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Characterisation of a glycosylated alkyl polyglycoside produced by a cyclodextrin glycosyltransferase by HPLC–ELSD and –MS

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ABSTRACT

A transglycosylation reaction between an alkyl polyglycoside and α-cyclodextrin catalysed by cyclodextrin glycosyltransferase (CGTase) from *Bacillus macerans* was investigated. The reaction products were identified by comparison with standards generated by CGTase catalysed modification of pure alkyl glycosides using HPLC–ELSD and –MS analysis. The main products were alkyl glucopyranosides (substrates present in the alkyl polyglycoside) glycosylated with 6 (primary coupling products) or 12 (secondary coupling products) glucose residues. Both α and β anomers were glycosylated.

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1. Introduction

Alkyl glycosides constitute a very potent group of nonionic surfactants. On commercial scale, alkyl glycosides are available as alkyl polyglycosides (APG). APGs are low in toxicity and biodegradable and they are used in a wide range of applications especially within detergency and personal care [1,2]. The industrial production of these surfactants is usually based on Fischer glycosylation, where a fatty alcohol (C8–C16) is reacted with a carbohydrate (typically starch or glucose) through a condensation reaction. The Fischer process gives a very complex mixture of alkyl glycosides due to the polyfunctionality of carbohydrates.

We have developed a method using a *Bacillus macerans* cyclodextrin glycosyltransferase (CGTase) to glycosylate the carbohydrate part of alkyl glycosides, thereby producing new surfactants [3,4]. CGTase is an enzyme capable of catalysing four different reactions: cyclisation, coupling, disproportionation and hydrolysis [5]. Cyclisation is an intramolecular reaction in which cyclodextrin is synthesized from starch. In the coupling reaction, the reverse of cyclisation, a cyclodextrin is coupled to an acceptor, typically a linear glycoside adding six or more glucose residues, whereas disproportionation (intermolecular) is a reaction in which monosaccharide residue(s) are transferred between two linear carbohydrates and/or glycosides. The two latter reactions are of great interest because they can be used to add glucose residues to acceptors such as alkyl glycosides and thereby change their properties, for example increase their water solubility or even reduce cytotoxicity. We have previously carried out the coupling reaction between α-cyclodextrin and pure alkyl glycosides having 1–3 glucose residues [3,4]. In the present study we probed the acceptor specificity of the *B. macerans* CGTase for the components of a commercial APG. Due to the complexity of the original and especially the glycosylated APG, a new analytical method was developed in order to analyse the reaction mixtures.

A considerable number of papers have dealt with analysis of APG using several different techniques: colorimetric determination [6], thin-layer chromatography [7], GC–MS [8], several HPLC methods [9,10], HPLC–MS [11–13], MALDI–TOF-MS [9] and both static and LC–NMR [12]. Reversed phase HPLC has been shown useful for the separation of alkyl glycosides with regard to alkyl chain length, DP and isomer composition (α- vs. β-anomers, pyranosides vs. furanosides and (1–4) vs. (1–6) binding) [11–13].

In the present study CGTase catalysed reactions between α-cyclodextrin and commercial alkyl polyglycoside, was studied using HPLC with ELSD and MS-detection. Reaction products were identified by comparison with products obtained in the corresponding reactions with pure alkyl glycosides as acceptors and/or by detailed HPLC–MS analysis.

2. Experimental

2.1. Materials

B. macerans CGTase (EC 2.4.1.19) was purchased from Amano Enzyme Europe Ltd. (Milton Keynes, UK). Alkyl polyglycoside mixture (C10–C16, Glucopon® 600UP) was kindly

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supplied by Cognis (Monheim, Germany). *n*-Dodecyl-(1,4)-α-maltopyranoside ($C_{12}G_2\alpha$), *n*-dodecyl-(1,4)-β-maltopyranoside ($C_{12}G_2\beta$), *n*-tetradecyl-(1,4)-β-maltopyranoside ($C_{14}G_2\beta$) all of ANAGRADE® grade (Anatrace Inc., Maumee, OH, USA) and alpha-cyclodextrin ≥98% purity (α-CD) (Sigma–Aldrich, St. Louis, USA) were kindly provided by AstraZeneca AB (Lund, Sweden). Other chemicals were of pro-analysis grade from VWR International (Stockholm, Sweden). MilliQ H_2O was used throughout the study.

