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Note

Enzymatic preparation of radiolabeled linear maltodextrins and cyclodextrins of high specific activity from [^{14}C] maltose using amylomaltase, cyclodextrin glucosyltransferase and cyclodextrinase

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Abstract

Radiolabeled linear and cyclic maltodextrins of high specific radioactivity were prepared using enzymes involved in maltodextrin metabolism. ^{14}C-Labeled maltose was the starting material yielding products of identical specific radioactivity with respect to glucosyl residues. The enzymatic steps involved: i) Formation of linear ^{14}C-labeled maltodexrins (< maltooctaose) using amylomaltase from *Escherischia coli*; ii) Cyclisation to α-cyclodextrin using cyclodextrin-glucosyltransferase of *Klebsiella oxytoca* M5a1; iii) Removal of the remaining linear dextrins by amyloglucosidase. The products were purified by paper chromatography, or maltohexaose was specifically obtained from purified α-cyclodextrin by the action of cyclodextrinase of *K. oxytoca* M5a1.

Keywords: Enzymatic synthesis; Maltodextrins; Cyclodextrins; [^{14}C]-Labeling; Transport

Radiolabeled linear and cyclic maltodextrins [(1→4)-linked α-D-glucopyranosyl oligosaccharides, α-cyclodextrins] are essential tools for the investigation of the specificity and activity of the components involved in cellular uptake and metabolism of these compounds. They can be synthesized from radiolabeled starch by degradation via specific maltodextrin-producing enzymes [1] and, for the synthesis of cyclic oligomers (cyclodextrins, CDs), by cyclization of the linear dextrins via the activity of cyclodextrin glucosyltransferases (EC 2.4.1.19) [2]. A disadvantage of this method is the attainable low specific radioactivity of starch and also the lack of information on the labeling pattern of the carbon atoms in the end product. This paper describes a method which circumvents this disadvantage since the oligomers are synthesized from uniformly labeled [^{14}C] maltose of high specific radioactivity.

The synthesis of [^{14}C] cyclomaltohexaose (α-cyclodextrin) is illustrated in Fig. 1. It began with

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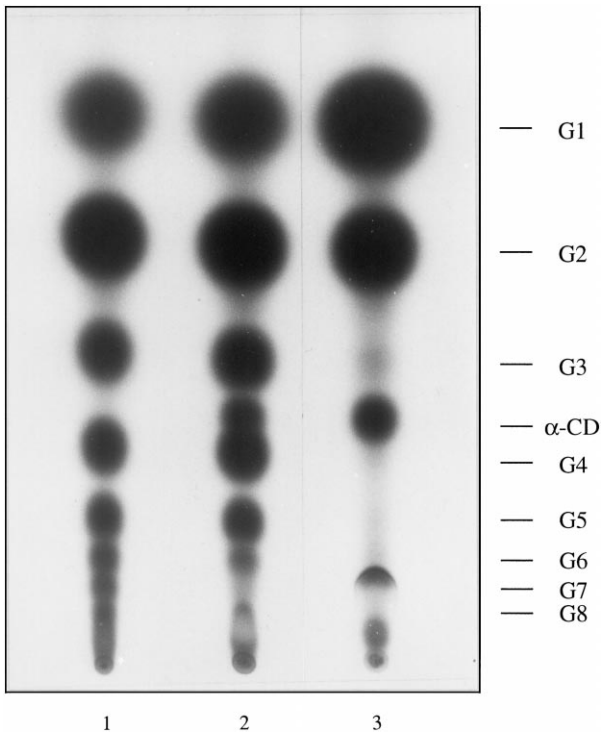

Fig. 1. Synthesis of [^{14}C] α-cyclodextrin. Lane 1: products of the reaction of amylomaltase with [^{14}C] maltose, separated by TLC and autoradiographed. Lane 2: incubation of the products of the amylomaltase reaction with cyclodextrin-glucosyltransferase from *K. oxytoca*. Lane 3: amyloglucosidase treatment of the products of the cyclodextrin–glucosyltransferase reaction. Standards: G1 D-glucose, G2 maltose etc. *a*-CD: α-cyclodextrin.

