fig. 2 shows the product yields from $(GlcNAc)_2$ at temperatures of between
138 180–220ºC as a function of the reaction time. For all reaction temperatures, the yield of
139 $(GlcNAc)_2$ decreased with increasing reaction time. For reaction temperatures 180-210ºC the
140 yield of GND increased with time until reaching a maximum. Thereafter it decreased as it

was hydrolyzed to GlcNAc, Chromogen I and III. The maximum yield of GND at 180ºC, 190ºC, 200ºC, and 210ºC was 23.6%, 22.7%, 21.4%, and 24.7%, respectively. Small amounts of GNL were formed; its highest yield was 2.6% at 200ºC and 28 sec. At all reaction temperatures, the yield of GlcNAc initially increased with reaction time and then decreased as it was dehydrated to Chromogen I and III. The maximum yield of GlcNAc at 190ºC and 200ºC was 7.5% and 8.3%, respectively. The yields of Chromogen I and III increased with the decrease in yield of GlcNAc. The highest yield of Chromogen I and III was 5.4% (at 190ºC and 33 sec) and 8.0% (at 200ºC and 35 sec), respectively. The total yield of identified compounds was low for reaction times of around 40 sec evidenced by the peak areas of unidentified products on HPLC increasing with reaction time.

The reaction pathway determined on the basis of the product distribution is shown in Scheme 1. When $(GlcNAc)_2$ dissolves in water, it exists as a pyranose ring and an open chain at the reducing end. The dehydration proceeds between H-2 and OH-3 of the open chain, as the electron-withdrawing *N*-acetyl group facilitates the elimination of H-2. The dehydrated open chain forms GND through a ring closure reaction. GND is readily oxidized to afford GNL or hydrolyzed to give GlcNAc and Chromogen I. GNL can be hydrolyzed to GlcNAc and Chromogen I dehydrated to Chromogen III while both may also react further to yield decomposition products. The dehydration and hydrolysis reactions generally require acid catalysis, but proceed under non-catalytic conditions in high-temperature water.[12, 13, 21, 22] The

reason is the higher ion product constant of water ($K_w = [H^+][OH^-]$) at 180–220ºC than that at ambient temperature. The K_w increases with temperature up to around 300ºC; for example, at 25 MPa, the K_w at 180 and 220ºC is 5.2×10^{-12} and 8.4×10^{-12} $mol^2 \cdot kg^{-2}$, respectively.[12] The dissociation of water molecules into H^+ and OH^- ions is an endothermic process; therefore the equilibrium constant for this process increases with temperature. The combined effects of high H^+ or OH^- concentrations and high temperature are probably responsible for the dehydration observed in the absence of added acid or base.

The reactions of $(GlcNAc)_2$ are different from those of cellobiose in high-temperature water. For cellobiose, both the hydrolysis to form glucose[14, 15, 18, 19] and the glucose dehydration reaction[12] occur at around 200ºC in high-temperature water. For $(GlcNAc)_2$, first dehydration occurs between H-2 and OH-3 of the reducing end GlcNAc, and the ring closure reaction then proceeds between C-1 and C-4. This difference arises because the *N*-acetyl group at C-2 in the reducing end GlcNAc facilitates the elimination of H-2 as mentioned above. These results indicate that the position and type of substituent group in the oligosaccharide affects their reactions. Additional studies on this topic will be required to synthesize amino oligosaccharide derivatives effectively.

In comparison with the GlcNAc and chitin studies with catalysts condition, the non-catalytic dehydration proceeded mildly in high-temperature water. In the presence of boric and alkaline chroride in *N*-methyl-2-pyrrolidone solvent, the dehydration of GlcNAc

179 unit in chitin mainly converted to 3-acetamido-5-acetylfulan,[10] which is formed from the
180 additional dehydration of Chromogen III between H-5 and OH-6. However, from the
181 previous studies,[22] the dehydration between H-5 and OH-6 would not proceed at from 180 to
182 220ºC within 1 min without any catalyst. Therefore, the formation of
183 3-acetamido-5-acetylfulan was not observed in this study. As mentioned above,
184 high-temperature water provides slightly higher H^+ and OH^- concentrations condition and the
185 stepwise dehydration of GlcNAc structure could be achieved.