2.2. Enzymatic synthesis and purification of APGs with long carbohydrate chained head groups

The synthesis of modified APG mixture catalysed by *B. macerans* CGTase (EC 2.4.1.19) was performed in a 50 ml round-bottomed flask with a reaction volume of 10 ml containing 46 g/L APG (dry weight) and 391 g/L α-CD dissolved in 10 mM Na-citrate pH 5.2 containing 2 mM $CaCl_2$. Temperature was kept at 60 °C using a temperature controlled paraffin oil bath (Heidolph MR 3001K/EKT 3001, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) and the reaction mixture was agitated using a magnetic stirrer. The reaction was initiated by adding 50 μl of enzyme solution to a final concentration of 0.0219 g/L. The reaction was terminated after 60 min in boiling water for 5 min, whereupon the mixture immediately was diluted to 50 ml with 10% (v/v) methanol in H_2O. The product mixture was applied onto a C18 RP flash column (i.d.: 2.0 cm h: 20 cm) preconditioned with 10% (v/v) methanol in H_2O. The remaining unreacted carbohydrates were eluted with 300 ml of 10% (v/v) methanol in H_2O followed by 200 ml of 100% methanol to elute the modified APG product and unreacted APG acceptor. The synthesis and purification were continuously followed by means of HPLC analysis (HPLC analysis section, results not presented). From the fraction containing the product, the solvent was evaporated using a rotary evaporator (Büchi RE 111 Rotavapor, Flawil, Switzerland) at 45 °C. The precipitate was dispersed in 50 ml methanol to further remove moisture. Finally, the product was recovered by evaporating the methanol followed by precipitation using 30 ml acetone. The white crystals were isolated and placed to dry in a vacuum desiccator equipped with molecular sieves (3 Å). The yield was 0.65 g/g.

2.3. Enzymatic synthesis of products from pure alkyl glycoside acceptors

All reactions were carried out in 10 mM Na-citrate buffer pH 5.15 with an addition of 2 mM $CaCl_2$, using either 50 mM $C_{12}G_2\alpha$, $C_{12}G_2\beta$, or $C_{14}G_2\beta$ and 400 mM α-CD. The reactions were performed in 4 ml septum-capped vials using a HTMR-131 thermomixer (HLC, Bovenden, Germany) set to 60 °C and 600 rpm and initiated by adding 5 μl of *B. macerans* CGTase solution to a final concentration of 0.0219 g/L. 30 μl samples were withdrawn and diluted in 970 μl 55% (v/v) methanol in H_2O prior to heat treatment (1 min in boiling water) followed by HPLC analysis.

2.4. HPLC–ELSD-analysis

Samples collected during the experiments including the original APG substrate were analysed using a Lachrom HPLC system from Merck Hitachi (pump L-7100, interface L-7000, Autosampler L-7250 with a 20 μl injection loop), Hitachi, Ltd., Tokyo, Japan) provided with a C-8 RP column (Kromasil 100-5C8, L: 25 cm, i.d.: 4.6 mm, Eka Chemicals AB, Separation Products, Bohus, Sweden) connected to an Evaporative Light Scattering Detector (ELSD) (Alltech 500 ELSD, Alltech Associates, Inc., Deerfield, IL, USA) operating at 91 °C with a nebuliser gas flow of 2.34 standard litres per minute (SLPM). The column was eluted using a linear gradient (1 ml/min) methanol (A) and 0.1% (v/v) acetic acid in water (B). The proportion of A was increased from 55 to 98% during a period of 45 min, followed by a hold period of 1 min at 98% A. Thereafter, the amount of A was reduced to 55% A during 1 min, and kept constant for 5 min, before the next sample was injected.

