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Preparation and reactivity of a novel disaccharide, glucosyl 1,5-anhydro-D-fructose (1,5-anhydro-3-*O*-α-glucopyranosyl-D-fructose)

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Abstract

A novel disaccharide, glucosyl 1,5-anhydro-D-fructose (1,5-anhydro-3-*O*-α-glucopyranosyl-D-fructose, GAF) was enzymatically prepared from 1,5-anhydro-D-fructose (1,5-AF) and cyclomaltoheptaose (β-cyclodextrin). Cyclodextrin glucanotransferase transferred various sizes of maltooligosaccharide to 1,5-AF. Glucoamylase digested the maltooligosyl chain of the products to a glucosyl residue giving a final product, GAF. An NMR analysis of GAF elucidated that the glucose residue was linked to C-3 of the 1,5-AF residue with an ether linkage. Reactivity on the aminocarbonyl reaction of GAF with bovine serum albumin was lower than that of 1,5-AF, but was higher than that of glucose.

Keywords: 1,5-Anhydro-D-fructose; Aminocarbonyl reaction; Cyclodextrin glucanotransferase

1. Introduction

1,5-Anhydro-D-fructose (1,5-AF) is the product formed when α-1,4-glucan lyase acts on an α-1,4-D-glucan.[1,2] This sugar is thought to be in equilibrium between the forms keto, enol, endiol and hydrate in water. However, the last form is predominant as observed by NMR in water.[3,4] The active forms, such as enol, could be dominant with disequilibrium by dehydration. Glycine was modified with 1,5-AF under mild dry conditions, but the reaction does not proceed in dilute solution.[5] Under the established conditions, several proteins are easily glycosylated to improve their solubility and emulsification. Glycosylation with 1,5-AF may provide a new tool to improve the quality of food proteins such as solubility, stability and resistance to proteases.

The sugar, 1,5-AF, has antioxidative activity[6] and effectively prevents the oxidation of linoleic acid better than ascorbic acid.[4] As a valuable material for the food and other industries, bulk and cheap production of 1,5-AF is accomplished by the degradation of starch with α-1,4-glucan lyase. The biochemical and chemical aspects of this sugar have been recently documented by Andersen and coworkers.[7]

A number of new oligosaccharides are produced by the action of enzymes. For example, isomaltooligosaccharides are synthesized by the transglucosylation activity of α-glucosidase[8] and branched cyclodextrins are prepared by the reversed action of isoamylase.[9] Cyclodextrin glucanotransferase (CGTase) is often used for the production of maltooligosaccharides having a radioisotopic label at their reducing-terminal glucosyl residues by the transfer and disproportionating actions of the enzyme.[10] Since the enzyme is reported to accommodate other saccharides such as ascorbic acid[11] and inositol[12] as acceptors, the enzyme may recognize 1,5-AF and produce new oligosaccharides having 1,5-AF at their reducing ends. Production of these oligo-

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saccharides will allow development of a new way of utilizing 1,5-AF.

In this study, glucosyl 1,5-anhydro-D-fructose was produced from cyclodextrin and 1,5-AF by the action of CGTase, and its structure was determined. In addition, the reactivity of the new sugar was compared with 1,5-AF and glucose.

2. Results and discussion

To prepare the novel, hetero oligosaccharides enzymatically, the transfer action of cyclodextrin glucanotransferase (CGTase) was used with cyclomaltoheptaose (β-CD) as the sugar donor and 1,5-AF as the acceptor. After 48 h at 35 °C, an analysis of the reaction mixture by high-performance liquid chromatography (HPLC) showed ten peaks (Fig. 1) in addition to peaks from unreacted 1,5-AF (peak 1 in Fig. 1). The retention time of each product suggested that those oligosaccharides were malto-oligosyl 1,5-AFs of DP 2–10 because CGTase generally transfers malto-oligosaccharides to an acceptor and each peak material eluted slightly faster than the malto-oligosaccharide of DP 2–10. For the linkage analysis between glucosyl residue and 1,5-AF, a disaccharide was obtained by trimming the malto-oligosyl chains of the products to a glucosyl residue by glucoamylase. The purified product had a purity of more than 98% by HPLC (Fig. 2(A)). The product was resistant against further reaction with glucoamylase (100 U/g substrate, 35 °C, 4 h) (Fig. 2(B)). Under these conditions, maltose was completely degraded into glucose. On the other hand, the product was hydrolyzed with α-glucosidase into an equimolar amount of glucose and 1,5-AF (Fig. 2(C)), confirming that this oligosaccharide was glucosyl 1,5-AF (GAF).