186 The maximum yield of GND was 24.7%, which was higher than that previously
187 reported using sodium borate catalysts.[8] In addition, this new method using high-temperature
188 water does not require elimination of the sodium borate catalyst before the products can be
189 used as functional food additives or medicines. The reaction time of this method was less
190 than 1 min, which is significantly shorter than the timescale of a few hours reported for
191 sodium borate catalysis. This means a continuous and compact process for the synthesis of
192 amino oligosaccharide derivatives is possible. Therefore, the discovery of non-catalytic
193 dehydration of $(GlcNAc)_2$ using only water is highly significant for practical applications of
194 amino oligosaccharide derivatives. As mentioned in the introduction, the market price of
195 $(GlcNAc)_2$ is about 10,000 times higher than that of GlcNAc. Therefore, the efficient
196 conversion of $(GlcNAc)_2$ to GND, retaining the disaccharide structure, is essential; to produce
197 Chromogen I and III, the starting material should be GlcNAc.

198 In order to determine the optimum reaction conditions for producing GND, we
199 developed a kinetic model as shown in Scheme 2. To simplify the kinetic model, we grouped
200 the concentrations of GND and GNL as [GNDL] and those of GlcNAc, Chromogen I, and
201 Chromogen III as [G1]. [G2] and [D] refer to the concentrations of $(GlcNAc)_2$ and the
202 decomposition products, respectively. The total product yields were less than 100% after
203 extended reaction times and we assumed a decomposition pathway from [G2] and [G1] to
204 decomposition products [D]. From the experimental results of Fig. 1 and 2, we assumed the
205 consecutive reactions of [G2], [GNDL], and [G1] and neglected the reverse reactions. In this
206 analysis we assumed that each reaction was first order with respect to the substrate.

207 We determined the reaction rate constants (k_1 – k_4) by considering the reaction
208 pathways shown in Scheme 2. The rates were as follows:

209 $$d\,[G2]/dt = -k_1[G2] - 2k_4[G2] \quad (3)$$

210 $$d\,[GNDL]/dt = k_1[G2] - 2k_2[GNDL] \quad (4)$$

211 $$d\,[G1]/dt = 2k_2[GNDL] - k_3[G1] \quad (5)$$

212 $$d\,[D]/dt = k_3[G1] + 2k_4[G2] \quad (6)$$

213 where the units of k_1 – k_4 are s^{-1}. The concentration of the decomposition products [D] was
214 calculated by Eq. (8) assuming a closed system with respect to materials.

215 $$[D] = 2[G2]_0 - 2[G2] - 2[GNDL] - [G1] \quad (7)$$

216 We fitted the models to the experimental data obtained at 180–220ºC including

reaction time. The preexponential factors ($A_1 \sim A_4$) and the activation energies ($E_{a1} \sim E_{a4}$) in Eq. (8) were obtained by an optimal fit of the predicted product concentrations to the experimental data using least-square analysis.

$$k_n = A_n \exp(-E_{an}/RT) \qquad (n = 1\sim4) \qquad (8)$$

where A_n is the preexponential factor, E_{an} is the activation energy, and R is 8.314 [$J \cdot K^{-1} \cdot mol^{-1}$]. The Simplex routine was used to minimize the absolute errors in concentrations. The fitted preexponential factors and activation energies are summarized as follows:

$$k_1 = 10^{6.79} \exp(-7.46\times10^4/RT) \qquad (9)$$

$$k_2 = 10^{1.32} \exp(-2.62\times10^4/RT) \qquad (10)$$

$$k_3 = 10^{7.25} \exp(-8.52\times10^4/RT) \qquad (11)$$

$$k_4 = 10^{7.53} \exp(-7.81\times10^4/RT) \qquad (12)$$

Analysis of the residuals between the kinetic model and the experimental data gave standard deviations of 3.8%, 4.1%, 3.0%, and 7.7% for the yields of [G2], [GNDL], [G1], and [D], respectively. On the whole, the calculated results of eq. (9)–(12), given by the solid lines in Figure 3, show a good fit between the model and the experimental results.

