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Epimerization and Decomposition of Kojibiose and Sophorose by Heat Treatment under Neutral pH Conditions

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Abstract: We evaluated the stabilities of kojibiose and sophorose when heated under neutral pH conditions. Kojibiose and sophorose epimerized at the C-2 position of glucose on the reducing end, resulting in the production of 2-*O*-α-D-glucopyranosyl-D-mannose and 2-*O*-β-D-glucopyranosyl-D-mannose, respectively. Under weak alkaline conditions, kojibiose was decomposed due to heating into its mono-dehydrated derivatives, including 3-deoxy-2,3-unsaturated compounds and bicyclic 3,6-anhydro compounds. Following these experiments, we propose a kinetic model for the epimerization and decomposition of kojibiose and sophorose by heat treatment under neutral pH and alkaline conditions. The proposed model shows a good fit with the experimental data collected in this study. The rate constants of a reversible epimerization of kojibiose at pH 7.5 and 90 °C were $(1.6 \pm 0.1) \times 10^{-5}$ s^{-1} and $(3.2 \pm 0.2) \times 10^{-5}$ s^{-1} for the forward and reverse reactions, respectively, and were almost identical to those [$(1.5 \pm 0.1) \times 10^{-5}$ s^{-1} and $(3.5 \pm 0.4) \times 10^{-5}$ s^{-1}] of sophorose. The rate constant of the decomposition reaction for kojibiose was $(4.7 \pm 1.1) \times 10^{-7}$ s^{-1} whereas that for sophorose [$(3.7 \pm 0.2) \times 10^{-6}$ s^{-1}] was about ten times higher. The epimerization reaction was not significantly affected by the variation in the buffer except for a borate buffer, and depended instead upon the pH value (concentration of hydroxide ions), indicating that epimerization occurred as a function of the hydroxide ion. These instabilities are an extension of the neutral pH conditions for keto-enol tautomerization that are often observed under strong alkaline conditions.

Key words: epimerization, β-elimination, glucopyranosyl-D-mannose, keto-enol tautomerization, kojibiose, sophorose

INTRODUCTION

Oligosaccharides have the potential to improve the quality of various kinds of food, either as functional food ingredients or as additives.[1)2)3)4)] Some of them provide useful modifications to food flavor and physicochemical characteristics including textural and organoleptic properties and further afford beneficial properties for human health, stimulating the growth of beneficial bacteria in the colon (prebiotic effects)[2)5)] and boosting the human immune system.[6)7)] Therefore, efforts are being focused on developing economical technologies for the production and application of oligosaccharides with beneficial properties for foods.

Together with the recent popularization of functional foods, the opportunities for use of food materials containing functional oligosaccharides, and the addition of oligosaccharides to foods before their heat processes will inevitably increase.[4)5)] Although a heating process can decrease microbiological risk and improve both shelf life and digestibility of food, it can also cause decomposition of the oligosaccharides with non-enzymatic "browning" by caramelization or the Maillard reaction. The reactions include enolization, water elimination, fragmentation, redox, and polymerization, bringing about the formation of a wide range of compounds that affect the flavor and color of foods and through which the beneficial biological function of the oligosaccharide may be lost during the decomposition of oligosaccharide, and an undesired color may occur in the

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Abbreviations: CHES, *N*-Cyclohexyl-2-aminoethanesulfonic acid; DQF-COSY, double-quantum filtered correlation spectroscopy; *Ea*, energy of activation; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSQC, heteronuclear single-quantum coherence; HMBC, heteronuclear multiple-bond correlation; JRES, *J*-resolved; LC-ESIMS, liquid chromatography-electrospray ionization mass spectrometry; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; and Tris, tris(hydroxymethyl)aminomethane.

foods. Therefore, understanding and controlling the non-enzymatic browning of oligosaccharides in foods is an important issue in food processing.

The aim of this research is to elucidate the heat decomposition and transformation reactions through their kinetic parameters, and understand the temperature and pH dependency of these rates. Oligosaccharides offer a wide diversity in sugar compositions and the types of *O*-linkage. We first investigated the heat stability of disaccharides comprising carbohydrates commonly found in foods at temperatures below 100 °C under neutral pH conditions. Our previous work characterized the heat decomposition reaction of 3-*O*-β-D-galactopyranosyl-D-2-acetamido-2-deoxy-D-glucose (lacto-*N*-biose I) under neutral pH conditions.[8] Lacto-*N*-biose I is considered to be a growth-promoting factor of infant-specific bifidobacteria in human milk[9][10] and has the potential to be used as a food or a food additive. Lacto-*N*-biose I, however, decomposed rapidly with water elimination during heating under neutral pH conditions, in which glycosyl linkages are generally believed to be stable.[8] Lacto-*N*-biose I was mostly stable at pH levels below 5.5 at 90 °C for 1 h (remaining amount >95 %) and its decomposition was undetectable at temperatures below 55 °C after introduction in a 100 mM sodium phosphate buffer (pH 7.5) for 1 h.[8] Some oligosaccharides containing structures of 3-*O*-substituted reducing 2-acetamido-2-deoxy-hexoses or aldoses showed further decomposition, resulting in the formation of unsaturated sugars.[8][11][12][13][14] These reactions proceed until an alkaline-stable glycosidic linkage appears at the reducing end, and they are thus, sometimes called "peeling" reactions. The thermal instability of lacto-*N*-biose I under neutral pH conditions is an extension of the alkaline instability of oligosaccharides with a 3-*O*-substituted reducing aldose including nigerose (3-*O*-α-D-glucopyranosyl-D-glucose) and laminaribiose (3-*O*-β-D-glucopyranosyl-D-glucose).[8] In addition, 1,5-anhydro-4-*O*-β-D-glucopyranosyl-D-fructose that was synthesized using cellobiose phosphorylase[15] or 3-keto-levoglucosan that was synthesized using pyranose oxidase[16] were also unstable in aqueous solution, with half lives of 16 or 17 h, respectively, at pH 7.0 and 30 °C.