the incubation of [^{14}C] maltose with a dialyzed crude extract of an *Escherichia coli* strain overproducing amylomaltase (EC 2.4.1.25), the *malQ* gene product (lane 1). This enzyme catalyses the reaction:

$$Glc_n + Glc_m <\!\!\longrightarrow Glc_{n-x} + Glc_{m+x} \qquad (1)$$

where n is larger than 2 and m and x are equal to or larger than 1 [3]. A series of maltodextrins was resolved on TLC in addition to D-glucose and the starting material, maltose. Amylomaltase thus catalyses the formation of long chain maltodextrins. These are required for the synthesis of cyclodextrins, since the cyclodextrin glucosyltransferase in the cyclisation reaction requires maltooligosaccharides of 8 or more D-glucose units. The yield of maltodextrins is also favored by the fact that the extract from the MalQ overproducing strain possesses lesions in the *malP*, *malS* and *malZ* genes whose products are involved in degradation of maltodextrins [4–6]. The maltodextrin primers required by amylomaltase are apparently present in the crude but dialyzed extract, most likely still bound to amylomaltase. The amount of these enzyme-bound maltodextrins is minimal and consists mainly of maltodextrins longer than maltotetraose. This can be seen by incubating the extract with [^{14}C] D-glucose. Trace amounts of [^{14}C]-labeled maltodextrins can be observed in TLC which are formed by the transfer of unlabeled maltodextrinyl residues to [^{14}C]-glucose (data not shown).

The mixture of glucose and maltodextrins generated by amylomaltase [3] was then incubated with cyclodextrin glucosyltransferase from *Klebsiella oxytoca* [7,8] (Fig. 1, lane 2). This enzyme catalyses a disproportionation reaction according to Eq. 2:

$$Glc_n + Glc_m <\!\!\longrightarrow Glc_{n-x} + Glc_{m+x} \quad \text{(disproportionation)} \qquad (2)$$

where n is equal to or larger than 3 and m and x are equal to or larger than 1. With maltodextrins of a chain length larger than 8, cyclodextrins are produced according to Eq. 3

$$G_n \rightarrow G_{n-x} + cG_x \text{ (cyclisation)} \qquad (3)$$

The enzyme from *K. oxytoca* produces cG_6 (α-cyclodextrin) as the kinetically favored product which is converted later on into the thermodynamically more stable cG_7 (β-cyclodextrin). Lane 2 shows that, indeed, α-cyclodextrin is produced at the expense of long chain maltooligosaccharides.

To facilitate purification, the reaction mixture was then incubated with commercial amyloglucosidase (EC 3.2.13) which degrades the linear but not the cyclic dextrins (lane 3). The lack of cleavage of maltose was unexpected and might be due to the fact that the pH was kept at 7.2 rather than at the optimal value of 5.0. The reaction mixture was separated by preparative paper chromatography and the cyclodextrins were eluted and concentrated by lyophilization. The purity and authenticity of the [^{14}C] α-cyclodextrin was tested by degradation with purified cyclodextrinase (EC 3.2.1.54) from *K. oxytoca* [9]. This enzyme opens cyclodextrins and consecutively removes maltose units from the maltodextrin, yielding maltose as the end product in the case of α-cyclodextrin [9].

The labeled compound synthesized behaves like authentic α-cyclodextrin as shown in Fig. 2 where the degradation of [^{14}C] α-cyclodextrin was followed with non-labeled authentic α-cyclodextrin added as carrier. The chemically detected pattern of the formed products on the TLC plate was superimposable with the position of the radioactive spots seen on the autoradiogram.

The total yield of [^{14}C] cyclomaltohexaose with respect to the starting material [^{14}C] maltose was about 2%. The specific activity of the formed [^{14}C] α-cyclodextrin remained essentially the same as the starting [^{14}C] maltose, in respect to the glucosyl residues. To assume this constancy extensive dialysis of the amylomaltase-containing extract was done and only an amount sufficient to reach the equilibrium of maltodextrin formation in less than 10 min was used. Alternatively, to avoid the appearance of even traces of unlabeled glucose in the final product, the extract could be dialyzed again after the first reaction of amylomaltase with [^{14}C] maltose has taken place. This enzyme preparation contained only radiolabeled maltodextrins as primers and addition of fresh [^{14}C] maltose did not reduce the specific activity of the final product.