From the kinetic model, the optimum conditions for [GNDL], yielding 25%, is 330ºC and 0.5 sec within the calculation temperature of 120–400ºC. The activation energy E_{a1} (74.6 $kJ \cdot mol^{-1}$) is higher than E_{a2} (26.2 $kJ \cdot mol^{-1}$), indicating higher reaction temperatures and shorter reaction times favor the formation of GND. Currently, we cannot

conduct an experiment at 330ºC for 0.5 sec using our flow-type apparatus, but this will be the subject of future work. We obtained the highest GND yield of 26.3%, but, the activation energy E_{a1} is almost the same as E_{a4} (78.1 kJ·mol^{-1}), indicating that the selective formation of GND from $(GlcNAc)_2$ is difficult under non-catalytic conditions. Nevertheless, the processes of neutralizing and eliminating the sodium borate catalyst are not needed in the high-temperature water method and thus it is a greener process. The activation energies E_{a1} and E_{a4} of $(GlcNAc)_2$ hydrolysis and decomposition are lower than those of the hydrolysis and the retro-aldol condensation of cellobiose (105 and 123 kJ·mol^{-1}) in high-temperature water.[16] This result indicates that the *N*-acetyl group promotes reaction of the original carbohydrate structure. Indeed, the reactions of $(GlcNAc)_2$ occur in lower-temperature water as compared with cellobiose. As mentioned before, the *N*-acetyl group is an electron-withdrawing group and so the elimination of H-2 occurs more easily as compared with the OH group in cellobiose.

Conclusions

This study has demonstrated, for the first time, the reaction of $(GlcNAc)_2$ in high-temperature water above 120ºC. Non-catalytic dehydration of $(GlcNAc)_2$ affords GND within 39 sec, which is a significantly shorter reaction time than the few hours reported in previous studies using sodium borate catalysts below 100ºC. The highest yield of GND obtained was 24.7%,

255 which is higher than that reported in earlier studies using sodium borate catalysts.
256 Furthermore, this new method, using only water, does not necessitate the elimination of
257 catalysts. The current study has shown that non-catalytic $(GlcNAc)_2$ conversion in
258 high-temperature water is an environmentally-benign method to utilize amino
259 oligosaccharide contained in chitin biomass resources. The reaction model deduced for the
260 successive reaction of $(GlcNAc)_2$ was shown to be suitable for predicting the product yields
261 in high-temperature water. The activation energy of $(GlcNAc)_2$ decomposition was lower
262 than that of cellobiose, and the presence of the *N*-acetyl group is crucial for the dehydration.
263 In order to synthesize amino oligosaccharide derivatives effectively, the effect of both the
264 position and type of substituent group in the amino oligosaccharide on the reaction should be
265 considered.

266

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310 **Figure legends**

311

312 Fig. 1 (a) Effect of temperature on the reaction of $(GlcNAc)_2$ in high-temperature water at

313 25 MPa and residence times from 32 to 39 sec. (b) Magnification of part of (a).

314

315 Fig. 2 Product yields of $(GlcNAc)_2$ reaction in high-temperature water at 25 MPa as a

316 function of reaction time. Error bars are based on one standard deviation.

317

318 Fig. 3 Comparison of calculated and experimental product yields of $(GlcNAc)_2$ reaction in

319 high-temperature water.

320

321 Scheme 1 Reaction pathway of $(GlcNAc)_2$ in high-temperature water.

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323 Scheme 2 Reaction pathway of $(GlcNAc)_2$ for kinetic calculations in high-temperature

324 water.

