ガラクトシルマルトースの哺乳類α-アミラーゼ基質アナログ阻害剤としてのガラクトシルマルトビオノラクトンへの酵素的変換

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Note

Facile Enzymatic Transformation of 4^2-*O*-β-D-Galactosylmaltose into 4^2-*O*-β-D-Galactosylmaltobionolactone as a Substrate Analogue Inhibitor for Mammalian α-Amylase

Masayasu Takada, Koichi Ogawa, Takeomi Murata[1] and Taichi Usui[1,*]

Nihon Shokuhin Kakou Co., Ltd. (30, Tajima, Fuji 417–8530, Japan)
[1]*Department of Applied Biological Chemistry, Shizuoka University (836, Ohya, Shizuoka 422–8529, Japan)*

Enzymatic transformation of 4^2-*O*-β-D-galactosylmaltose (**1**) into 4^2-*O*-β-D-galactosylmaltobionolactone (**2**) was carried out by the use of oligosaccharide oxidase (OSOD). The anomer hydroxyl group of glucose residue at the reducing end of **1** was readily oxidized to the corresponding lactone on a large scale, and the product was conveniently isolated by the sequential column chromatographies of Amberlite IR-120B and Toyopearl HW-40S. The desired compound, **2**, was obtained in a high yield (80%) based on the addition of **1**. The jar-fermentor was included in this reaction system to facilitate the maintenance of pH and supply of air to the reaction mixture. As a result, **2** obtained enzymatically was shown to be identical to that obtained chemically.

We have previously reported that the end-modified maltooligosaccharide derivative 4^2-*O*-β-D-galactosyl maltobionolactone (**2**) is useful as a novel substrate analogue inhibitor for mammalian α-amylases.[1] In the present report, the anomeric hydroxyl group of 4^2-*O*-β-D-galactosylmaltose (**1**) was enzymatically oxidized with oligosaccharide oxidase (OSOD) instead of conventional chemical oxidation with iodine, because the enzymatic method is much more suited for process administration than the chemical method. OSOD, which oxidizes maltooligosaccharides (DP 2–7), cellobiose and lactose (data not shown), has a broad substrate specificity.[2] On the basis of the above data, **1** was oxidized by the enzyme as follows: 100 g of **1** was dissolved in 1.5 L of 50 mM potassium phosphate buffer (pH 7.1) and the reaction mixture was incubated with 600 U of catalase, 2500 ppm of antiform and 3000 U of OSOD at 40°C for 12 h. During the entire course of the reaction, the maintenance of the pH and supply of oxygen were needed to complete the reaction. A jar-fermentor was employed as a reaction system for facilitating these processes. The pH was adjusted to 7.1 with 2 N NaOH and air was supplied by stirring at the speed of 0.45 L/min. Figure 1 shows the time course of enzymatic conversion of **1** into 4^2-*O*-β-D-galactosylmaltobionic acid (**3**) by HPLC analysis according to our previous method.[1] The starting material, **1**, was oxidized completely to the corresponding sodium lactonate after 12 h. The resulting product was then neutralized by passing through on a Amberlite IR-120B (H^+) column (5.0×30 cm). The acidic eluate (4.5 L) was collected and concentrated to 200 mL. A 1/5 volume of the solution was loaded onto a Toyopearl HW-40S (20×120 cm) column. The elution was monitored by RI. The eluate gave one main peak. The remaining aliquots were similarly processed as above. The fractions corresponding to one main peak were collected, concentrated and lyophilized to afford **2** (80 g) at a yield of 80% (w/w) based on the addition of **1**.

The structure of **2** was confirmed from the ^{1}H- and ^{13}C-NMR spectra. The spectral data were iden-

*To whom correspondence should be addressed.
Abbreviations: **1**, 4^2-*O*-β-D-galactosyl maltose; **2**, 4^2-*O*-β-D-galactosylmaltobionolactone; **3**, 4^2-*O*-β-D-galactosylmaltobionic acid; OSOD, oligosaccharide oxidase.

