ELSEVIER

Carbohydrate Research 261 (1994) 157–162

CARBOHYDRATE RESEARCH

Note

A convenient enzymatic synthesis of 4^2-α-isomaltosylisomaltose using *Thermoactinomyces vulgaris* R-47 alpha-amylase II (TVA II)

Takashi Tonozuka [a], Hiroshi Sakai [b], Takahisa Ohta [b], Yoshiyuki Sakano[a] *

[a] *Department of Applied Biological Science, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8, Saiwai-cho, Fuchu, Tokyo 183, Japan*

[b] *Department of Agricultural Chemistry, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113, Japan*

(Received January 12th, 1994; accepted in revised form March 4th, 1994)

Enzymes that hydrolyze pullulan (**1**), such as pullulanases [1,2], isopullulanase [3], neopullulanases [4–6], and *Thermoactinomyces vulgaris* alpha-amylases [7,8] are useful in the production of various oligosaccharides from starch or pullulan. Recently, because of the commercial significance, many of their amino acid sequences have been reported. Comparisons between the amino acid sequences and the substrate specificities may provide clues about their pullulan-hydrolyzing mechanism.

Oligosaccharides with α-(1 → 6)-glucosidic linkages are indispensable for the study of their substrate specificities. *Thermoactinomyces vulgaris* R-47 produces two alpha-amylases, TVA I and TVA II, which hydrolyze α-(1 → 4)-glucosidic linkages of **1** to produce panose (**2**). These enzymes can hydrolyze not only α-(1 → 4)-glucosidic linkages of **1**, but also the α-(1 → 6)-glucosidic linkage of isopanose [Glc-α-(1 → 4)-Glc-α-(1 → 6)-Glc] [8]. Thus they should also transglycosylate both α-(1 → 4)- and α-(1 → 6)-glucosidic linkages. Similar reactions have been reported in neopullulanase [4] and *Bacillus licheniformis* alpha-amylase [9]. In this paper, we report convenient methods of preparing 4^2-α-isomaltosylisomaltose (**4**) using TVA II.

We used a mixture of **1** and glucose as the starting materials. In this system, TVA II should produce a hydrolysate (**2**) and two tetrasaccharides, 6^3-α-gluco-

* Corresponding author.

SSDI 0008-6215(94)00096-X

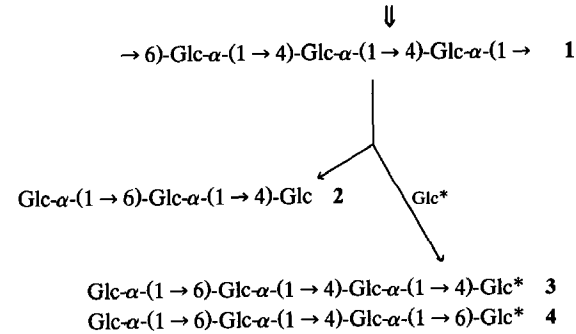

Fig. 1. Scheme of the pattern of action of *Thermoactinomyces vulgaris* alpha-amylases for pullulan and glucose. Abbreviations: asterisk, radioactive glucose residue; bold arrow, site of enzymatic attack.

sylmaltotriose (**3**) and 4^2-α-isomaltosylisomaltose (**4**) (Fig. 1). To confirm this prediction, D-[U-^{14}C]glucose was used as the acceptor for the reaction, and the mixtures were analyzed by TLC. As shown in Fig. 2, two radioactive oligosaccharides, spots a and b, were detected as expected. The mobility of an oligosaccharide having α-(1 → 6)-glucosidic linkages is less than that of equal molecular weight having α-(1 → 4)-glucosidic linkages [10]. Therefore, spots a and b should be **3** and **4**, respectively. (D-[U-^{14}C]Glucose was also detected at the spot of pullulan, but this may be a nonspecific binding of glucose to pullulan.) Both **3** and **4** were produced in the initial stage of the reaction, but as the reaction proceeded, **3** was degraded and **4** accumulated (Fig. 2), since the enzyme activity hydrolyzing α-(1 → 4)-linkages is higher than that toward α-(1 → 6)-linkages.