2.5. HPLC–MS-analysis

A HPLC system from PerkinElmer (two PerkinElmer Series 200 Micro pumps and a PerkinElmer Series 200 Autosampler, PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA, USA) connected via a C-8 RP column (see HPLC section) and a split (1/5 of the flow goes through the MS-unit) to a API QSTAR™ Pulsar Hybrid LC/MS/MS system (a high performance hybrid quadrupole time-of-flight mass spectrometer equipped with an electrospray ionisation unit, Applied Biosystems, Foster City, CA, USA) run in positive mode was used. The mass range was set to m/z 300–2500. The ESI-voltage was set at 5.0 kV and nitrogen was used both as a nebuliser gas and as drying gas (250 °C). The same flow rate, mobile phase and gradient were used as in the HPLC–ELSD analysis. Samples of the original APG mixture received from Cognis and the enzymatically modified APG mixture were prepared by dissolving 1 mg/ml of sample in 55% (v/v) methanol in 0.1% (v/v) acetic acid in H_2O. The injection volume was set to 4 μl.

3. Results and discussions

3.1. HPLC–ELSD – development of HPLC method and characterisation

The aim of this investigation was to examine the product specificity of CGTase from *B. macerans* via transglycosylation of an APG mixture, containing alkyl glycosides with alkyl chains ranging from C10 to C16 and a carbohydrate part containing primarily one glucose residue but also minor amounts of alkyl glycosides having up to seven residues (Glucopon® 600UP, Cognis, Monheim, Germany), and α-CD. A HPLC method was developed for the qualitative analysis of the reaction mixtures based on reversed phase separation on a RP C8 column using a gradient elution and an evaporative light scattering detector.

Alkyl glycosides were separated according to their alkyl chain length and their degree of polymerisation. Furthermore, α- and β-anomers of alkyl glycosides having more than 2 glucose residues were separated because the β-anomers are slightly more hydrophobic [14]. In Fig. 1 the elution profiles for Glucopon® 600UP and enzymatically modified Glucopon® 600UP are shown. The original APG mixture is predominately made up of C_{12}-/C_{14}-glucopyranosides and only very limited amounts of C_{12}-/C_{14}-disaccharides, and other saccharides (Fig. 1A). The α- and β-anomers of the alkyl glucopyranosides were not separated and thus only single peaks were detected (peaks 1 and 2).

The product profile of modified APG is quite complex (Fig. 1B). In order to identify the major peaks, CGTase catalysed reactions between α-cyclodextrin and pure alkyl glycosides were performed. Initially coupling products were produced, with six additional glucose residues after each coupling step. However, the reactions were continued long enough for disproportionation reactions to generate complete ranges of products with one to about 20 glucose residues. The reaction based on dodecyl-(1,4)-α-maltoside ($C_{12}G_2\alpha$) thus generated a series of α-glycosides dominated by the substrate and the coupling products (primary (G_8), secondary (G_{14}) and tertiary (G_{20})), but also showing the intermediate glycosides (Fig. 2A). Likewise the other maltosides used ($C_{12}G_2\beta$ and $C_{14}G_2\beta$) generated series of products (Fig. 2B and C). In Fig. 2 the chromatograms of enzymatically modified APG and standard mixtures obtained from $C_{12}G_2\alpha$, $C_{12}G_2\beta$ and $C_{14}G_2\beta$ were aligned to enable peak