The structure of GAF was elucidated by NMR spectroscopy and fast atom bombardment mass spectrometry (FAB-MS). In order to determine the position of substituted by the glucosyl residue, all signals in the spectrum of GAF were assigned using $^{1}H–^{1}H$ COSY and $^{1}H–^{13}C$ COSY in D_2O at 35 °C. Fig. 3 shows the

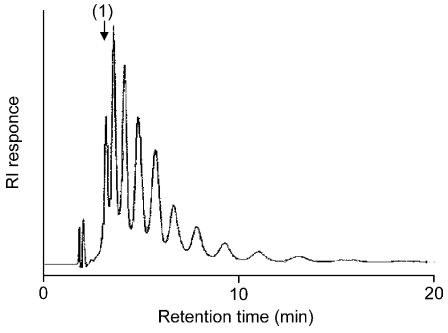

Fig. 1. Chromatogram of product by HPLC (Tosoh TSKgel NH_2-60). Peak (1) represents 1,5-anhydro-D-fructose.

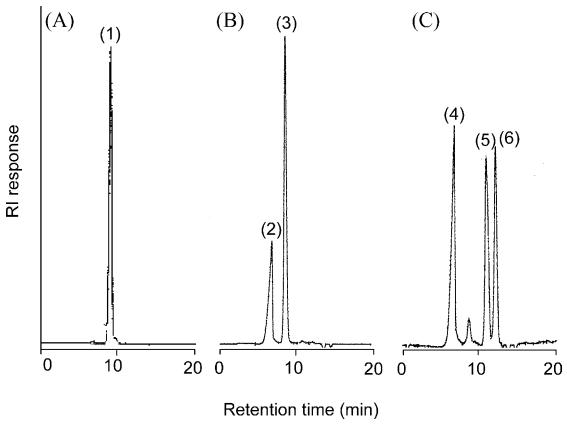

Fig. 2. Enzymatic digestion of purified product by glucoamylase or α-glucosidase. (A) purified product, (B) glucoamylase digestion, and (C) α-glucosidase digestion. Peaks indicate the following: (1) and (3) product, (2) and (4) salt, (5) glucose, and (6) 1,5-AF.

$^{1}H–^{13}C$ COSY spectrum of GAF, and the ^{13}C- and ^{1}H-chemical shifts are presented in Tables 1 and 2, respectively. In the ^{13}C NMR spectrum of GAF, the signals at 208 ppm and at around 130–150 ppm, assigned to the keto form and double bond, respectively, were not detected. These data suggest that the glucose was not transferred to the keto, enol, or endiol forms of 1,5-AF, and consequently, was transferred to the hydrate form. Only the C-3 signal of the 1,5-AF residue shifted downward to 86.53 ppm from the C-3 signal (79.70 ppm) of 1,5-AF. In the ^{1}H NMR spectrum of GAF, the H-1 signal of the glucose residue was observed at σ 5.32 (d, $J_{1,2}$ 3.85 Hz). In the FAB-MS spectrum of GAF, ion peaks in the negative-ion mode were observed at m/z 323 $[M-H_2O-1]^-$, m/z 415 $[M+\text{glycerol}-H_2O-1]^-$, and m/z 647 $[2M-2H_2O-1]^-$. Thus, the structure and molecular mass of GAF were determined to be that of glucose linked to the hydrate form of 1,5-AF, that is, m/z 342. Hence, CGTase transfers the glucose residue to the hydroxyl group on C-3 of the 1,5-AF having the α-(1→3) linkage (Fig. 4).

GAF is expected to be more reactive toward amino groups than other disaccharides since the reducing terminal sugar is 1,5-AF residue (hydrate form). The high reactivity of 1,5-AF is due to the tautomerization of this sugar,[7] thus the 1,5-AF residue, the reducing end of GAF, may be highly reactive since the hydroxyl group bonding C-3 of the 1,5-AF is not concerned with the tautomerization. The reactivity of GAF with BSA on amino-carbonyl reaction was compared with that of 1,5-AF and glucose. The mixture of GAF, 1,5-AF or glucose and BSA was incubated at 30 °C and 7.6% relative humidity. The rate of modification with 1,5-AF was highest at 24 h. After a 72-h reaction, the modified BSA with 1,5-AF became insoluble and determination of the free amino groups in BSA was therefore not