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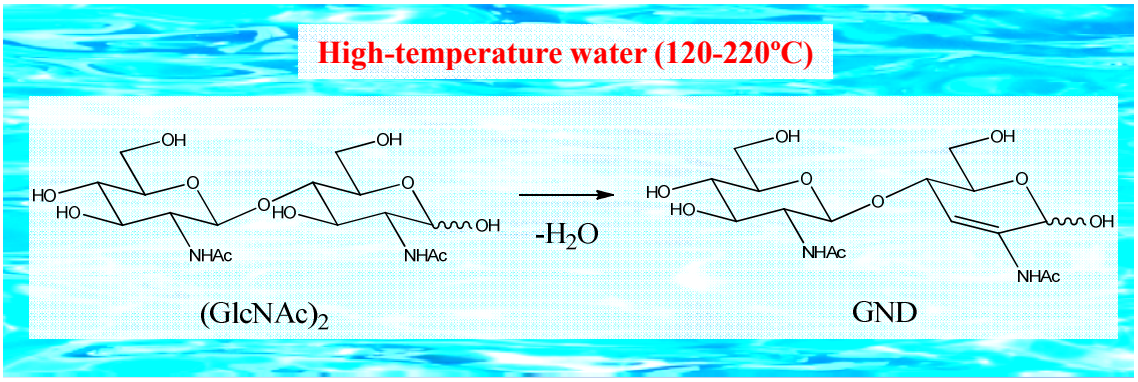
Graphical abstract



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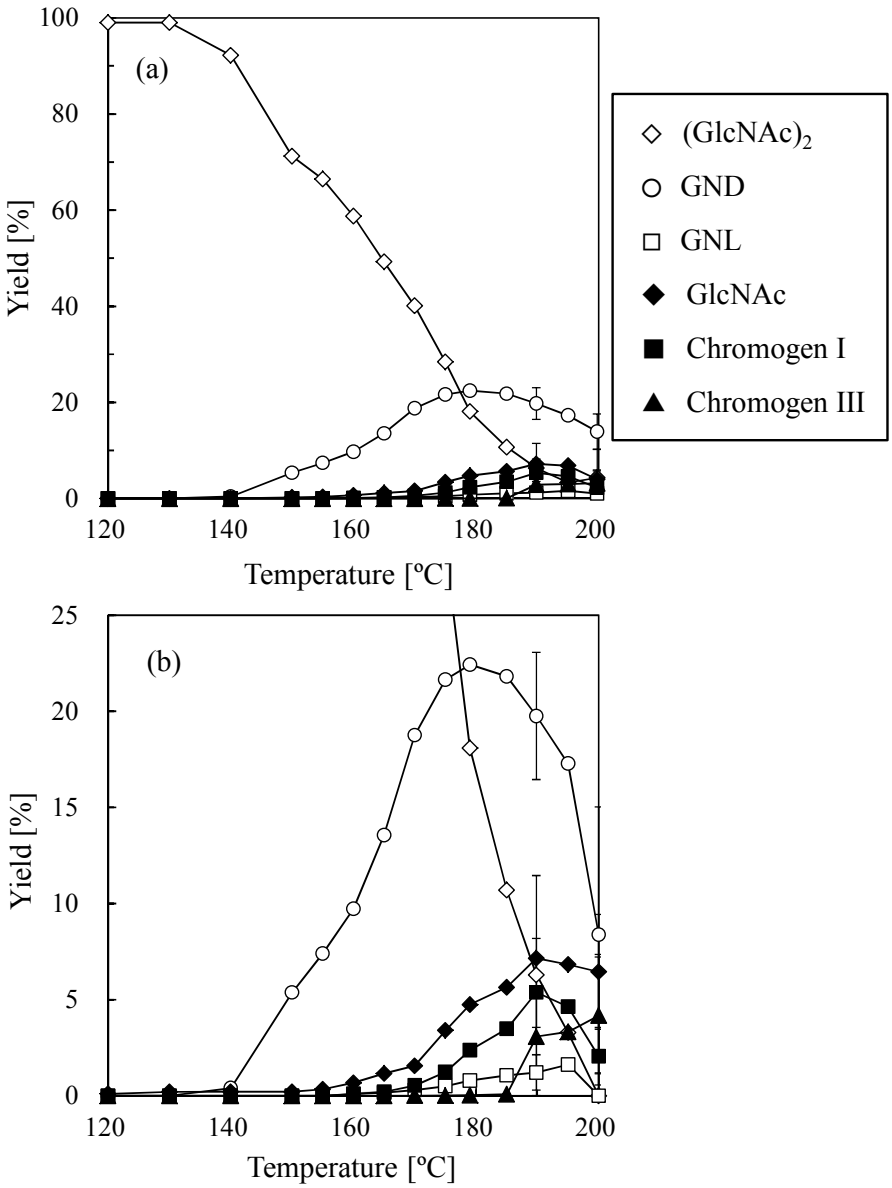