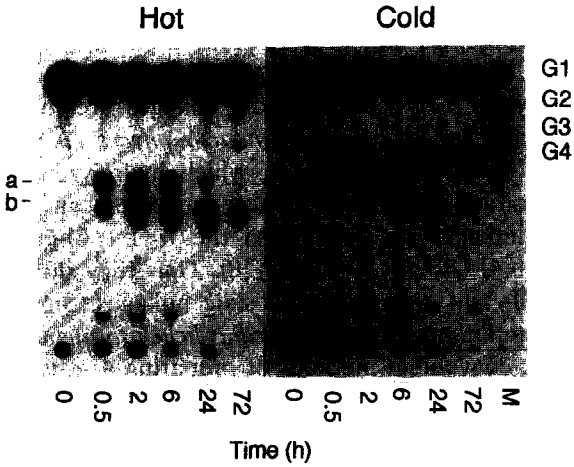

Fig. 2. A thin layer chromatogram showing the products from the incubation of pullulan and glucose with TVA I or TVA II. After development spots were detected by charring with H_2SO_4 (cold) or by a Fuji Film BAS2000 image analyzer (hot). M, Maltooligosaccharide series; G1, G2, G3, and G4, glucose, maltose, maltotriose, and maltotetraose, respectively; spots a and b, transglycosylation products.

To identify the oligosaccharides, we performed the reaction with TVA II on a large scale. Under condition *A*, described in the Experimental section, the mixture showed a pattern similar to the "2 h" lane in TLC (Fig. 2). We fractionated the mixture using a Toyopearl HW-40S gel filtration column, but could not separate the products corresponding to spots a and b. This supports the idea that these two products have similar molecular weights. To further characterize these products, the sample obtained by gel filtration was subjected to two-dimensional TLC using isomalto-dextranase. Isomalto-dextranase is an exo-type dextranase that hydrolyzes α-(1 → 2)-, α-(1 → 3)-, α-(1 → 4)-, and α-(1 → 6)-glucosidic linkages of a nonreducing isomaltose unit [11]. After the sample was applied and developed in the first direction, the lane containing the sample was uniformly sprayed with a solution of isomalto-dextranase (0.6 U/mL). Spot a released isomaltose and maltose, while spot b released only isomaltose (data not shown). These results suggest that spots a and b were Glc-α-(1 → 6)-Glc-α-(1 → *n*)-Glc-α-(1 → 4)-Glc and Glc-α-(1 → 6)-Glc-α-(1 → *n*)-Glc-α-(1 → 6)-Glc, respectively (*n* = 2, 3, 4, or 6). These sugars should include a Glc-α-(1 → 6)-Glc-α-(1 → 4)-Glc unit because one of the starting materials in this synthesis was **1**. Thus spots a and b should be Glc-α-(1 → 6)-Glc-α-(1 → 4)-Glc-α-(1 → 4)-Glc and Glc-α-(1 → 6)-Glc-α-(1 → 4)-Glc-α-(1 → 6)Glc, respectively.

The tetrasaccharide **3** was easily obtained by the method described in the Experimental section, thus we intended to obtain **4**. We therefore added a larger amount of the enzyme to promote the reaction. Under condition *B*, the TLC pattern of the mixture was similar to the 24 h lane in Fig. 2. To remove **3** completely, we carefully fractionated the eluate from the Toyopearl HW-40S column, and found that **4** eluted slightly faster than **3**.

We also employed ^{1}H NMR spectroscopy to elucidate the structure of **4**. The H-1 proton signals of the glucooligosaccharide were observed at 4.6–5.4 ppm [4,12], and these were compared with those of an authentic sample of **3**. The ^{1}H NMR spectrum of **3** is shown in Fig. 3A. The four H-1 protons gave signals labeled a–d. Tetrasaccharide **3** has one reducing centre, two α-(1 → 4)-linkages, and one α-(1 → 6)-linkage, and the ratio of the peak areas of a–d was 2:0.4:1:0.6. Peaks b and d were assigned to the α- and β-anomeric protons at the reducing end, respectively [12]. Peaks a and c were assigned to two α-(1 → 4)-linkages and one α-(1 → 6)-linkage, respectively. The ^{1}H NMR spectrum of synthetic oligosaccharide **4** is shown in Fig. 3B. Similar chemical shifts were observed for peaks a–d and a′–d′, respectively. However, the ratio of the peak areas of a′–d′ was 1:0.4:2:0.6. These results suggest this compound has one α-(1 → 4)-linkage (peak a′) and two α-(1 → 6)-linkages (peak c′). These data are consistent those expected for 4^2-α-isomaltosylisomaltose.