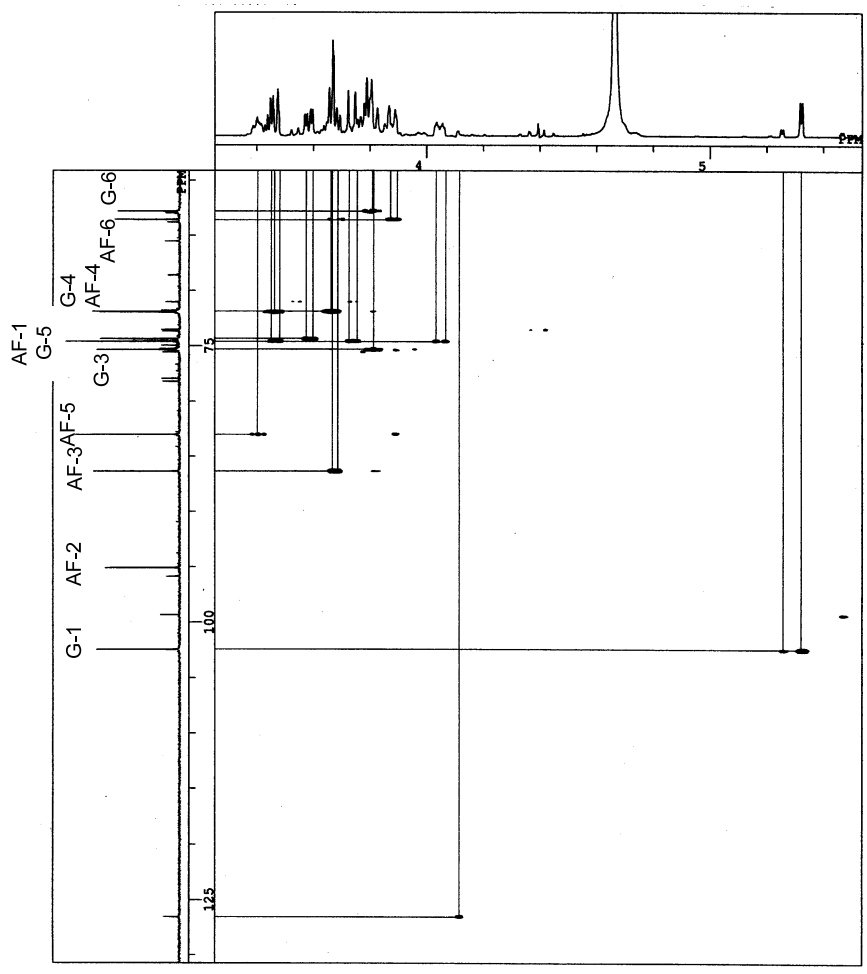

Fig. 3. $^{1}H-^{13}C$ COSY NMR spectrum of glucosyl 1,5-anhydro-D-fructose in D_2O at 500 MHz.

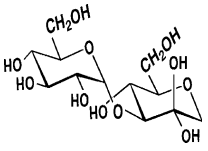

Fig. 4. Possible structure of glucosyl 1,5-anhydro-D-fructose.

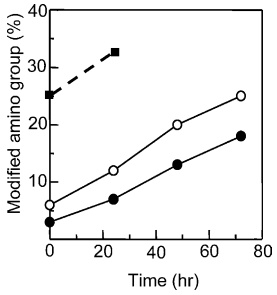

Fig. 5. Modification of bovine serum albumin with sugars. Symbols: ■ 1,5-AF; ○ GAF; ● glucose.

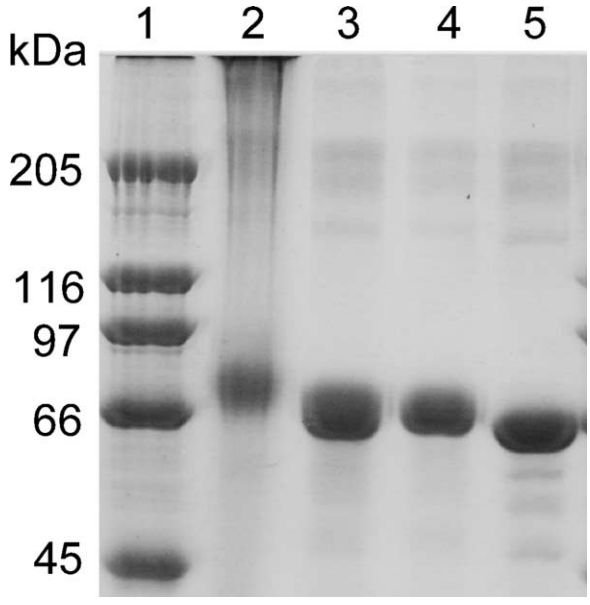

Fig. 6. SDS-PAGE of modified BSA. Lane 1, protein standard; lane 2, modified with 1,5-AF; lane 3, modified with glucose; lane 4, modified with GAF; and lane 5, control BSA.