In this study, we investigate the thermal stabilities of two disaccharides comprising two glucose units with 1,2-glycosidic linkage: kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose) and sophorose (2-*O*-β-D-glucopyranosyl-D-glucose). Kojibiose is found in *sake,*[17] its moromi fermentation mash,[17] and honey.[18] Kojibiose and its derived oligosaccharides are largely resistant to the activity of enzymes in the human digestive tract, but can be cleaved by lactobacilli[19][20][21] and have been reported to stimulate the growth of bifidobacteria.[22] Sophorose was reported as a structural unit of sophorolipid, a surface-active glycolipid synthesized by certain species of nonpathogenic yeasts.[23] It is also a building unit of cyclic β-1,2-glucan, which is an extracellular polysaccharide produced by species of *Agrobacterium* and *Rhizobium*.[24] Recently, large-scale production of kojibiose and 1,2-β-glucan has been reported, by using sucrose phosphorylase variants[25][26] and by using 1,2-β-oligoglucan phosphorylase,[27] respectively. In addition, the discovery of 1,2-oligomannan phosphorylase and β-1,2-mannobiose phosphorylase[28][29] may allow the enzymatically driven large-scale production of 1,2-oligomannan in the near future. The versatility of the use of oligosaccharides with 1,2-glycosidic bonds is therefore, expected to increase. Hence, we herein determine the kinetic parameters and temperature or pH dependence for their thermal transformation reactions of kojibiose and sophorose.

RESULTS

Heat treatment of kojibiose and sophorose under neutral pH conditions.

Various reducing sugars (50 mM) were heated in a 100 mM sodium phosphate buffer (pH 7.5) at 90 °C. After heating, the changes in the color and pH of the solutions were measured. Only kojibiose (substrate **1**) and sophorose (substrate **2**) did not undergo the changes of browning indices and pH values after 12 h incubation (Table 1); however, HPLC analysis of the reaction solutions showed that the amount of kojibiose or sophorose had gradually reduced (Fig. 1).

In kojibiose, a new major peak was observed (retention time 9.5 min in Fig. 1) in addition to the peak corresponding to kojibiose (retention time 10.6 min in Fig. 1) on the HPLC chromatogram. The major peak (retention time 9.5

Table 1. Browning indices and pH values when reducing sugars (50 mM) were incubated in 100 mM sodium phosphate (initial pH value 7.5).

	Browning index (A_{420})		pH value	
	Incubation time		Incubation time	
	3 h	12 h	3 h	12 h
Kojibiose	0.000	0.000	7.5	7.5
Nigerose	0.116	0.216	6.8	6.4
Maltose	0.039	0.098	7.2	6.7
Isomaltose	0.028	0.074	7.2	6.8
Sophorose	0.000	0.005	7.5	7.3
Glucose	0.016	0.046	7.2	6.8
Fructose	0.037	0.065	7.1	6.7
Blank (Sodium phosphate)	0.000	0.000	7.5	7.5

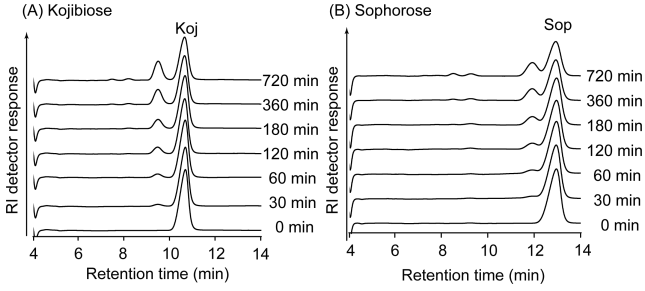


Fig. 1. Transformation with time of kojibiose (A) and sophorose (B) under heat treatment (90 °C) at neutral pH conditions (pH 7.5).

The sugars were determined by HPLC systems equipped with a refractive index detector using a Shodex HILICpak VG-50 column at 40 °C with a mobile phase comprising 75 % acetonitrile (for kojibiose) and 80 % acetonitrile (for sophorose) in water.

Table 2. Chemical shifts in ^{1}H and ^{13}C NMR spectrum of the reaction product 3 obtained from the heat treatment of kojibiose under neutral pH condition.

Sugar ring	Position	α-Pyranose (93 %)				β-Pyranose (7 %)			
		^{13}C	^{1}H			^{13}C	^{1}H		
		δ (ppm)	δ (ppm)	Shape	J (Hz)	δ (ppm)	δ (ppm)	Shape	J (Hz)
I (Man)	1	95.3	5.45	d	2.3	96.2	4.93	d	1.3
	2	**83.2**	***3.96***	dd	2.3, 3.1	**82.8**	***4.07***	dd	1.3, 3.1
	3	73.1	3.96	dd	3.1, 8.7	76.5	3.67	dd	3.1, 10.0
	4	70.0	3.78	dd	8.7, 9.9	71.4	3.50	dd	9.0, 10.0
	5	75.3	3.82	ddd	2.0, 5.6, 9.9	79.4	3.42	ddd	2.3, 5.6, 9.0
	6	63.8	3.88	dd	2.0, 11.9	63.8	3.93	dd	2.3, 12.5
	6′		3.78	dd	5.6, 11.9		3.76	dd	5.6, 12.5
II (αGlc)	1	***103.7***	**5.13**	d	3.8	**103.2**	***5.20***	d	3.9
	2	74.7	3.56	dd	3.8, 10.0	74.8	3.61	dd	3.9, 9.8
	3	75.6	3.76	dd	8.8, 10.0	75.7	3.82	dd	9.1, 9.8
	4	72.5	3.41	dd	8.8, 9.9	72.2	3.45	dd	9.1, 10.3
	5	75.1	3.85	ddd	2.2, 5.9, 9.9	75.0	4.07	ddd	2.4, 4.7, 10.3
	6	63.5	3.85	dd	2.2, 12.9	63.2	3.83	dd	2.4, 13.1
	6′		3.75	dd	5.9, 12.9		3.79	dd	4.7, 13.1

The terms I and II indicate the first (D-mannose) and second (α-D-glucopyranose) residues from the reducing end, respectively. Bolds and bold italics represent HMBC correlations between the anomeric protons and carbons of α-D-glucopyranose and the carbons and protons of D-mannose, respectively. The $^{1}J_{C1\text{-}H1}$ coupling constant of δ_C 95.5/δ_H 5.45 and δ_C 96.3/δ_H 4.93 were 172 Hz and 161 Hz, respectively. The ^{1}H and ^{13}C NMR spectra were shown in Supplementary material; Figs. S1 and S2.