1. Experimental

General methods.—TLC was performed on Merck Kieselgel 60 with 5:5:3 1-BuOH–EtOH–H_2O and spots were detected by charring with H_2SO_4. When

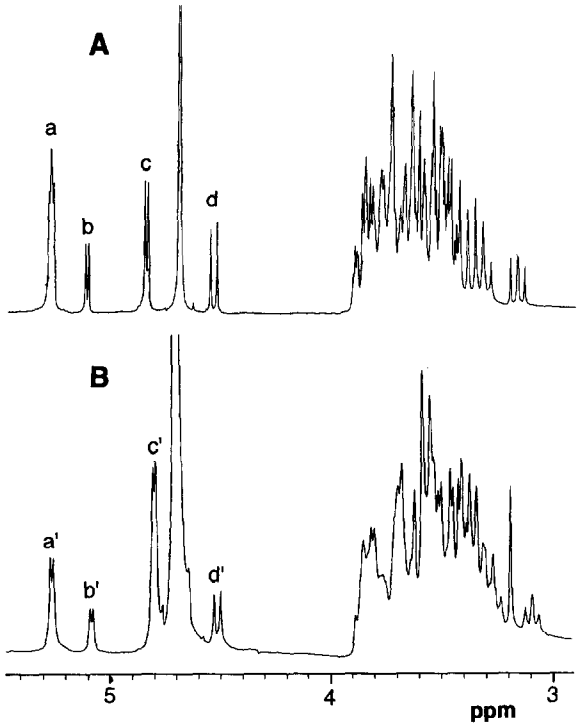

Fig. 3. ^{1}H NMR spectra of **3** and **4**. The signals of four H-1 protons were observed at 4.6–5.4 ppm (a–d and a'–d'). The peak at 4.7 ppm is due to HDO. (A) Spectrum of **3**. (B) Spectrum of **4** (the synthesized oligosaccharide).

D-[U-^{14}C]glucose was used, autoradiographical detection was achieved with a Fuji Film BAS 2000 image analyzer. ^{1}H and ^{13}C NMR spectra were recorded with a Jeol JNM-GX 270 spectrometer (270 MHz) in D_2O at room temperature. Optical rotations were determined with a Jasco DIP-360 polarimeter. Melting points were determined with a Mitamura Riken melting-point apparatus and are uncorrected. Synthetic oligosaccharides were purified on a column ($2 \times 10 \times 100$ cm) of Toyopearl HW-40S hydrophilic synthetic gel-permeation resin at 60°C. Water was the eluent at a flow rate of 10 mL/min, and the columns fractions were monitored with a differential refractometric detector (Shodex RI SE-52, Showa Denko Co., Japan)

Materials.—Cloned TVA I and TVA II were prepared from the cultures of *Escherichia coli* MV1184 (pTV93 and pTN1, respectively) as described previously [8]. Isomalto-dextranase was prepared as described by Sawai et al. [13] Pullulan (**1**) was obtained from Hayashibara Biochemical Laboratories Inc., Japan. 6^3-α-glucosylmaltotriose [**3**; Glc-α-(1 → 6)-Glc-α-(1 → 4)-Glc-α-(1 → 4)-Glc) was prepared according to the following procedure (a more detailed description will be published elsewhere). Pullulan (**1**; 25 g) was dissolved in sodium acetate buffer (1 L, pH 5.0), and digested partially using of TVA I (1.5 mL, 0.5 mg/mL) at 40°C for 10 h.

Crude Glc-α-(1 → 6)-Glc-α-(1 → 4)-Glc-α-(1 → 4)-Glc-α-(1 → 6)-Glc-α-(1 → 4)-Glc was obtained. The mixture was fractionated on the Toyopearl HW-40S column. This hexasaccharide (5 g) was then dissolved in sodium phosphate buffer (250 mL, pH 6.0), and digested using *Klebsiella pneumoniae* pullulanase (10 mL, 5 U/mL) (Hayashibara Biochemical Laboratories Inc., Japan) at 40°C overnight. A mixture of **3** [Glc-α-(1 → 6)-Glc-α-(1 → 4)-Glc-α-(1 → 4)-Glc] and maltose [Glc-α-(1 → 4)-Glc] was obtained, and was fractionated on the same column under the same conditions. The purity of **3** was confirmed by TLC and ^{1}H NMR; ^{1}H NMR data (D_2O): δ 5.29 (m, 2 H, H-1′ and H-1″), 5.13 (d, 0.4 H, $J_{1,2}$ 3.9 Hz, H-1α), 4.86 (d, 1 H, $J_{1''',2'''}$ 3.6 Hz, H-1‴), and 4.55 (d, 0.6 H, $J_{1,2}$ 8.0 Hz, H-1β).