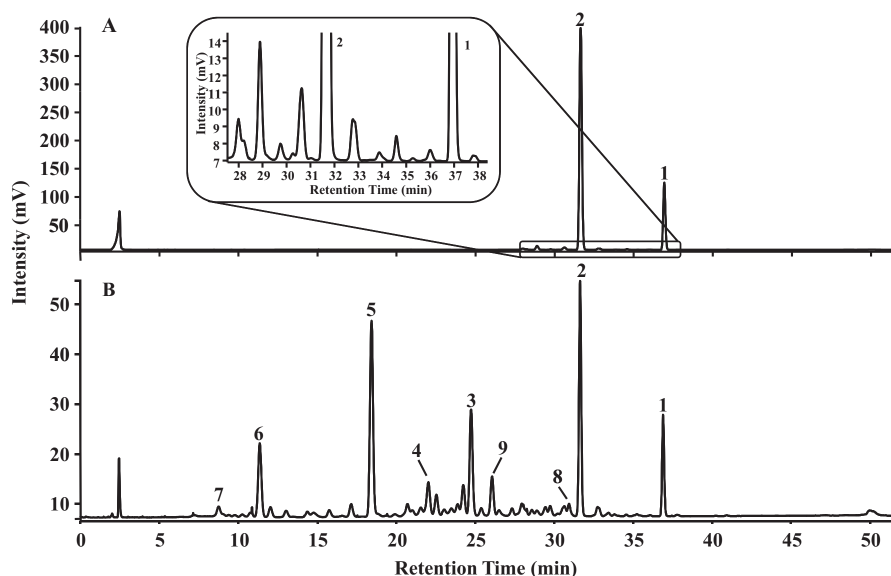

Fig. 1. HPLC–ELSD analysis of Glucopon® 600UP before (A) and after (B) enzymatic modification (20 µl injection volume, 5 mg/ml). Peak identification: (1) *n*-tetradecyl-glucopyranoside (α and β); (2) *n*-dodecylglucopyranoside (α and β); (3) *n*-dodecyl-β-maltoheptaoside; (4) *n*-dodecyl-β-maltotridecaoside; (5) *n*-dodecyl-α-maltoheptaoside; (6) *n*-dodecyl-α-maltotridecaoside; (7) *n*-dodecyl-α-maltononadecaoside; (8) *n*-tetradecyl-β-maltoheptaoside; (9) unidentified. See Figs. 3 and 4 and the text for further details.

identification. It should be noted the main products generated from the pure alkyl maltosides had 8, 14 and 20 glucose residues, while the main products from APG had 7, 13 and 19 glucose residues. It is thus the minor peaks eluting just after the major ones in Fig. 2A–C, which are supposed to coincide with the peaks in Fig. 2D. The peaks 3, 4, 5, 6 and 7 were thus tentatively identified as $C_{12}G_7\beta$, $C_{12}G_{13}\beta$, $C_{12}G_7\alpha$, $C_{12}G_{13}\alpha$ and $C_{12}G_{19}\alpha$ respectively. Peaks 3 and 4 are the first and second coupling products of $C_{12}G_1\beta$ respectively and 5–7 are the coupling products of $C_{12}G_1\alpha$. There is just a small peak (8) of the coupling product ($C_{14}G_7\beta$) of $C_{14}G_1\beta$ in the APG mixture. This is at least partly due to that the initial concentration of $C_{14}G_1\beta$ is low. Furthermore, there is a peak (9) that can be derived from $C_{14}G_1\alpha$, but according to the MS results (see below) several compounds co elute with these coupling products. Therefore it is difficult to determine the contribution of each compound.

3.2. HPLC–MS characterisation

In order to identify the products in the enzymatically modified APG with certainty, HPLC–MS analyses were carried out. The complex peak patterns of the total ion chromatograms (TIC) of the APG before and after enzymatic modification (data not shown) were resolved by extracted ion chromatograms (XIC) for each of the calculated monoisotopic *m*/*z* values for $[M+Na]^+$ and the doubly charged $[M+2Na]^{2+}$ adducts. The proportion of doubly charged adducts $[M+2Na]^{2+}$ increased with increasing degree of polymerisation (DP). This could probably be explained by that hydroxyl groups and ring oxygens are working as acceptor sites of sodium ions. [13]. In the XIC of *m*/*z* 371, corresponding to C12 monoglycosides, two peaks with almost the same height are seen (Fig. 3). As mentioned above the HPLC method cannot separate α- and β-anomers. This means that the second peak is a different isomer, which could be a glucofuranoside (G_F in Fig. 3). Eichhorn and