Table 3. Chemical shifts in ^{1}H and ^{13}C NMR spectrum of the reaction product **4** obtained from the heat treatment of sophorose under neutral pH condition.

Sugar ring	Position	α-Pyranose (81 %)				β-Pyranose (19 %)			
		^{13}C	^{1}H			^{13}C	^{1}H		
		δ (ppm)	δ (ppm)	Shape	J (Hz)	δ (ppm)	δ (ppm)	Shape	J (Hz)
I (Man)	1	95.2	5.30	d	1.6	96.8	4.99	d	1.3
	2	**81.3**	***4.10***	dd	1.6, 3.5	**83.3**	***4.18***	dd	1.3, 3.5
	3	72.4	3.90	dd	3.5, 9.8	75.1	3.69	dd	3.5, 9.7
	4	70.0	3.70	dd	9.6, 9.8	70.0	3.56	dd	9.7, 9.9
	5	75.3	3.84	ddd	1.5, 4.5, 9.6	79.3	3.42	ddd	1.8, 5.6, 9.4
	6	63.4	3.86	dd	1.5, 11.2	63.8	3.92	dd	1.8, 12.5
	6′		3.82	dd	4.5, 11.2		3.75	dd	5.6, 12.5
II (βGlc)	1	***104.8***	**4.52**	d	7.9	***106.9***	**4.63**	d	7.9
	2	75.7	3.35	dd	7.9, 9.5	76.5	3.42	dd	7.9, 9.5
	3	78.4	3.51	dd	8.9, 9.5	78.4	3.53	dd	9.1, 9.5
	4	72.5	3.43	dd	8.9, 9.9	72.5	3.46	dd	9.1, 9.8
	5	78.9	3.45	ddd	2.1, 5.7, 9.9	79.0	3.48	ddd	2.2, 5.8, 9.8
	6	63.6	3.92	dd	2.1, 12.4	63.6	3.91	dd	2.2, 12.4
	6′		3.75	dd	5.7, 12.4		3.74	dd	5.8, 12.4

The terms I and II indicate the first (D-mannose) and second (β-D-glucopyranose) residues from the reducing end, respectively. Letter a represent that the chemical shift was read from HSQC cross peak. Bolds and bold italics represent HMBC correlations between the anomeric proton and carbon of β-D-glucopyranose and the carbon and proton of D-mannose, respectively. The $^{1}J_{C1\text{-}H1}$ coupling constant of δ_C 95.19/δ_H 5.30 and δ_C 96.83/δ_H 4.99 were 171 Hz and 161 Hz, respectively. The ^{1}H and ^{13}C NMR spectra were shown in Supplementary material; Figs. S3 and S4.

min in Fig. 1) was fractionated by column chromatography using a Shodex HILICpak VG-50 column. The liquid chromatography-electrospray ionization mass spectrometry (LC-ESIMS) peak was observed at *m/z* 341.3, which corresponds to the mass value of a deprotonated molecular ion $[M–H]^-$ of disaccharide, indicating that the product is an isomer of kojibiose. Using ^{1}H and ^{13}C NMR spectroscopy, its structure was identified as 2-*O*-α-D-glucopyranosyl-D-mannose (product **3**). The doublet peaks at δ_H 5.36 ($J_{1,2}$ 2.3 Hz) and δ_H 4.84 ($J_{1,2}$ 1.3 Hz) were respectively assigned to the H-1 of the α-anomer and β-anomer (α/β = 19:1) of reducing mannose residue at reducing end. The assignments of the ^{1}H and ^{13}C NMR chemical shifts of the product **3** are summarized in Table 2. In the HPLC analysis, approximately 28 % of kojibiose was epimerized to 2-*O*-α-D-glucopyranosyl-D-mannose after the 12-h incubation in a 100 mM sodium phosphate buffer (pH 7.5) at 90 °C.

Sophorose was also epimerized at the C-2 position of the reducing terminal glucose into 2-*O*-β-D-glucopyranosyl-D-mannose (product **4**), of which the assigned ^{1}H and ^{13}C NMR chemical shifts are summarized in Table 3. In the HPLC analysis, approximately 24 % of sophorose was epimerized to 2-*O*-β-D-glucopyranosyl-D-mannose after the 12-h incubation in a 100 mM sodium phosphate buffer (pH 7.5) at 90 °C.

Table 4. Chemical shifts of product **5** in ^{1}H and ^{13}C NMR spectrum of Fraction 2 obtained from the heat treatment of kojibiose under weak alkaline condition.

Sugar ring	Position	α-Furanose (54 %)				β-Furanose (26 %)				α-Pyranose (15%)				β-Pyranose (5 %)			
		^{13}C	^{1}H			^{13}C	^{1}H			^{13}C	^{1}H			^{13}C	^{1}H		
		δ (ppm)	δ (ppm)	Shape	J (Hz)	δ (ppm)	δ (ppm)	Shape	J (Hz)	δ (ppm)	δ (ppm)	Shape	J (Hz)	δ (ppm)	δ (ppm)	Shape	J (Hz)
I	1	101.3	5.37	dd	0.8, 2.1	102.4	5.45	dd	0.8, 1.9	90.6	5.29	dd	0.4, 1.6	91.9	5.43[a]		
	2	**154.5**	—			**153.9**	—			**152.9**	—			153.2	—		
	3	100.6	5.87	dd	0.8, 3.9	100.5	5.84	dd	0.8, 1.4	107.1	5.35	dd	1.6. 2.2	107.0	5.43[a]		
	4	86.4	5.04	ddd	2.1, 3.6, 3.9	85.9	4.81	dd	1.4, 1.9, 5.2	66.1	4.29	dd	2.2, 9.3	65.9	4.27[a]		
	5	76.2	3.79	ddd	3.6, 4.0, 7.2	76.6	3.71	ddd	3.6, 5.2, 6.6	75.5	3.67[a]			81.3	3.75[a]		
	6	65.0	3.70	dd	4.0, 11.9	65.1	3.74	dd	3.6, 11.8	63.5	3.89	dd	2.3, 12.1	64.1	3.83[a]		
	6′		3.56	dd	7.2, 11.9		3.61	dd	6.7, 11.8		3.75[a]				3.75[a]		
II (αGlc)	1	100.5	**5.48**	d	3.6	101.1	**5.48**	d	3.6	98.3	**5.51**	d	4.2	98.9	5.51[a]		
	2	73.7	3.70	dd	3.6, 9.1	73.9	3.69	dd	3.6, 9.8	75.3	3.67	dd	3.7, 9.8	75.3	3.64[a]		
	3	75.6	3.82	dd	9.1, 10.5	75.7	3.82	dd	9.8, 10.5	75.7	3.81[a]			75.7	nd		
	4	72.0	3.50	dd	9.2, 10.2	72.0	3.50	m		72.1	3.47	dd	9.2, 10.1	72.1	nd		
	5	75.5	3.63	ddd	2.5, 5.0, 9.2	74.5	3.63	m		75.5	3.80[a]			75.5	nd		
	6	63.2	3.84	dd	2.5, 12.5	63.1	3.83	dd	2.6, 12.4	63.3	3.82[a]			63.3	nd		
	6′		3.77	dd	5.0, 12.5		3.77	m			3.77[a]				nd		