The rate of the linearization reaction by cyclodextrinase is higher than the subsequent cleavage of the maltodextrin produced. As a consequence, maltohexaose accumulates in the case of α-cyclodextrin which can be exploited for the production of the radiolabeled compound (Fig. 3). In this experiment, about 50% of the α-cyclodextrin was converted to maltohexaose with only minor further degradation (lane 2). Separation by preparative paper chromatography gave an essentially pure compound (lane 3).

The technique described should be applicable to the synthesis of radiolabeled β- and γ-cyclodextrins using a cyclodextrin glucosyltransferase with an appropriate product specificity [2]. Moreover, β- and γ-cyclodextrins could be used for the formation of labeled maltoheptaose and maltooctaose by linearization with cyclodextrinase.

By applying preparative paper chromatography to the reaction mixture of amylomaltase with [^{14}C] maltose (lane 1 in Fig. 1), one can easily separate and purify maltotriose and maltotetraose and obtain these compounds with the same specific radioactivity as the [^{14}C] maltose used as substrate.

In conclusion, the use of amylomaltase, cyclodextrin glucosyltransferase and cyclodextrinase provides a convenient procedure for the preparation of radiolabeled *a*-,β and γ-cyclodextrins and

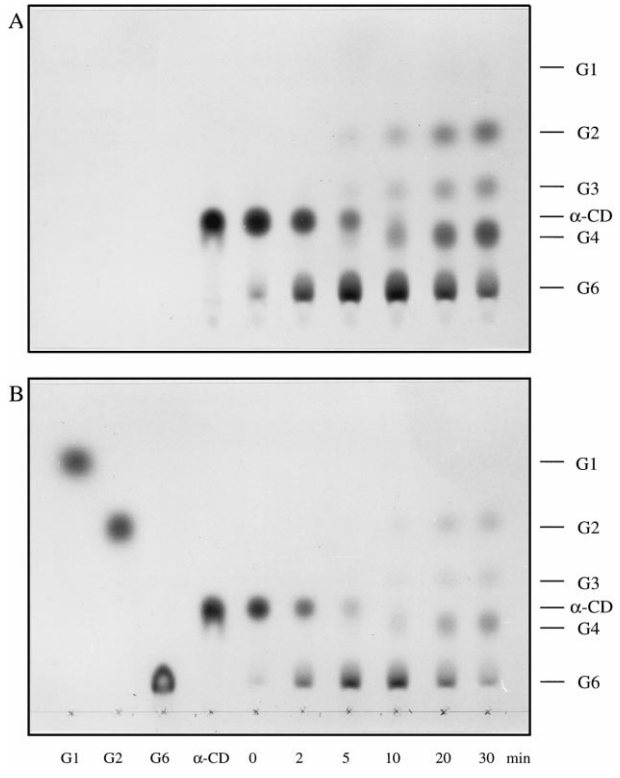

Fig. 2. Authenticity of the [^{14}C] α-cyclodextrin synthesized. A sample of [^{14}C] α-cyclodextrin was mixed with non-labeled α-cyclodextrin and incubated with cyclodextrinase from *K. oxytoca* for the times indicated. The reaction products were separated by TLC together with standards. A. Autoradiograph, B. TLC plate developed with methanol/sulfuric acid.

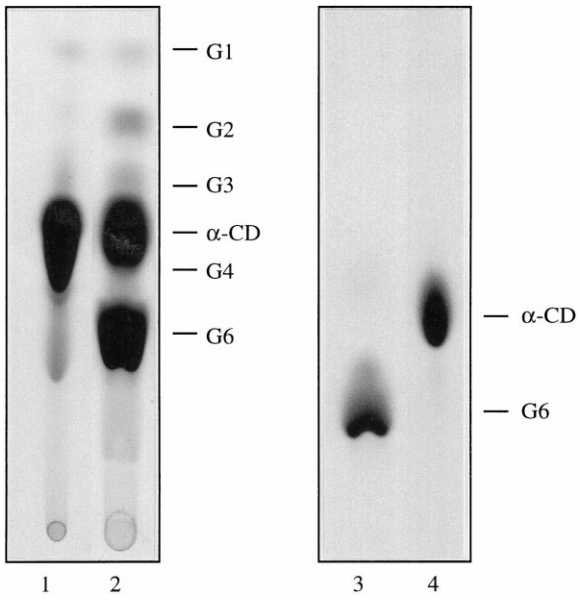

Fig. 3. Synthesis of [^{14}C] maltohexaose from [^{14}C]-α-cyclodextrin. Lanes 1 and 4: authentic α-cyclodextrin as standard; lane 2: products of the cyclodextrinase reaction; lane 3: [^{14}C] maltohexaose after preparative paper chromatography.