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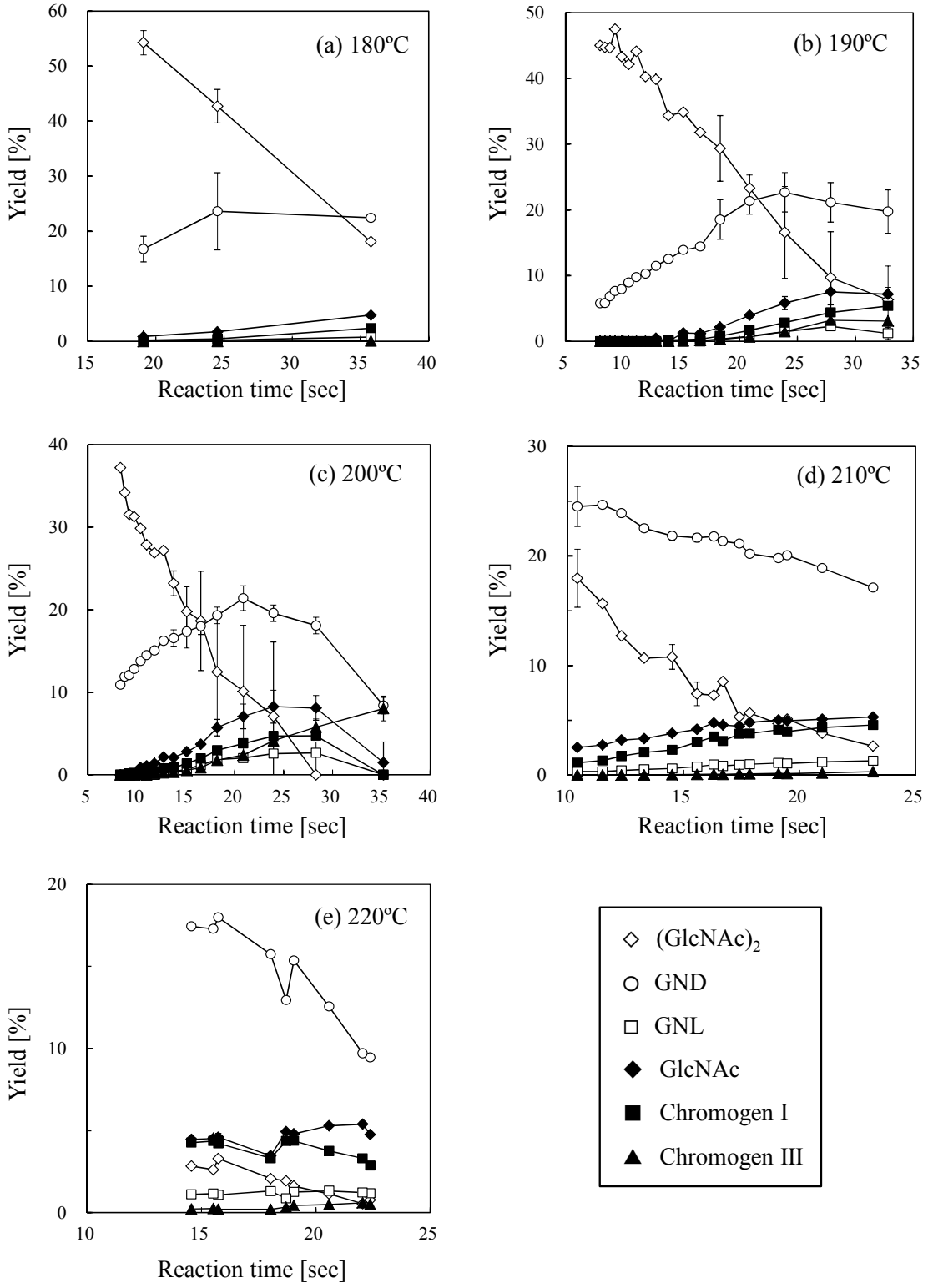