The terms I and II indicate the first (3-deoxy-D-erythro-hex-2-enose) and the second (α-D-glucopyranose) residues from the reducing end, respectively. Bolds represent HMBC correlations between the anomeric protons of α-D-glucopyranose and the carbons of 3-deoxy-D-erythro-hex-2-enose, respectively. nd, these values cannot be determined due to overlapping signals. [a], these signals were assigned by HSQC spectrum. The ^{1}H and ^{13}C NMR spectra were shown in Supplementary material; Figs. S5 and S6.

Heat treatment of kojibiose under weak alkaline conditions.

After the incubation of kojibiose at 90 °C for 3 h in a 100 mM sodium phosphate buffer (pH 7.5), two minor peaks (retention time 7.5 and 8.2 min) were detected on the HPLC chromatogram (Fig. 1). These two peaks increased gradually with increasing incubation time. Higher pH resulted in an increase rate of formation in these compounds. After kojibiose (50 mM) was incubated at 90 °C for 3 h in a 100 mM sodium carbonate buffer (pH 10.0), the fractions corresponding to the two peaks were separated by column chromatography using a TOYOPEARL HW40 column and a Wakosil 5SIL column. LC-MS peaks of the fractions were observed at *m/z* 323.1, indicating that the products are mono-dehydrated derivatives of the corresponding disaccharide. The structure of the compounds contained in Fraction 1 and 2 (retention times 7.5 and 8.2 min in Fig. 1) was identified using ^{1}H and ^{13}C NMR spectroscopy.

Fraction 2 (retention time 8.2 min in Fig. 1) mainly contained 2-*O*-α-D-glucopyranosyl-3-deoxy-D-*erythro*-hex-2-enose (product **5**) existing as α-furanose (54 %), β-furanose (26 %), α-pyranose (15 %), and β-pyranose (5 %). Product **5** was cyclized to furanose (80 %) and pyranose (20 %) rings in solution. The anomeric proton (H-1) of product **5** with a 3-deoxy-2,3-unsaturated structure gave a characteristic doublet of doublets (dd; furanose ring of H-1α, $J_{1,3}$ 0.8 Hz, $J_{1,4}$ 2.1 Hz; furanose ring of H-1β; $J_{1,3}$ 0.8 Hz, $J_{1,4}$ 1.9 Hz; and pyranose ring of H-1α, $J_{1,3}$ 0.4 Hz, $J_{1,4}$ 1.6 Hz), consistent with previous reports.[8] The H-3 of product **5** was deduced by determining cross peaks with H-3 and H-4 using nuclear Overhauser effect spectroscopy (NOESY). The heteronuclear multiple-bond correlation (HMBC) spec-

Table 5. Chemical shifts of the product **6** in ^{1}H and ^{13}C NMR spectrum of Fraction 2 obtained from the heat treatment of kojibiose under weak alkaline condition.

Sugar ring	Position	α-Furanose (29 %)				β-Furanose (71 %)			
		^{13}C	^{1}H			^{13}C	^{1}H		
		δ (ppm)	δ (ppm)	Shape	J (Hz)	δ (ppm)	δ (ppm)	Shape	J (Hz)
I	1	98.2	5.49	d	5.2	103.6	5.40	d	5.2
	2	**80.5**	***4.23***	t-like	5.0	**84.0**	***4.14***	t-like	5.0
	3	81.9	4.71	t-like	4.6	82.0	4.75	t-like	4.7
	4	83.8	4.64	t-like	4.6	83.1	4.78	t-like	4.8
	5	74.0	4.44[a]	m		74.2	4.40[a]	m	
	6	74.1	3.99	dd	7.8, 8.6	74.4	4.06	dd	6.5, 9.0
	6′		3.93	dd	6.8, 8.6		3.64[a]	m	
II (αGlc)	1	***102.0***	**5.12**	d	3.6	***100.7***	**5.08**	d	3.7
	2	75.7	3.57[a]			76.2	3.56[a]		
	3	75.2	3.84[a]			74.9	3.76[a]		
	4	72.3	3.44[a]			72.2	3.47[a]		
	5	75.4	nd			75.4	3.67[a]		
	6	65.0	nd			65.0	3.85[a]		
	6′		nd				3.80[a]		

The terms I and II indicate the first (3,6-anhydro-D-mannofuranose) and second (α-D-glucopyranose) residues from the reducing end, respectively. Bolds and bold italics represent HMBC correlations between the anomeric proton and carbon of α-D-glucopyranose and the carbon and proton of 3,6-anhydro-D-mannofurnose, respectively. nd, these values cannot be determined due to overlapping signals. [a], these signals were assigned by HSQC spectrum. The ^{1}H and ^{13}C NMR spectra were shown in Supplementary material; Figs. S5 and S6.

