Enzymatic route to alkyl glycosides having oligomeric head groups

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Cyclodextrin glycosyl transferase (CGTase) from *Bacillus macerans* was used to catalyse the coupling of α-cyclodextrin to alkyl β-glycosides. The acceptor substrate dodecyl β-maltoside was thus converted to dodecyl β-D-maltooctaoside. Further coupling steps and disproportionation reactions occurred, but by optimisation of the reaction time, a yield of 50% of the primary coupling product was obtained. The method worked well for a range of acceptors with different length of the carbohydrate part (1–3 glucose residues) and the hydrocarbon chain (10–14 carbon atoms). With respect to the principles of green chemistry, the method is superior to previously used methods involving protection/deprotection reactions.

Introduction

There is an increasing demand for environmentally benign surfactants and those having a carbohydrate moiety as the hydrophilic part constitute an attractive group. In addition to their excellent technical properties as surfactants, they generally have low toxicity and good biodegradability.[1] The hydrophobic part of the surfactant can be either a fatty acid residue (in carbohydrate esters) or an alcohol (in alkyl glycosides). Glycosides are preferred over carbohydrate esters in many applications because of their superior chemical stability, especially under alkaline conditions. If well-defined alkyl glycosides are desired in pure form, chemical synthesis involving protection/deprotection reactions are possible to apply. Typically, hydroxyl groups are protected by acetylation using acetic anhydride/sodium acetate at 140 °C,[2] or by protection of vicinal hydroxyl groups using acetone or phenylboronic acid as reagents. In order to synthesize the alkyl glycoside, the protected carbohydrate is then dissolved in an organic solvent in the presence of a Lewis acid catalyst. Typical catalysts used are $FeCl_3$[2] and $SnCl_4$.[3] However, due to the low reactivity of the protected oligosaccharides, more active heteropolyacids, such as phosphotungstic acid or phosphomolybdic acid have been used as catalysts instead.[2] In any case, large amounts of catalyst (50–100% by weight compared to the amount of oligosaccharide) must be used, the reaction temperature must be high (80 °C) and the yields are moderate (40–76%).[2] Furthermore, the synthesis produces a mixture of α- and β-anomers, even when a pure α anomer of the protected oligosaccharide is used as starting material. After the reaction, the protecting groups are removed, typically using sodium methoxide in methanol.[2] It is obvious that there is a lot to be gained in terms of atom economy, reduced waste generation, use of safer solvents, reduced energy consumption, etc., if these protection/deprotection reactions are replaced by enzymatic reactions in water.

Enzymes can be used as catalysts for the preparation of both carbohydrate esters and alkyl glycosides. Lipases have been used to prepare carbohydrate esters by direct esterification[4,5] or by transesterification using vinyl esters as acyl donors.[6] The reactions are relatively favourable since lipases express satisfactory catalytic activity at low water activity, which is required for a sufficiently favourable equilibrium position. Typically, rather polar solvents, such as mixtures of dimethyl sulfoxide and 2-methyl-2-butanol, are required to dissolve enough of the carbohydrate to convert it efficiently.[6] However, water must be avoided, or else hydrolytic side reactions will interfere. Recently, ionic liquids in combination with t-butanol have been used as medium for glucose ester synthesis catalysed by polyethylene glycol modified lipase.[7] Enzymatic carbohydrate ester synthesis works well with monosaccharides, but yields decrease drastically with increasing length of the carbohydrate, and trisaccharides like maltotriose seem to be the upper limit.[6]

In a strategy analogous to the one applied in the synthesis of carbohydrate esters, glycosidases can be used to make alkyl glycosides by condensation of a carbohydrate and an alcohol.[8] These reactions are hampered by solubility problems in a similar way as in carbohydrate ester synthesis, and the yield decreases with increasing chain length of the alcohol.[9] Furthermore, glycosidases require considerably higher water activity than the lipases to be catalytically active,[10,11] and as a consequence the yields are relatively low. Using dextransucrase or alternansucrase as catalyst and sucrose as glucosyl donor, the carbohydrate group of alkyl glucosides can be extended with one glucose residue at a time.[12] With this approach, butyl- and octyl-α-D- glucopyranoside were extended, but rather complex product mixtures were obtained containing alkyl glycosides with up to 4 glucose units.