Preliminary study using D-*[^{14}C]glucose.*—A mixture (50 μL) containing **1** (0.6 mg) and unlabeled D-glucose (0.6 mg) in sodium citrate buffer (50 mM, pH 6.0) was prepared, and 10 μl D-[U-^{14}C]glucose (10 μL; 74 kBq, 0.013%, Amersham Corp.) and TVA II (6 μL, 13 U/mL) were added. The mixture was kept at 40°C, and samples (2 μL) of the mixture were taken at appropriate times and analyzed by TLC.

4^2-α-Isomaltosylisomaltose (**4**).—Pullulan (**1**, 10 g) and D-glucose (10 g) were dissolved in sodium phosphate buffer (10 mM, 200 mL, pH 6.0). To this solution TVA II was added and the mixture was kept at 40°C under the following conditions: *A*, 1.5 mL of TVA II (29 U/mL) for 11 h; *B*, 5 mL of TVA II (29 U/mL) for 34 h. The reactions were halted by autoclaving. Under condition *A*, **2**, **3**, and **4** were obtained, and the samples were evaporated and fractionated on the Toyopearl HW-40S column. Under condition *B*, the sample contained mostly **2** and **4**. The solution was evaporated, and the resulting syrup was fractionated on the Toyopearl HW-40S column. We obtained 0.33 g of pure **4** by collection of the first one-third of the peak, as **4** eluted slightly faster than **3**; $[\alpha]_D^{20}$ +153.4° (*c* 1.0, H_2O); mp 156–157°C; ^{1}H NMR data (D_2O): δ 5.27 (d, 1 H, $J_{1'',2''}$ 4.0 Hz, H-1″), 5.09 (d, 0.4 H, $J_{1,2}$ 3.9 Hz, H-1α), 4.80 (m, 2 H, H-1′ and H-1‴), 4.52 (d, 0.6 H, $J_{1,2}$ 8.0 Hz, H-1β); ^{13}C NMR data (acetone-d_6): δ 99.3 (C-1″), 97.6 and 97.3 (C-1′ and C-1‴), 95.6 (β anomer of C-1), 91.7 (α anomer of C-1), and 76.7–60.0 (16 peaks).

Acknowledgments

We thank Professor Hiroshi Matsuzawa, The University of Tokyo, and Professor Daisaburo Fujimoto, Tokyo University of Agriculture and Technology, for useful advice and discussion. This work was supported in part by a Grant-in-Aid for Scientific Research (no. 05303010) from the Ministry of Education, Science and Culture of Japan.

References

[1] N. Katsuragi, N. Takizawa, and Y. Murooka, *J. Bacteriol.*, 169 (1987) 2301–2306.
[2] M.G. Kornacker and A.P. Pugsley, *Mol. Microbiol.*, 4 (1989) 73–85.

[3] Y. Sakano, M. Masuda, and T. Kobayashi, *Agric. Biol. Chem.*, 35 (1971) 971–973.
[4] H. Takata, T. Kuriki, S. Okada, S. Takesada, M. Iizuka, N. Minamimura, and T. Imanaka, *J. Biol. Chem.*, 267 (1992) 18447–18452.
[5] K. Igarashi, K. Ara, K. Saeki, K. Ozaki, S. Kawai, and S. Ito, *Biosci. Biochem. Biotechnol.*, 56 (1992) 514–516.
[6] K.A. Smith and A.A. Salyers *J. Bacteriol.*, 173 (1991) 2962–2968.
[7] Y. Sakano, S. Hiraiwa, J. Fukushima, and T. Kobayashi, *Agric. Biol. Chem.*, 46 (1982) 1121–1129.
[8] T. Tonozuka, M. Ohtsuka, S. Mogi, H. Sakai, T. Ohta, and Y. Sakano, *Biosci. Biochem. Biotechnol.*, 57 (1993) 395–401.
[9] I.-C. Kim, J.-H. Cha, J.-R. Kim, S.-Y. Jang, B.-C. Seo, T.-K. Cheong, D.S. Lee, Y.D. Choi, and K.-H. Park, *J. Biol. Chem.*, 267 (1992) 22108–22114.
[10] D. French and G.M. Wild, *J. Am. Chem. Soc.*, 75 (1953) 2612–2616.
[11] T. Matsuo, K. Sakakibara, A. Misaki, and T. Sawai, *Biochem. Biophys. Res. Commun.*, 70 (1976) 459–464.
[12] T. Usui, M. Yokoyama, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama, and S. Seto, *Carbohydr. Res.*, 33 (1974) 105–116.
[13] T. Sawai, K. Toriyama, and K. Yano, *J. Biochem. (Tokyo)*, 75 (1974) 105–112.