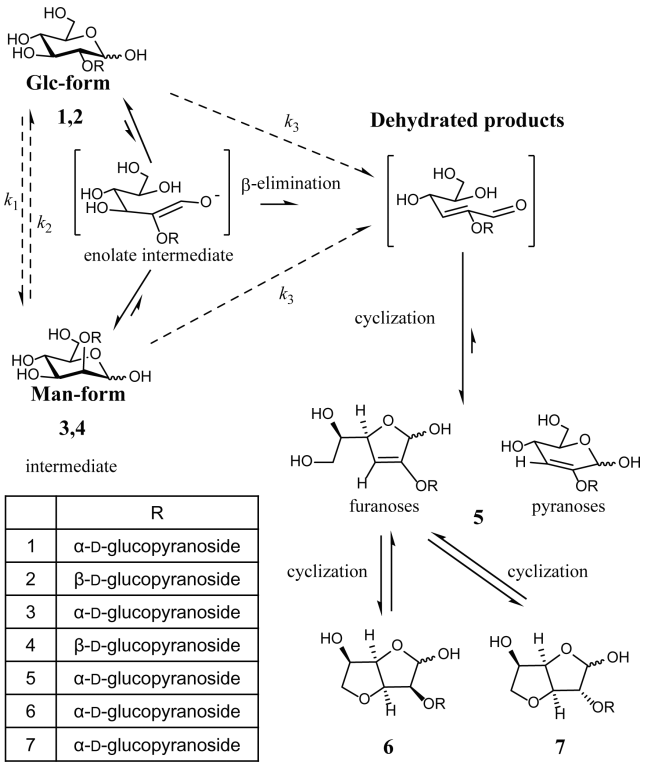
trum exhibited a long-range C–H coupling correlation of C-4–H-1 at δ_C 86.4/δ_H 5.37 (α-anomer) and δ_C 85.9/δ_H 5.45 (β-anomer), corresponding to adapted 2-enofuranose, and a long-range C–H coupling correlation of C-5–H-1 at δ_C 75.5/δ_H 5.29 (α-anomer) and δ_C 81.3/δ_H 5.43 (β-anomer), corresponding to the adapted 2-enopyranose. Fraction 2 (retention time 8.2 min in Fig 1) also contained both the α and β anomers of 2-*O*-α-D-glucopyranosyl-3,6-anhydro-D-mannofuranose (product **6**) as minor products. The bicyclic structure of the reducing end was indicated by the HMBC correlation of C-4–H-1 at δ_C 83.8/δ_H 5.49 (α-anomer) and δ_C 83.1/δ_H 5.40 (β-anomer) and C-6–H-3 at δ_C 74.1/δ_H 4.71 (α-anomer) and δ_C 74.4/δ_H 4.75 (β-anomer). Fraction 2 contained 89 % of product **5** and 11 % of product **6.**

Fraction 1 (retention time 7.5 min in Fig. 1) contained both the α and β anomers of 2-*O*-α-D-glucopyranosyl-3,6-anhydro-D-glucofuranose (product **7**). HMBC correlation of C-4–H-1 at δ_C 81.8/δ_H 5.61 (α-anomer) and δ_C 85.6/δ_H 5.55 (β-anomer) and C-6–H-3 at δ_C 73.1/δ_H 4.75 (α-anomer) and δ_C 73.8/δ_H 4.68 (β-anomer) indicated the bicyclic

Table 6. Chemical shifts of product **7** in ^{1}H and ^{13}C NMR spectrum of Fraction 1 obtained from the heat treatment of kojibiose under weak alkaline condition.

Sugar ring	Position	α-Furanose (54 %)				β-Furanose (46 %)			
		^{13}C	^{1}H			^{13}C	^{1}H		
		δ (ppm)	δ (ppm)	Shape	J (Hz)	δ (ppm)	δ (ppm)	Shape	J (Hz)
I	1	100.6	5.61	d	4.1	104.9	5.55	d	0.9
	2	**85.0**	***4.22***	dd	3.2, 4.1	**87.9**	***4.18***	dd	0.9, 1.5
	3	88.0	4.75	dd	3.2, 5.5	88.2	4.68	dd	1.5, 4.7
	4	81.8	4.77	dd	5.5, 5.9	85.6	4.81	dd	4.7, 5.3
	5	73.2	4.31	dt	5.9, 7.5	73.5	4.40	ddd	5.3, 6.8, 8.7
	6	73.1	3.99	dd	5.9, 9.3	73.8	3.97	dd	6.8, 8.7
	6′		3.60	dd	7.5, 9.3		3.83	t-like	8.7
II (αGlc)	1	***102.0***	**5.06**	d	3.8	***101.1***	**5.11**	d	3.8
	2	74.3	3.57	dd	3.8, 9.9	73.9	3.58	dd	3.8, 9.5
	3	75.6	3.77	dd	9.6, 9.9	75.6	3.68	dd	9.4, 9.5
	4	72.2	3.46	t-like	9.6	72.3	3.43	dd	9.4, 9.5
	5	75.4	3.76	ddd	2.2, 5.1, 9.6	75.4	3.69	ddd	2.3, 7.6, 9.5
	6	63.2	3.86	dd	2.2, 12.2	63.3	3.87	dd	2.3, 12.3
	6′		3.79	dd	5.1, 12.4		3.83	dd	7.6, 12.3

The terms I and II indicate the first (3,6-anhydro-D-glucofuranose) and second (α-D-glucopyranose) residues from the reducing end, respectively. Bolds and bold italics represent HMBC correlations between the anomeric proton and carbon of α-D-glucopyranose and the carbon and proton of 3,6-anhydro-D-glucofuranose, respectively. The ^{1}H and ^{13}C NMR spectra were shown in Supplementary material; Figs. S7 and S8.



	R
1	α-D-glucopyranoside
2	β-D-glucopyranoside
3	α-D-glucopyranoside
4	β-D-glucopyranoside
5	α-D-glucopyranoside
6	α-D-glucopyranoside
7	α-D-glucopyranoside

Fig. 2. Proposed mechanism of epimerization and decomposition of kojibiose and sophorose.
Arrows show in the reaction pathways, while dashed arrows show the rate constant of the reactions.

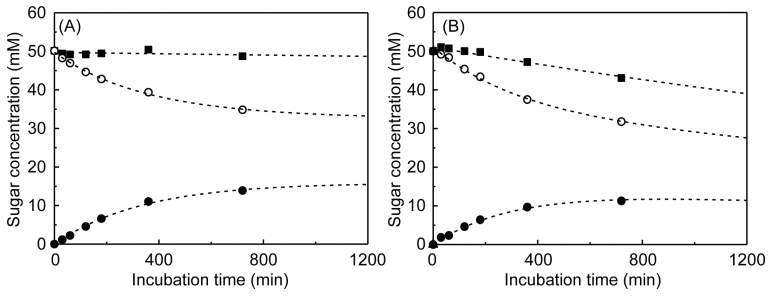


Fig. 3. Epimerization of kojibiose (A) and sophorose (B) under heat treatment (90 °C) in neutral pH conditions (pH 7.5).
Symbols show experimental data for the decomposition of disaccharides (closed square), the reduction of kojibiose or sophorose (open circle), and the production of products **3** and **4** (closed circle), while broken lines show the theoretical fitting curves calculated using Equations (1–3).