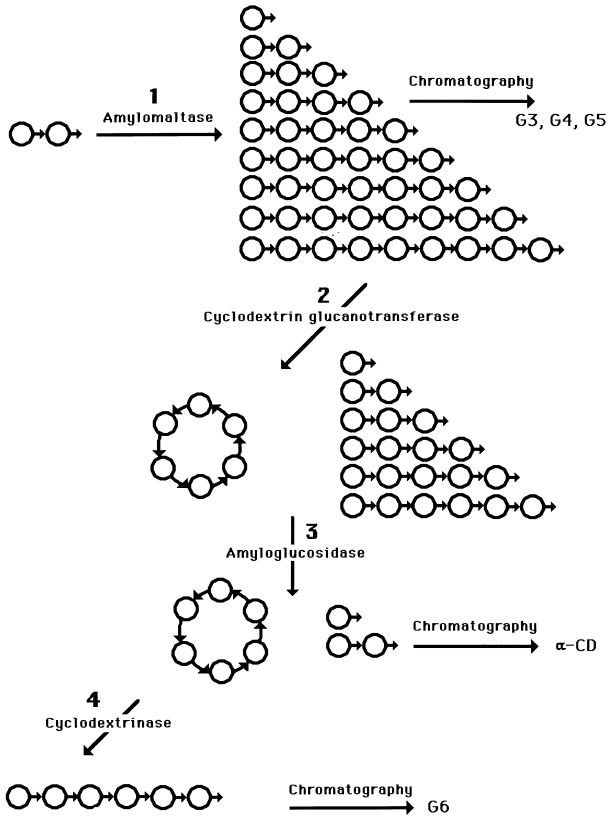

Fig. 4. Schematic representation of the enzymatic synthesis of [^{14}C]-labeled α-cyclodextrins and linear maltooligosaccharides. The circles with the arrow attached indicate α-linked D-glucose residues. Numbers 1–4 designate the different enzymatic reactions. α-CD is α-cyclodextrin; G3, G4, G5, G6, indicate maltotriose, maltotetraose, maltopentaose and maltohexaose. Reactions 1–3 of the actual experiment are represented by TLC analysis in Fig. 1, lanes 1–3.

linear maltodextrins from maltotriose up to maltooctaose. The streamlined procedure is shown in Fig. 4. The product possesses, per glucose unit, the same specific radioactivity as the substrate maltose and displays an identical carbon labeling pattern. The yield of α-CD depends on the amount of maltodextrin larger than G6 obtained in the first reaction. The above procedure gives a rather low final yield of α-CD. Improvements could be achieved by combining reactions 1 and 2 and at the same time removing the massive amounts of D-glucose formed by the amylomaltase reaction, for instance by glucokinase-dependent phosphorylation. Since the reaction of amylomaltase consists essentially of a reshuffling of α-glucosidic linkages [3], the formation of larger maltodextrins followed by cyclization to α-CD could be driven to completion by removing D-glucose from the equilibrium. Under these conditions, six molecules of maltose should form one molecule of α-CD.

1. Experimental

Reagents and materials.—All chemicals were reagent grade unless otherwise stated. The non-radioactively labeled α-cyclodextrin as well as the [^{14}C] α-cyclodextrin of low specific radioactivity (2 mCi mmol^{-1}) and the purified cyclodextrin-glucosyltransferase of *K. oxytoca* M5a1 were gifts from Wacker Chemicals (München). Linear maltodextrins as standards for thin-layer chromatography (TLC) were purchased from Sigma Chemicals (Deisenhofen), [^{14}C] maltose was supplied from Amersham (Little Chalfont, UK). Amyloglucosidase was a product from Roth (Karlsruhe), whereas the cyclodextrinase of *K. oxytoca* M5a1 was purified as described in [9]. TLC was performed on Silica Gel 60 plates (E. Merck, Darmstadt).