In the present article, we present an enzymatic route to alkyl glycosides having seven or more monosaccharide residues, using a reaction catalyzed by cyclodextrin glycosyltransferase (CGTase) to extend the carbohydrate part of an alkyl glycoside originally having one to three monosaccharide residues. CGTase belongs to the α-amylase family (glycoside hydrolase family 13),[13] and their most well-known reaction is the production of cyclodextrins from starch. However, they can also use

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cyclodextrins as substrates and add them to suitable acceptors of carbohydrate nature in a coupling reaction[14](Fig. 1). Furthermore, CGTases can transfer oligosaccharides from a linear oligosaccharide to an acceptor (disproportionation reaction) and, to a limited extent, also hydrolyse cyclodextrins and linear substrates. The coupling reaction works quite well with pure carbohydrates as acceptors, with elongation of maltose to maltooctaose, maltotetradecaose etc. as a typical example.[15] Also derivatised carbohydrates have been shown to function as acceptors in CGTase catalysed coupling reactions. Recently, the coupling reactions between α-cyclodextrin and methyl-D-glucopyranosides (α and β) and phenyl-D-glucopyranosides (α and β) were optimized. However, the yields of the primary coupling products, methyl maltoheptaosides were below 5% and for the phenyl maltoheptaosides below 2%.[16] A CGTase from *Bacillus stearothermophilus* has been evaluated for elongation of alkyl maltosides, but it seems that no coupling reaction was achieved since the products obtained contained only 3–4 glucose units.[17]

Here, we use a CGTase from *Bacillus macerans* to catalyse a coupling reaction for the synthesis of dodecyl glycosides having carbohydrate parts containing 7 or more glucose residues.

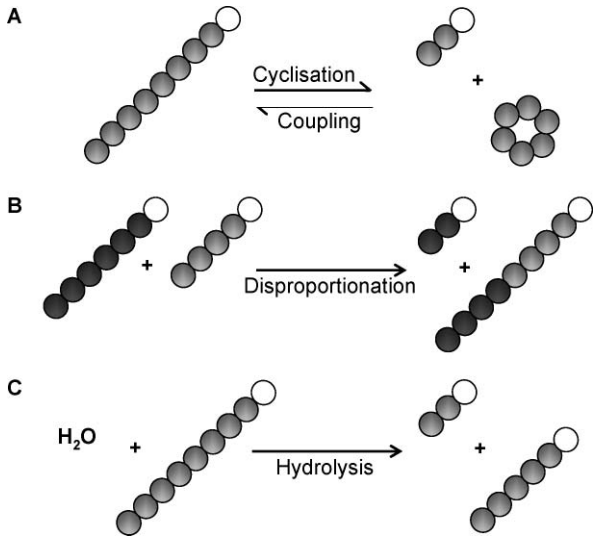


Fig. 1 CGTase catalysed reactions. White circles represent reducing end glucose residues. The other glucose residues are drawn light grey or dark grey (the two shades of grey indicate from which substrate they originate in the disproportionation reaction).

Results and discussion

CGTase catalysed elongation of alkyl glycosides was first evaluated using α-cyclodextrin (**1**) as glycosyl donor and dodecyl-β-D-maltoside (**2a**) as acceptor. After a preliminary screening of a few commercially available CGTases, the one from *Bacillus macerans* was selected because it efficiently catalysed the desired coupling reactions. Identification of the products by HPLC-MS showed that the major product was dodecyl-β-D-maltooctaoside (**2b**), the expected primary coupling product (Fig. 2 and 3). In addition, the secondary coupling products, dodecyl-β-D-maltotetradecaoside (**2c**) and dodecyl- β-D-maltoeicosaoside (**2d**) were detected. Several minor products were identified as dodecyl-β-D-maltooligosaccharides carrying various numbers of glucose residues, formed in disproportionation reactions catalysed by CGTase. After 75 minutes reaction time, individual peaks for all dodecyl-β-D-maltooligosaccharides having between 1 and 18 glucose residues were detected (Fig. 2B). For some practical applications the complex mixtures can be useful. In other cases, it will be beneficial to stop the reaction at an earlier stage to maximize the formation of one specific reaction product. The time course of a typical reaction with a clear maximum for the primary