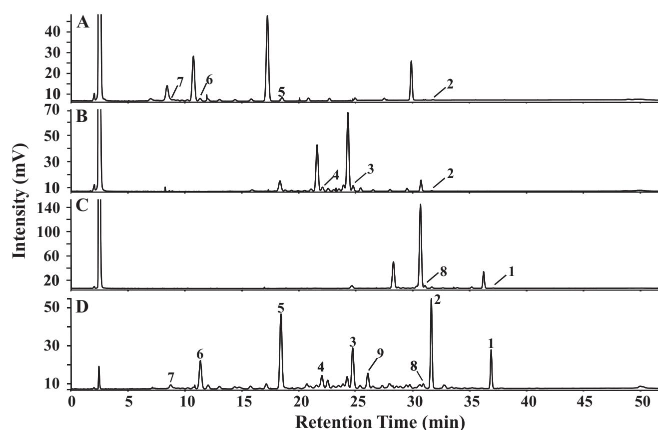

Fig. 2. Transglycosylation using *B. macerans* CGTase (0.0219 g/L) in Na-citrate buffer pH. 5.15 at 60 °C with 400 mM α-CD (donor) and four acceptors: (A) $C_{12}G_2\alpha$, (B) $C_{12}G_2\beta$, (C) $C_{14}G_2\beta$ and (D) Glucopon® 600UP. It should be noted that the main products in A–C originates from maltosides (C_nG_2) whereas the main products of D from glucosides (C_nG_1). It is thus the peak eluting subsequent to the main peaks of A–C that should be aligned with the main product peaks of D. Peak identification according to Fig. 1.

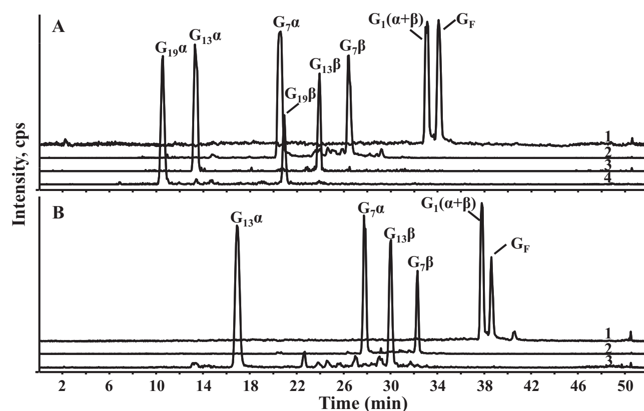

Fig. 3. (A) XIC of $C_{12}G_1$ from Glucopon® 600UP and its coupling products: (1) monoglucosides *m*/*z* 371, (2) heptaosides *m*/*z* 1343, (3) tridecaoside *m*/*z* 1167 double charged and (4) nonadecaoside *m*/*z* 1653 double charged. (B) $C_{14}G_1$ and its coupling products: (1) monoglucosides *m*/*z* 399, (2) heptaosides *m*/*z* 1371 and (3) tridecaoside *m*/*z* 1181 double charged. G_F: glucofuranoside. Due to a difference in dead volume of the two HPLC systems, the retention times were approximately 2 min longer in this analysis compared to the ELSD system (Figs. 1 and 2).

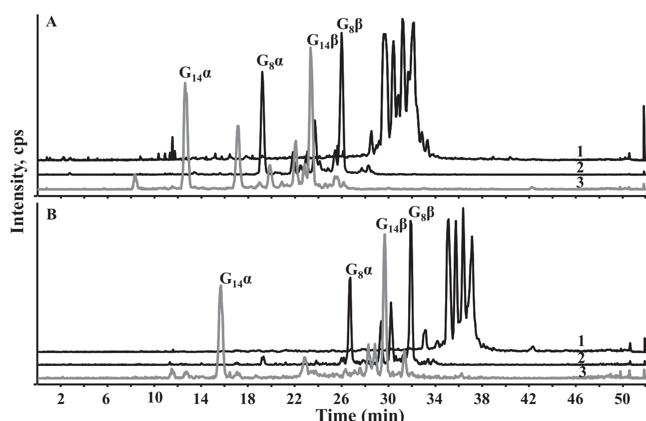

Fig. 4. (A) XIC of $C_{12}G_2$ from Glucopon® 600UP and its coupling products: (1) diglucosides *m/z* 533, (2) octaosides *m/z* 1505 and (3) tetradecaoside *m/z* 1247 double charged. (B) $C_{14}G_2$ and its coupling products: (1) diglucosides *m/z* 561, (2) octaosides *m/z* 1533 and (3) tetradecaoside *m/z* 1261 double charged. Due to a difference in dead volume of the two HPLC systems, the retention times were approximately 2 min longer in this analysis compared to the ELSD system.