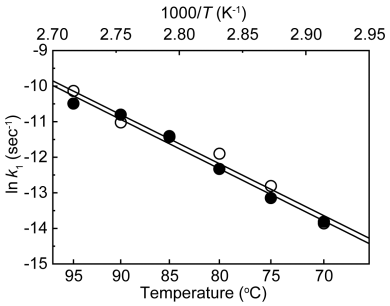


Fig. 4. The Arrhenius plot on the decompositions of kojibiose and sophorose.

The following relations for kojibiose (open circle) and sophorose (closed circle) were derived from a linear plot with correlation coefficients of 0.97 and 0.98, respectively. (A), $y = 17.7x - 38$ ($x = [1000/T]$; $y = [\ln k_1]$); (B), $y = 17.8x - 38$ ($x = [1000/T]$; $y = [\ln k_1]$).

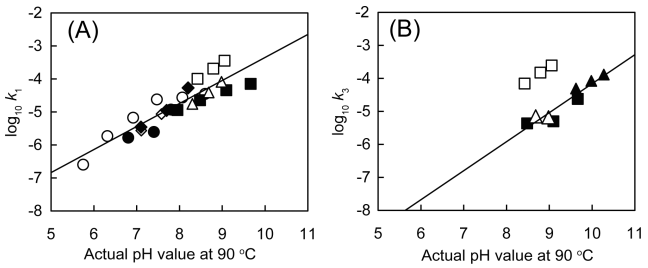


Fig. 5. The relation between rate constants and pH during epimerization (A) and elimination (B) reactions of kojibiose under the heat treatment.

The following relations for the epimerization (A) and elimination (B) of kojibiose were derived from a linear plot with correlation coefficients of 0.89 and 0.90, respectively. (A), $y = 0.77x - 9.2$ (x = [pH value]; $y = \log_{10}[k_1]$); (B), $y = 0.88x - 11$ (x = [pH value]; $y = \log_{10}[k_3]$). Buffers used are as follows: open circle, 100 mM sodium phosphate buffer (pH 5.7, 6.3, 6.9, 7.5, 8.1, and 8.6); closed circle, 100 mM HEPES-sodium hydroxide buffer (pH 6.8, 7.4, and 7.8); open diamond, 100 mM Tris-sodium hydroxide buffer (pH 7.1 and 7.6); closed diamond, 100 mM tricine-sodium hydroxide buffer (pH 7.1, 7.7, and 8.2); open square, 100 mM sodium borate buffer (pH 8.4, 8.8, and 9.1); closed square, 100 mM glycine-sodium hydroxide buffer (pH 8.0, 8.5, 9.1, and 9.7); open triangle, 100 mM CHES-sodium hydroxide buffer (pH 8.7 and 9.0); and closed triangle, 100 mM sodium bicarbonate buffer (pH 9.6, 10.0, and 10.3). The pH values of these buffers were measured at the initial stage of heating at 90 °C.

structure of the reducing end. The assignments of the ^{1}H and ^{13}C NMR chemical shifts of the products **5**–**7** are summarized in Tables 4–6.

Reaction mechanism and kinetics of epimerization and decomposition of kojibiose and sophorose.

Herein, we propose a mechanism for the epimerization and decomposition of kojibiose (substrate **1**) and sophorose (substrate **2**) through an enol intermediate, as illustrated in Fig. 2. In the cases of kojibiose and sophorose, glucose–mannose isomerization occurs through the enolate intermediate, resulting in the production of 2-*O*-α-D-glucopyranosyl-D-mannose (product **3**) and 2-*O*-β-D-glucopyranosyl-D-mannose (product **4**). In addition, some decomposition of kojibiose occurs via the β-elimination of the 3-*O*-hydroxyl group resulting in the generation of a 3-deoxy-2,3-unsaturated compound (product **5**) and two bicyclic 3,6-anhydro compounds (products **6** and **7**).

Both the epimerization and β-elimination proceed through the enol intermediate. The scheme was simplified as shown by the dotted arrows in Fig. 2. Using the rate constants, the following formulae (1–3) were constructed:

$$[\text{Glc-form}]_t+[\text{Man-form}]_t = [\text{Glc-form}]_0\exp(-k_3t) \quad (1)$$

$$[\text{Man-form}]_t=[\text{Glc-form}]_0\exp(-k_3t) \times(1-\{k_2+k_1\exp[-(k_1+k_2)t]\}/[k_1+k_2]) \quad (2)$$

$$[\text{Glc-form}]_t=[\text{Glc-form}]_0\exp(-k_3t) \times\{k_2+k_1\exp[-(k_1+k_2)t]\}/(k_1+k_2) \quad (3)$$

The proposed model shows a good fit with the experimental data collected in this study (Fig. 3). The rate constants k_1 and k_2 of the epimerization of kojibiose under the neutral pH condition (pH 7.5) at 90 °C were (1.6 ± 0.1) × 10^{-5} s^{-1} and (3.2 ± 0.2) × 10^{-5} s^{-1} and were almost identical to those of sophorose, which were (1.5 ± 0.1) × 10^{-5} s^{-1} and (3.5 ± 0.4) × 10^{-5} s^{-1}, respectively. The epimerization rates were not significantly affected by the concentration of sodium phosphate buffer between 20 mM to 100 mM. The rate constant k_3 of the decomposition reaction for kojibiose was (4.7 ± 1.1) × 10^{-7} s^{-1}, and that for sophorose was about ten times higher [(3.7 ± 0.2) × 10^{-6} s^{-1}].