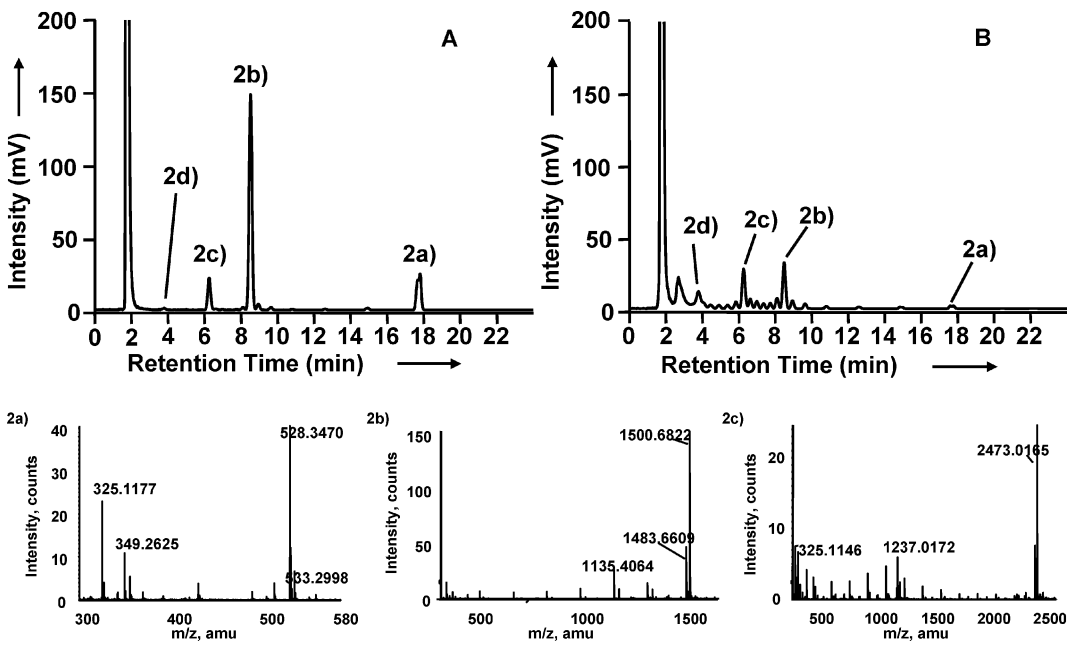
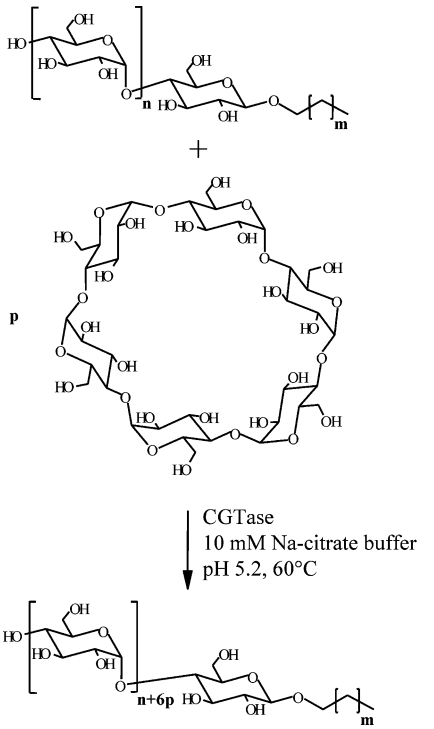


Fig. 2 HPLC chromatogram of reaction mixture from the reaction between **1**, (400 mM) and **2a** (50 mM) catalysed by CGTase at 60 °C and pH 5.2 after 25 min (A) and after 75 min (B). Mass spectra of peaks **2a**, **2b** and **2c** are shown. Products were detected as ammonium adducts.



	m	n	p	
2a	10	1	0	$C_{12}G_2$
2b	10	1	1	$C_{12}G_8$
2c	10	1	2	$C_{12}G_{14}$
2d	10	1	3	$C_{12}G_{20}$
3a	10	0	0	$C_{12}G_1$
3b	10	0	1	$C_{12}G_7$
3c	10	0	2	$C_{12}G_{13}$
4a	10	2	0	$C_{12}G_3$
4b	10	2	1	$C_{12}G_9$
4c	10	2	2	$C_{12}G_{15}$
5a	8	1	0	$C_{10}G_2$
5b	8	1	1	$C_{10}G_8$
5c	8	1	2	$C_{10}G_{14}$
6a	12	1	0	$C_{14}G_2$
6b	12	1	1	$C_{14}G_8$
6c	12	1	2	$C_{14}G_{14}$

Fig. 3 Reaction scheme. The acceptors (n = 0–2) react with **1** to form coupling products containing 6p (p = number of **1** added) additional glucose residues.

coupling product is shown in Fig. 4. The maximal concentration of the primary coupling product **2b** corresponded to a yield of 55%, and the isolated yield was 50%. It is thus clear that the *Bacillus macerans* CGTase used here is very effective in catalyzing coupling reactions.