Synthesis of [^{14}C] cyclomaltohexaose of high specific radioactivity.—[^{14}C]-Maltose (50 μCi 630 mCi mmol^{-1}) was lyophilized in its delivery container and dissolved in 150 μL crude cellular extract (routinely 10 mg protein mL^{-1} obtained from *E. coli* strain ME469 [10] defective in *malP* (encoding maltodextrin phosphorylase) [4], *malS* (encoding α-amylase) [5], and *malZ* (encoding maltodextrin glucosidase) [6] but containing plasmid pCHAP113 harboring *malQ* (encoding amylomaltase) under IPTG-inducible promoter control [11]. For preparation of the extract, the strain had been grown in Luria-Bertani broth to an optical density (at 600 nm) of 0.8 before 0.1 mM IPTG was added. After overnight growth, the cells were harvested, resuspended in 30 mM Tris/HCl, pH 7.2 and broken by a French Pressure cell. Cell debris were removed by centrifugation at 40,000 x g for 10 min at 4 °C. The supernatant (about 30 mg protein mL^{-1}) was dialyzed extensively against 30 mM Tris/HCl, pH 7.2, in order to remove unbound maltodextrins. In accordance with the original publication of the *malQ* harboring plasmid, the extract contained not less than 6000 Units (U) mg^{-1} protein (one unit is equal to 1 μmol D-glucose produced per min at room temperature under conditions of the published assay [11]). For the use as a source of amylomaltase, the extract was diluted such that in trial runs (using unlabeled maltose as substrate) the equilibrium of products was reached within 10 min (9000 U in 150 μL). After 30 min at room temperature, the solution was transferred into an Eppendorf tube and heated to 95 °C for 5 min. The precipitate was removed by

centrifugation and washed once with 100 μL water, the supernatants were combined and $CaCl_2$ was added to a final concentration of 5 mM. The solution was preincubated at 30 °C for 5 min, 4 μL α-cyclodextrin-glucosyltransferase (previously diluted 10-fold in 30 mM Tris/HCl, pH 7.2, to attain a concentration of 0.1 U/μl) was added and incubation continued at 30 °C for 10 min. After heating to 95 °C for 5 min, the solution was cooled to 30 °C, then 53 U of amyloglucosidase were added, followed by incubation for 15 min. Finally, the solution was heated again to 95 °C, clarified by centrifugation and the supernatant applied onto Whatman 3MM paper for descending chromatography with (5:3:2 butanol–ethanol–water) as solvent. After drying, the chromatogram was autoradiographed for 4 h. Paper strips containing α-cyclodextrin were eluted with water. The different steps in the synthesis of α-cyclodextrin were followed by TLC (1 μL samples applied) with the same solvent system as above. The dried plate was autoradiographed.

To check the authenticity of the [^{14}C] α-cyclodextrin synthesized, a sample of the radioactive compound was mixed with 10 mM unlabeled compound in 50 mM potassium phosphate buffer, pH 7.0, and incubated with cyclodextrinase. The products were analyzed by TLC in the system described above. The amount of enzyme was adjusted such that the formation of linear maltohexaose could be observed within 2 min. The thin layer chromatogram was first autoradiographed and then developed by charring. The plate was dipped into MeOH containing 2% concd H_2SO_4, dried and heated at 120 °C for 10 min.

Synthesis of [^{14}C] maltohexaose.—As starting material for the synthesis of [^{14}C] maltohexaose, [^{14}C] α-cyclodextrin of low specific radioactivity (2 mCi mmol^{-1}) was used which was hydrolyzed to [^{14}C] maltohexaose and subsequently to shorter labeled dextrins by the action of cyclodextrinase. The amount of [^{14}C] α-cyclodextrin was 6 μmol corresponding to 13 μCi. The reaction was performed in a vol of 500 μl, buffered with 25 mM potassium phosphate, pH 7.0, using 0.5 U of the cyclodextrinase. After a preincubation for 5 min at 23 °C, the enzyme was added to the soln and incubation was continued for 15 min. The soln was then heated at 80 °C for 10 min and applied onto Whatman 3MM paper for descending chromatography with 3:2:2, propanol-1–EtOAc–water as solvent. After drying, the chromatogram was autoradiographed overnight. Paper strips containing maltohexaose were eluted with water and the eluate was concentrated by lyophilization.

The use of purified enzymes is not obligatory in the above described syntheses. The plasmids encoding these enzymes [8,9] can be used to transform strain ME469 and the respective extracts may be used as source of the enzymes. Amyloglucosidase is available commercially. Both strain and plasmids are available from the authors.

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