The rate constants of epimerization increased exponentially with temperature. Neither the epimerization of kojibiose or sophorose was observed (remaining amount > 90 %) at temperatures of 70 °C within 24 h. The activation energy (E_a) value calculated for the epimerization of kojibiose was 147 ± 11 kJ mol^{-1}, identical to that of sophorose (148 ± 9 kJ mol^{-1})(Fig. 4).

pH dependence of epimerization and decomposition of kojibiose.

The pH dependence of the epimerization and decomposition of kojibiose was studied through treatment at 90 °C in buffers with a pH range of 6.0–10.5. The rate constants of the epimerization increased exponentially with the increase of pH. A high correlation coefficient (0.89) was observed between $\log_{10}k_1$ and pH, indicating that the reaction rate is dependent upon the concentration of the hydroxide ion (Fig. 5A), regardless of the type of buffer used. This suggests that epimerization is triggered by deprotonation at the C-2 position by hydroxide ions and the formation of an enol structure. The decomposition of kojibiose was mainly observed above pH 8.5. Borate buffers enhanced the elimination reaction of kojibiose compared with other buffers within a pH range of 8.5–10.5 (Fig. 5B). This result is in agreement with previous reports that the β-elimination of *N*-acetyl glucosamine facilely occurred during heating with borate[14]. We have herein confirmed that products **5**–**7** were isolated from the heat treatment of kojibiose in borate buffer (pH 8.5) (data not shown) and that the proposed model exhibits a good fit with experimental data collected in the presence of a borate buffer (Fig. S9). Kojibiose was very stable at pH 6.0 (remaining amount > 98 %).

DISCUSSION

We have herein evaluated the behavior of kojibiose and sophorose during heating at temperatures below 100 °C under neutral pH conditions in which glycosyl linkages are stable. The pH dependency of the reaction rates clearly indicates that the epimerization of both kojibiose and sophorose is caused by keto-enol tautomerization, which is often observed under alkaline conditions. The initial phase of alkaline degradation of D-glucose results in the formation of a mixture of D-glucose, D-fructose and D-mannose. The aldose-ketose isomerization is referred to as the Lobry de Bruyn/Alberda van Ekensterin transformation.[30] In the case of kojibiose and sophorose, the ketose form cannot be generated because the 2-*O*-hydroxy group is blocked, so that glucose residue directly transferred into mannose residue. The glucose epimerization into mannose occurs through enolate intermediate during which the stereochemical configuration at the C-2 position is lost. It has been reported that 2-*O*-α-D-glucosyl-D-mannose was generated from kojibiose by heating under strong alkaline condition with sodium hydroxide or potassium hydroxide.[31] The epimerization can be also observed under neutral pH and weak alkaline conditions, resulting in the formation of 2-*O*-α-D-glucopyranosyl-D-mannose from kojibiose and 2-*O*-β-D-glucopyranosyl-D-mannose from sophorose. The epimerization was observed to hold at the pH values in the reaction solution, supporting the fact that the epimerization proceeded by base catalyst.

When kojibiose was heated under weak alkaline conditions, β-elimination reactions occurred on one of the *O*-hydroxy groups on the C-3 of D-glucopyranose at the reducing end of kojibiose. We also characterized a 3-deoxy-2,3-unsaturated compound (product **5**) and two bicyclic 3,6-anhydrofuranose (products **6** and **7**), which included gluco- and manno-forms, as previously recognized.[8][14] Additionally, pyranose rings of 3-deoxy-2,3-unsaturated compound (product **5**) were identified.

The rate constants (k_1 and k_2) and E_a value of the epimerization of kojibiose were almost identical to those of sophorose, indicating that the epimerization rate was not affected by the configuration at the anomeric center (α- or β-linkage) on non-reducing glucose. While the rate constant (k_3) of the decomposition reaction for sophorose was about ten times higher than that for kojibiose. The reasons for this may be as follows: Kojibiose can adapt a structure that seem to stepwise and stacking conformation against the faces of non-reducing and reducing glucopyranose rings. But sophorose cannot adapt such a conformation. As the feature of kojibiose has potential to inhibit β-elimination of *O*-hydroxyl group on the C-3 of D-glucopyranose at the reducing end or generation of 3-deoxy-2,3-unsaturated compound, it may contribute to stabilization of kojibiose.

We conclude that 2-*O*-substituted reducing aldoses are generally epimerized via aldose–aldose isomerization and decomposed via β-elimination of *O*-hydroxyl group under neutral and alkaline conditions by heating. The information reported herein regarding the behavior of kojibiose and sophorose under heating will be useful in the development of production and application methods for oligosaccharides with 1,2-glycosidic bonds.

EXPERIMENTAL

Sugars used. Kojibiose and nigerose were purchased from Carbosynth Limited (Berkshire, UK). α-Sophorose monohydrate was purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany).

Heat treatment of sugars. The sugars were dissolved to approximately 50 mM in 100 mM sodium phosphate buffer (pH 7.5) as standard conditions of preparation. The solutions (25 μL) were placed into a 200 μL thermal tube and heated at 90 °C for 0, 30, 60, 120, 180, 360, and 720 min in an Astec PCR Thermal Cycler PC-708 (Astec Cell Science Research Laboratory, Kasuya, Fukuoka, Japan). The reaction mixture solutions were analyzed by HPLC to determine the starting substrate and its products.

Browning Index. The Browning index of each reaction solution was measured at 420 nm using a microvolume UV-Vis spectrophotometer Q5000 (Tomy Digital Biology, Tokyo, Japan). The optical path length was set up to be 1 mm.

HPLC analysis. The compositions of the sugars in solutions were determined by an HPLC system (Shimadzu, Kyoto, Japan) equipped with a RefractoMax 521 refractive index detector (ERC Inc., Saitama, Japan) using a Shodex HILICpak VG-50 column (4.6 mm i.d. × 250 mm; Showa Denko, Tokyo, Japan) at 40 °C under a constant flow (0.8 mL/min) of 75 % acetonitrile (for kojibiose) and 80 % acetonitrile (for sophorose) in water as the mobile phase.