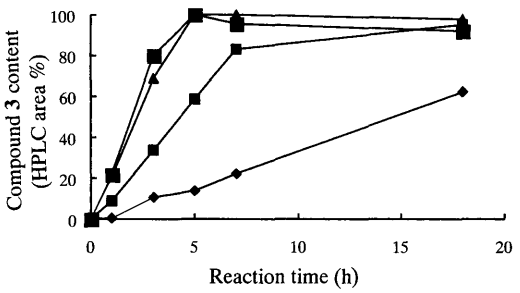

Fig. 1. Enzymatic oxidation of **1** by OSOD using a jar-fermentor.

Compound **3** content was determined by our HPLC method (See Ref. 1)). The amounts of OSOD added, 112 U; ▲, 56 U; ■, 28U; ◆, 7 U/g of **1**.

Table 1. Comparison of the ^{13}C-NMR chemical shifts between **2** oxidized chemically and **2** oxidized enzymatically.

		Enzymatic oxidation (δ, ppm[a])					
		C-1	C-2	C-3	C-4	C-5	C-6
I	Lactone	176.33	83.65	74.08	78.40	72.94	63.31
	GluA	178.76	75.02	74.81	84.62	74.41	64.89
II	Lactone	102.99	73.96	75.42	80.93	74.21	62.64
	GluA	102.53	73.96	75.52	80.93	74.21	62.64
III		105.73	73.84	75.42	71.45	78.22	63.92

		Chemical oxidation (δ, ppm[a])					
		C-1	C-2	C-3	C-4	C-5	C-6
I	Lactone	176.26	83.58	73.94	78.29	72.87	63.22
	GluA	178.45	74.91	74.72	84.47	74.32	64.78
II	Lactone	102.44	73.87	75.35	80.83	74.14	62.50
	GluA	103.06	73.87	75.45	80.83	74.14	62.50
III		105.81	73.76	75.35	71.36	78.15	63.84

[a]Measured in D_2O.

Table 2. Comparison of the K_i values (μM) of **2** synthesized by chemical and enzymatic oxidation.

	Chemical	OSOD
HSA	4.9	4.7
HPA	18.0	18.0
PPA	2.8	2.9

tical to those of chemically oxidized **2**, which exists in two forms; the lactone and aldonic acid forms shown in Table 1. In the present study, the proportion of lactone and aldonic acid forms in the ^{1}H-NMR spectrum was calculated to be 60 : 40, immediately after dissolution in D_2O (data not shown). This ratio is also the same as that of the chemically oxidized product. The identity of enzymatically and chemically oxidized products was further confirmed by comparison of the retention times and peak intensities due to lactone and aldonic acid forms in HPLC (data not shown). The inhibition activities toward human salivary α-amylase (HSA), human pancreatic α-amylase (HPA) and porcine pancreatic α-amylase (PPA) by **2** obtained enzymatically were also compared with those by chemically oxidized one as shown in Table 2. The assay was done using p-nitrophenyl α-maltopentaoside as the substrate according to our method.[1] The K_i values of **2** obtained enzymatically and those of **2** obtained chemically were very close to each other. As a result, **2** obtained enzymatically was shown to be identical to that of obtained chemically.

In conclusion, we have established a method for the facile enzymatic conversion of **1** into **2** by the use of OSOD. The yield (80% w/w) was sufficiently high for a practical method because of the simplicity of enzymatic synthesis.

EXPERIMENTAL

Materials. Compound **1** was prepared according to our method.[1] A series of p-nitrophenyl-maltooligosaccharides was purchased from Calbiochem-Boehring (USA). HSA and PPA were obtained from Sigma Chemical (USA), and purified further by the methods of Mayo *et al.*[3] and Schramn and Loyter,[4] respectively. Isozyme II (PPA-II) was isolated by the method of Sakano *et al.*[5] HPA, which was purified by the method of Matsuura *et al.*,[6] was a generous gift from Dr. Watatsu of Kokusai Siyaku (Japan). OSOD was a generous gift from Dr. Inukai of Biseiken (Japan). Other chemicals were of the highest quality available commercially.