Knepper have reported that in the analysis of a different APG mixture consisting mainly of C_8- and C_{10}-glycosides, there was evidence of glucofuranosides at approximately the same position in the chromatogram [11]. The concentrations of glucofuranosides are very low and therefore it is difficult to conclude anything about their acceptor quality in this system.

In Fig. 3 XIC chromatograms representing coupling products of $C_{12}G_1$ and $C_{14}G_1$ respectively are shown. Comparing these results with the results from the HPLC–ELSD study, the identity of the peaks 3–8 was confirmed. Due to a difference in dead volume of the two HPLC systems, the retention times were approximately 2 min longer in the case of MS-analysis. It should be noted that the MS-analysis did not provide information on whether the peaks belonged to the α- or β-series of products. This distinction is best done by comparison with products of known configuration (Fig. 2A–C).

Alkyl disaccharides were present in the original APG only in small amounts (Fig. 1A). It is still possible to investigate potential enzymatic modifications of these components due to the sensitivity of the HPLC–MS analysis. However, one has to realise that acceptors such as $C_nG_2\alpha$ and $C_nG_2\beta$ may not entirely originate from the original APG solution but also as products from disproportionation reaction between C_nG_1 and its coupling products. In the XICs of alkyl diglucosides $C_{12}G_2$ and $C_{14}G_2$ at *m/z* 533 and 561, a large number of incompletely resolved peaks were observed (Fig. 4). The complexity of the peak pattern of diglucosides originates from the high number of possible isomers including ring- and stereoisomers. Furthermore, the two glucose units may be linked by a (1,4)-, (1,6)-, (1,2)- or (1,3)-glucosidic bond. The XICs corresponding to the C12 and C14 octasaccharides and tetradecasaccharides are shown in Fig. 4. These compounds can be primary and secondary coupling products of alkyl disaccharides in the original APG and/or from products of disproportionation reactions between coupling products of C_nG_1 and C_nG_1. An alternative could be that these compounds are products of disproportionation reactions involving coupling products of the main substrates, $C_{12}G_1$ and $C_{14}G_1$. The primary coupling products $C_{12}G_7$ and $C_{14}G_7$, will most likely work as efficient donor molecules when present in high concentrations at long reaction times (60 min) [4]. The peaks assigned G8- and G14α or β in Fig. 4A and B, are likely to originate from a combination of the reaction mentioned.

The raw material, Glucopon® 600UP, does contain C10 and C16 glycosides although in very low concentrations. C10 glucopyranoside was detected in the raw material but not in the final product (XIC data not shown). C16 glycosides were detected in both the substrate and the final product as mono-, di- and triglucosides (XIC data not shown). However, there were no coupling products detected in the final product. According to our recent studies the acceptor selectivity of CGTase is not substantially affected by the alkyl chain length of the alkyl glycoside [3]. It is thus likely that C10 glucosides were indeed elongated in the reaction. However, the C10 glycosides are either present in too low concentrations to be detected in the analysis or they have been lost in the product purification step owing to the weak hydrophobicity of the C10 glycosides.

In conclusion, the combination of HPLC–ELSD and HPLC–MS was shown to be an excellent method to study complex mixtures of alkyl glycosides without the need for reprocessing of the samples. It was possible to identify all major peaks using commercially available standards and enzymatically produced standard mixtures. The data generated from the analyses clearly display that the *B. macerans* CGTase is prone to use both the α- and β-glucopyranosides as acceptors. The surfactant properties of the reaction products are currently studied.

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