Table 1
^{13}C NMR chemical shifts σ (ppm) of glucosyl 1,5-anhydro-D-fructose

	C-1	C-2	C-3	C-4	C-5	C-6
1,5-AF residue	74.94	95.19	86.53	72.05	83.20	63.76
Glucose residue	102.54	74.52	75.48	72.09	74.74	63.00

possible. The degree of modification of 20 and 17% were attained by GAF and glucose, respectively (Fig. 5). The polymerized BSA and broader band near 66 kDa appeared in the case of 1,5-AF modification. The GAF-BSA band migrated at a slower rate than that of glucose–BSA. Based on these results, the reactivity of GAF was lower than that of 1,5-AF, but higher than that of glucose.

1,5-AF acted as the acceptor for cyclodextrin glucanotransferase and the subsequent action of glucoamylase produced a new, highly reactive disaccharide. GAF is expected to be a base sugar for the creation of some specific sugar chains that are physiologically active, and the sugar chains will be attached to the proteins by amino carbonyl reaction under mild conditions (Fig. 6).

3. Experimental

3.1. Materials

1,5-AF was prepared from waxy corn starch.[4] CGTase from *Bacillus stearothermophilus*, crystal-grade glucoamylase from *Rhizopus* sp. and α-glucosidase from *Saccharomyces* sp. were purchased from Hayashibara Biochemical Laboratory, Seikagaku Kogyo and Wako Pure Chemicals, respectively. β-CD was provided from Nihon Syokuhin Kako Co., Japan, and was recrystallized four times before use. Bovine serum albumin was purchased from Sigma. The other materials used were of the highest grade.

Table 2
^{1}H NMR chemical shifts δ (ppm) and coupling constants (Hz) of glucosyl-1,5-anhydro-D-fructose

	1,5-AF residue	Glucose residue
H-1	3.46 (d, 13.25) 3.73 (d, 12.40)	5.32 (d, 3.85)
H-2		3.58 (dd, 3.85, 9.83)
H-3	3.67 (dd, 6.20, 12.61)	3.80 (t, 8.12)
H-4	3.67 (t, 6.53)	3.46 (t, 9.19)
H-5	3.40 (m)	4.04 (m)
H-6	3.68 [a] 3.88 (d, 10.26)	3.79 (d, 12.40)

[a] Due to spectral overlap, the coupling constant could not be accurately determined.

3.2. Methods

3.2.1. Preparation of GAF. The reaction mixture of β-CD (22 mM), 1,5-AF (62 mM) and CGTase (10 U/g β-CD) in 50 mM acetate buffer (pH 5.0) was incubated for 48 h at 35 °C. After the reaction was terminated by heating in a boiling-water bath for 10 min, glucoamylase (4 U/g β-CD) was added and then incubated at 35 °C for 24 h. After the reaction, the product was purified by HPLC (MCI GEL CK 08 S). Finally, 170 mg of GAF was obtained from 760 mg of β-CD and 300 mg of 1,5-AF.

3.2.2. High-performance liquid chromatography (HPLC). The reaction mixtures with enzyme(s) were analyzed by HPLC using a Mitsubishi Chemical MCI GEL CK 08 S column (eluent; water, 40 °C, 1 mL/min) and Tosoh TSKgel NH_2-60 column (eluent; 65% acetonitrile, room temperature (rt), 1 mL/min), respectively.

3.2.3. Structure analysis of GAF. NMR spectra data were recorded for a 10% solution in D_2O at 35 °C with a JEOL GSX-500 spectrometer. Chemical shifts were expressed in ppm downfield from the signal of tetramethylsilane (Me_4Si) referred to external 1,4-dioxane (67.40 ppm). The other conditions for ^{13}C NMR, ^{1}H–^{1}H COSY and H–^{13}C COSY measurements were as reported in a previous paper.[13]

FAB-MS was performed in the negative-ion mode on a JEOL JMS-DX 303 mass spectrometer using xenon atoms having a kinetic energy equivalent to 6 kV at an accelerating voltage of 3 kV. The mass marker was calibrated with perfluoroalkylphosphazine (Ultra Mark), and glycerol was used as the matrix.

3.2.4. Modification of BSA. The BSA and sugars were mixed in 100 mM phosphate buffer (pH 7.5) and dried under reduced pressure for 24 h at rt, and then incubated over saturated NaOH (relative humidity of 7.6% at 30 °C for 12–72 h). The extent of modification was calculated by the determination of a free amino group with trinitrobenzensulfonic acid (TNBS) reagent.[14]

3.2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weights of the modified and unmodified BSA were determined by

SDS-PAGE using egg albumin, triosephosphate isomerase and chicken egg-white lysozyme as molecular markers.

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