Analytical methods. HPLC analysis for sugars

was performed on a Shodex DC-613 column (6.0 ×150 mm) or Asahipak GS-220HQ column (7.5 ×300 mm) in a Hitachi 6000-series liquid chromatograph equipped with a Hitachi L-4000 ultraviolet detector and an L3350 RI monitor. Elution of the former column was performed with 30 : 70 (v/v) H_2O-acetonitrile at room temperature, and that of the latter with distilled water at 65°C. The flow rate in both cases was 0.9 mL/min. Analysis of oxidized sugars was also performed under the following conditions: Shodex DC-613 column (6.0 ×150 mm): mobile phase, 30 : 70 (v/v) H_2O-acetonitrile containing 0.1 M $NaNO_3$; flow rate, 0.8 mL/min; temperature, 30°C; and detection, RI. Before injection, 0.2 mL of the reaction digest containing about 2 mg of a lactone was mixed thoroughly with 0.2 mL of a 0.2 M $NaNO_3$ solution, and then the mixture was injected into the apparatus immediately. ^{13}C- and ^{1}H-NMR spectra were analyzed using the same conditions as our previous report.[1]

Enzyme activity assay. OSOD activity was determined as follows. First, the enzyme solution was prepared. One-hundred microliters of OSOD solution (50 mg/mL) was added to 300 μL of 0.1 M PIPES buffer (pH 7.0) and 10 μL of 4-aminoantyprine solution (30 mg/mL), and this solution was pre-incubated at 37°C for 30 min. Then, 2 mL of 0.1 M PIPES buffer (pH 7.0) was added to the solution (enzyme solution). Secondly, solution A was prepared. PIPES (300 mg), boric acid (900 mg), TOOS (30 mg), 4-aminoantypirine (10 mg), peroxidase (1300 U) and Triton X-100 (130 mg) were dissolved in water and volume increased to 100 mL with water, and the pH was adjusted to 7.0. Solution A (2.5 mL) was added to 500 μL of 0.5 M maltose solution and pre-incubated at 37°C for 10 min. The reaction was performed by adding the appropriate diluted enzyme solution (50 μL). The increase in optical density was followed at 550 nm for 5 min. One unit of enzyme activity was defined as the amount of enzyme which produced 1 μmol/min H_2O_2.

Assaying of inhibition activity. The inhibition of α-amylase activity by **2** was determined according to the previous paper.[1] The inhibition constants (K_i) for **2** were calculated from Dixon and Webb plots,[7] and the mode of inhibition was reconfirmed by the method of Lineweaver-Burk.[8]

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高田正保，小川浩一，村田健臣[1]，碓氷泰市[1]

日本食品化工株式会社
(417–8530 富士市田島 30)
[1] 静岡大学農学部応用生物化学科
(422–8529 静岡市大谷 836)

4^2-*O*-β-D-galactosylmaltose (**1**) にオリゴ糖酸化酵素(OSOD)を作用させると，哺乳類α-アミラーゼの基質アナログ阻害剤である 4^2-*O*-β-D-galactosylmaltobi-

onolactone (**2**) に容易に変換できた．すなわち，化合物 **1** の還元末端グルコース残基のアノマー水酸基を OSOD を用いて酸化し，生成物をカチオン交換樹脂およびトヨパール HW-40S ゲル濾過カラムによって順次精製が可能であった．本反応を円滑に行うためには全反応期間中 pH を維持し，また酸素を供給する必要があった．そのためにはジャーファメンターの導入が有効であり，化合物 **2** の大量調製を可能にした．調製した **2** の NMR による構造解析結果および哺乳類 α-アミラーゼに対する阻害活性を検討した結果，酵素的酸化法によって調製した **2** は化学的酸化法によって調製したものと同一物質であることを確認した．この結果，哺乳類 α-アミラーゼ基質アナログ阻害剤 **2** の工業レベルでの調製が可能になった．