Isolation of a major reaction product from kojibiose. Kojibiose (3.4 mg, 10 μmol) was dissolved in 200 μL of 100 mM sodium phosphate buffer (pH 7.5) and incubated at 90 °C for 12 h. The reaction mixture was desalted using Amberlite MB-4 (Organo, Tokyo, Japan), lyophilized, and dissolved with 75 % acetonitrile in distilled water. The reaction products were isolated using an HPLC system (Prominence; Shimadzu) equipped with a Shodex HILICpak VG-50 column (4.6 mm i.d. × 250 mm; Showa Denko) at 40 °C under a constant flow (0.8 mL/min) of 75 % acetonitrile in water as the mobile phase. The fractions containing the reaction products were collected and lyophilized. The remaining kojibiose and major reaction product **3** were 0.5 mg and 0.6 mg, respectively.

Isolation of a major reaction product from sophorose. Sophorose (3.4 mg, 10 μmol) was dissolved in 200 μL of 100 mM sodium phosphate buffer (pH 7.5) and incubated at 90 °C for 12 h. The reaction mixture was desalted using Amberlite MB-4 (Organo), lyophilized, and dissolved with 80 % acetonitrile in distilled water. The reaction products were isolated by using an HPLC system equipped with a Shodex HILICpak VG-50 column (4.6 mm i.d. × 250 mm; Showa Denko) at 40 °C under a constant flow (0.8 mL/min) of 80 % acetonitrile in water as the mobile phase. The fractions containing the reaction products were collected and lyophilized. The remaining sophorose and the major reaction product **4** were 0.7 mg and 0.6 mg, respectively.

Isolation of minor reaction products from kojibiose heated under weak alkaline conditions. Kojibiose (68 mg, 200

μmol) was dissolved in 4 mL of a 100 mM sodium bicarbonate buffer (pH 10) and the solution was incubated at 90 °C for 3 h. The reaction mixture was passed through a Sep-Pak® Plus C18 cartridge (360 mg of sorbent; Waters, Milford, MA), desalted using Amberlite MB-4 (Organo), lyophilized, and redissolved using distilled water. The solution was passed through a TOYOPEARL HW40S gel-filtration column (26 mm i.d. × 1000 mm) under a constant flow (1.0 mL/min) of water as the mobile phase. Separation of the products was confirmed by TLC analysis. The fractions containing decomposed products other than disaccharides were collected, lyophilized, and redissolved using 90 % acetonitrile in distilled water. The decomposed products were isolated using a HPLC system equipped with a Wakosil 5SIL column (10 mm i.d. × 250 mm; FUJIFILM Wako Pure Chemicals Corporation, Osaka, Japan) at 40 °C under a constant flow (2.0 mL/min) of 85 % acetonitrile in water as the mobile phase. The peaks eluted at 23.4 min (Fraction 1), 27.6 min (Fraction 2), and 32.7 min (Fraction 3) were collected and lyophilized. The amount of lyophilized products derived from Fractions 1, 2, and 3 were 3.6, 8.5, and 4.0 mg, respectively. The trace amounts of these lyophilized products were resolved in water and analyzed using an HPLC system equipped with a Shodex HILICpak VG-50 column (4.6 mm i.d. × 250 mm; Showa Denko) at 40 °C under a constant flow (0.8 mL/min) of 75 % acetonitrile in water as the mobile phase. The isolates from Fractions 1, 2, and 3 showed peaks at retention times of 7.5 min (product **7**), 8.2 min (the mixture of products **5** and **6**), and 9.5 min (product **3**), respectively.

LC-ESIMS analysis. LC-ESIMS spectra were recorded in negative-ion mode on an API 2000 LC/MS/MS (AB SCIEX, Foster, CA).

NMR analysis. One-dimensional (^{1}H and ^{13}C) and two-dimensional [double-quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), NOESY, heteronuclear single-quantum coherence (HSQC), HMBC, and homonuclear *J*-resolved (JRES)] NMR spectra of the products were acquired in deuterium oxide, using 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid as an internal standard (δ_H 0.00 and δ_C 0.00), using a Bruker Avance 800 spectrometer (Bruker Biospin GmbH, Karlsruhe, Germany). The proton signals were assigned based on the DQF-COSY, TOCSY, and NOESY spectra, and ^{13}C signals were assigned using the HSQC spectra based on the assignment of proton signals. The linkage position in each product was determined through detection of the inter-ring cross peaks in each HMBC spectrum. Coupling constants were read using the JRES spectra based on the assignment of proton signals. The anomeric configuration of mannose residue was confirmed based on the $^{1}J_{C1-H1}$ coupling constant that was extracted from the non-decoupled HSQC spectrum.

Determination of kinetic parameters. The rate constant of the dehydrating decomposition (k_3) was calculated by regressing the data set of ([Glc-form]$_t$ + [Man-form]$_t$) vs t in Equation (1) using Origin 2018 (LightStone Corp., Tokyo, Japan). Following this, the epimerization rate constants (k_1 and k_2) were calculated by regressing the data set of [Man-form]$_t$ vs t in Equation (2) with the k_3 value calculated using Origin 2018. [Glc-form]$_t$ was then computed using Equation (3) including the calculated the k_1, k_2, and k_3 values.

The apparent energy of activation (E_a) was obtained by regressing data with the Arrhenius Equation (4), where *A, R* and *T* are frequency factors, the universal gas constant, and absolute temperature (in Kelvin), respectively, again using Origin 2018:

$$\ln k = \ln A - E_a/R \times T \quad (4)$$

Influence of temperature and pH on epimerization of kojibiose. The effects of temperature on the epimerization rates of kojibiose and sophorose were measured under the standard condition described above at the temperatures of 70, 75, 80, 85, 90, and 95 °C. The effects of pH on the epimerization rate k_2 of kojibiose were measured under the standard condition described above using the following buffers: 100 mM sodium phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5); 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-sodium hydroxide buffer (pH 7.5, 8.0, and 8.5); 100 mM tricine-sodium hydroxide buffer (pH 8.0, 8.5, and 9.0); 100 mM tris(hydroxymethyl)aminomethane (Tris)-sodium hydroxide buffer (pH 8.5 and 9.0); 100 mM sodium borate buffer (pH 8.5, 9.0, and 9.5); 100 mM *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES)-sodium hydroxide buffer (pH 9.5 and 10.0); 100 mM sodium bicarbonate buffer (pH 9.6, 10.0, and 10.3); and 100 mM glycine-sodium hydroxide buffer (pH 9.0, 9.5, 10.0, and 10.5). The concentration of the kojibiose or sophorose reduced with the heat treatment was sequentially measured using HPLC methods described above.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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