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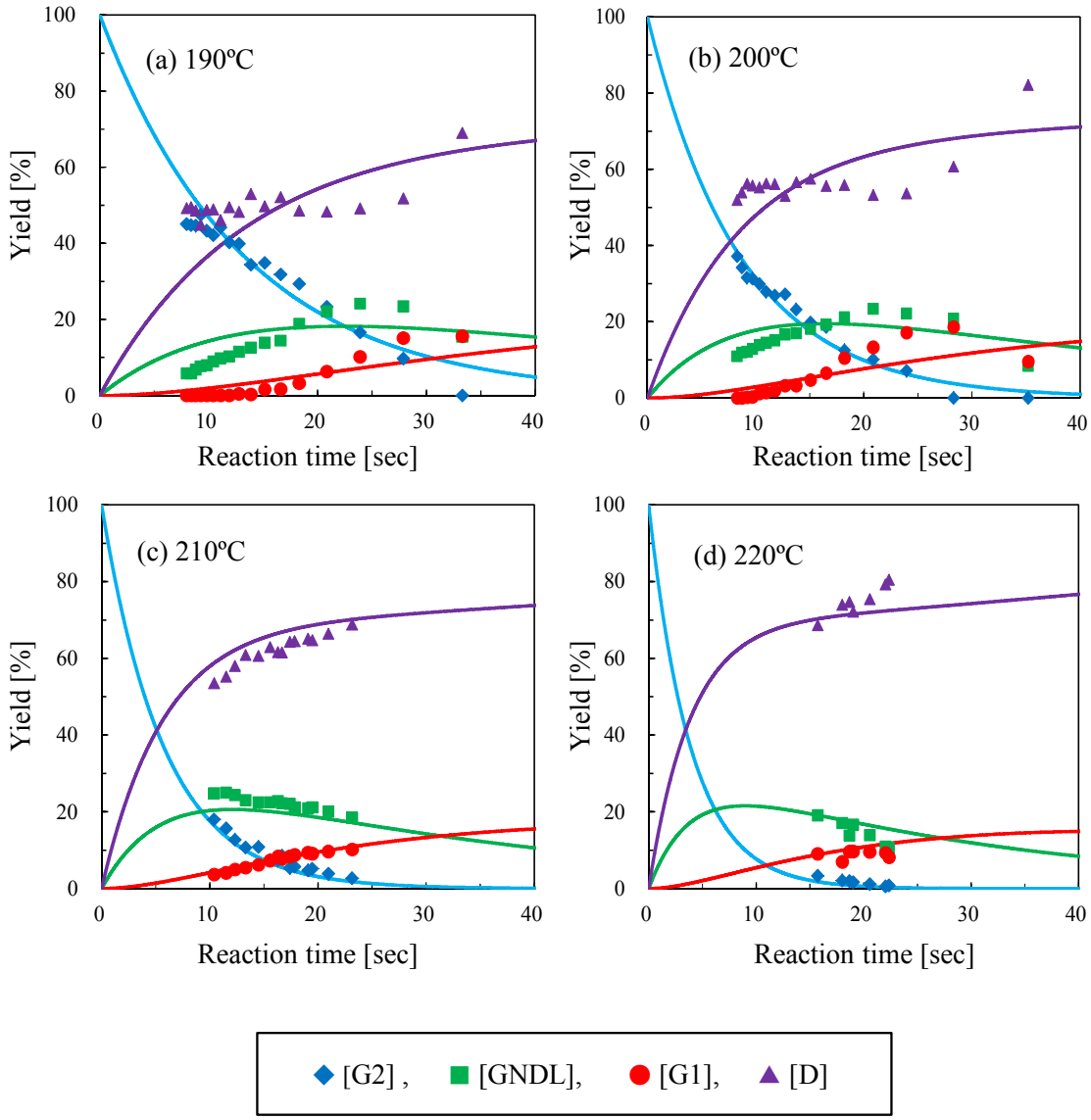
354 Fig. 2 Osada M. et al.



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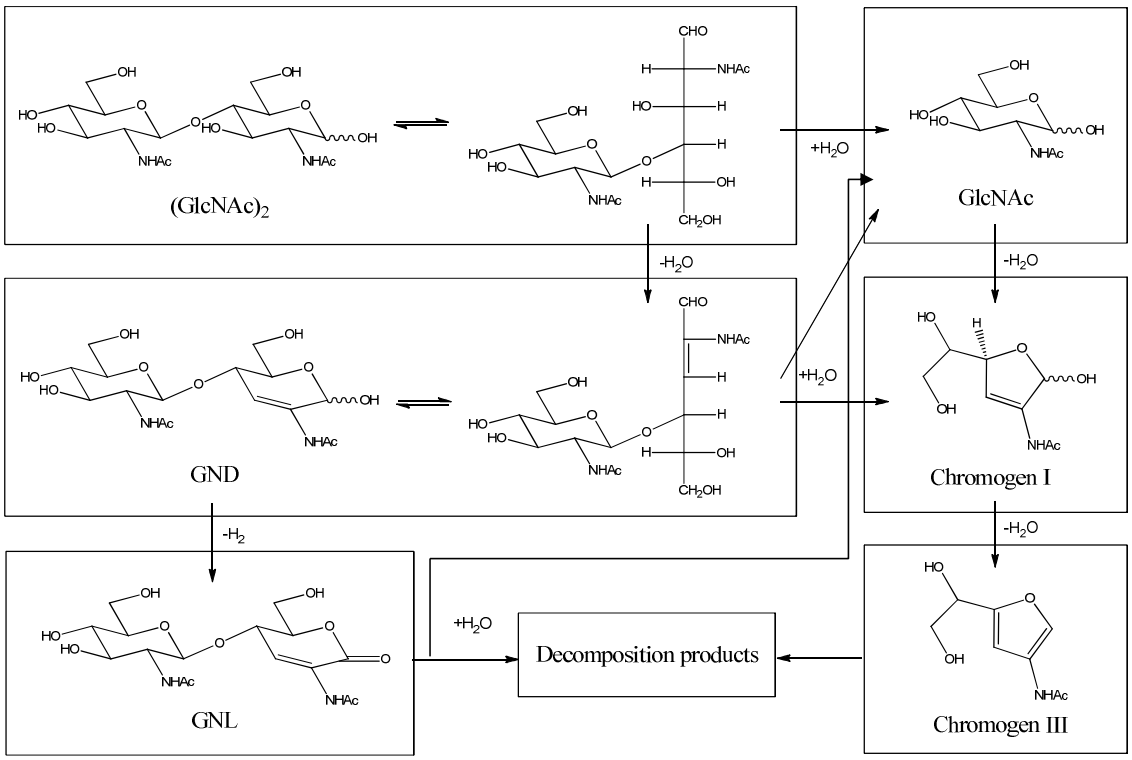
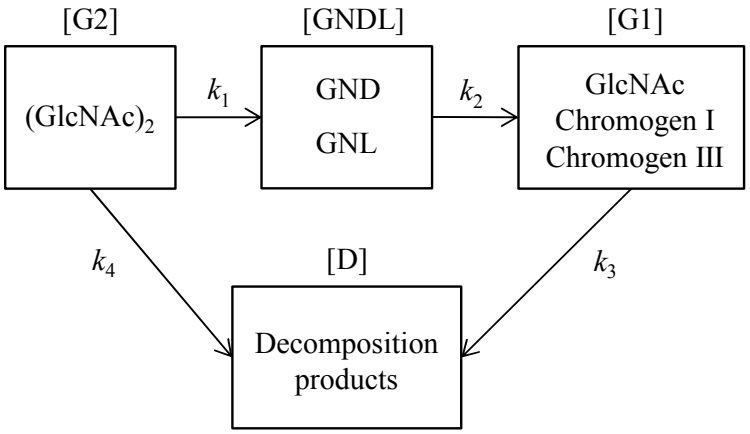


Scheme 1 Osada M. et al.



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388 Scheme 2 Osada M. et al.