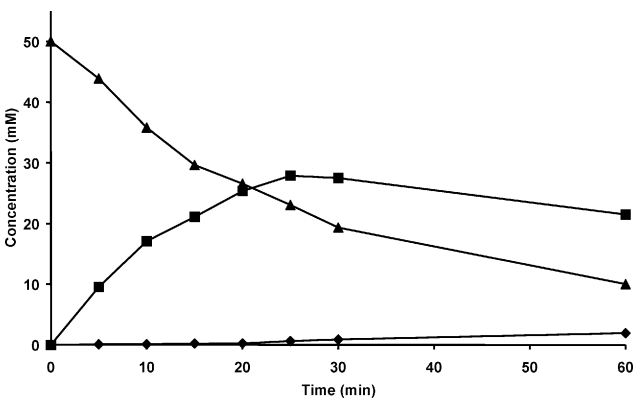


Fig. 4 Time course of the CGTase catalysed reaction between **1** (initial concentration: 400 mM) and **2a** (initial concentration: 50 mM, filled triangles), at 60 °C and pH 5.2, yielding **2b** (filled squares) and **2c** (filled diamonds).

Different technical applications call for different molecular properties of alkyl glycosides, particularly with respect to the length of the alkyl chain and of the carbohydrate part. It is thus of key importance to determine which acceptors can be used by the enzyme. The acceptor specificity was evaluated using alkyl glycosides having 1–3 carbohydrate residues since these are considerably easier to synthesize than longer ones and therefore represent reasonable starting materials for synthesis. In an experiment with equimolar amounts of three competing acceptors (dodecyl-β-D-glucoside (**3a**), **2a** and dodecyl-β-D-maltotrioside (**4a**)), all primary coupling products were detected. Among the acceptors, **2a** was converted with the highest rate, but also the other two acceptors reacted with reasonable rates (Table 1). The number of glucose residues in the acceptor is thus not of critical importance. It is of special interest that the glucoside **3a** was efficiently elongated, since this is the starting material most easily prepared by enzymatic synthesis. Likewise, the effect of the length of the hydrocarbon chain of the acceptor was evaluated. Alkyl-β-D-maltosides having hydrocarbon chains with between decyl (**5a**) and tetradecyl (**6a**) proved efficient acceptors, yielding both primary coupling products (maltooctaosides) and secondary ones (maltotetradecaosides) already after 10 minutes (Fig. 5). It is thus clear that the method presented works with a range of acceptors with differing length of both the carbohydrate and the hydrocarbon part.

Table 1 Initial rates in the CGTase catalysed reaction between **1** and a mixture of **2a**, **3a** and **4a**

Formed coupling product	Initial formation rate (μmol/(min*mg protein)
Dodecyl-β-D-maltoheptaoside (**3b**)	5.2
Dodecyl-β-D-maltooctaoside (**2b**)	10.1
Dodecyl-β-D-maltononaoside (**4b**)	6.0

In its present version, our method uses too expensive starting materials to be attractive on large scale. However, preliminary experiments show that the glycosyl donor α-cyclodextrin can

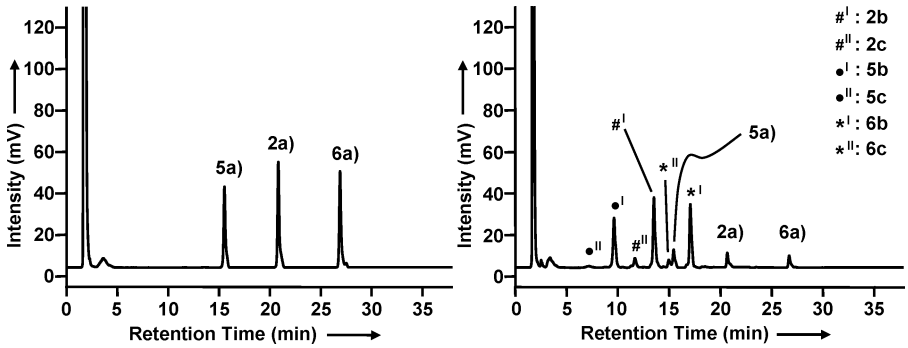


Fig. 5 Effect of hydrocarbon chain length. HPLC chromatograms before and after 10 minutes CGTase catalysed reaction between **1** and a mixture of **2a**, **5a** and **6a**. Primary coupling products **2b**, **5b** and **6b** were the main products and small amounts of secondary coupling products **2c**, **5c** and **6c** were formed as well.

be replaced by starch. In this case, the CGTase first forms α-cyclodextrin from starch and subsequently uses it in the coupling reaction as described here. However, in this case disproportionation reactions occurred to a much higher extent and the maximal yield of the primary coupling product did not exceed 10%. This approach is now under further investigation.

The method presented in the present article can be used to prepare alkyl glycosides having 7 or more glucose residues in a pure form and in good yields. By the addition of 6 glucose residues in one step, the broader product distribution obtained when one glucose residue at a time is added in reactions catalysed by dextransucrase and alternansucrase is avoided.[12] Compared to the chemical method for making alkyl glycosides having several glucose residues,[2] the method clearly represents a better alternative with respect to atom economy, waste generation, solvents characteristics, energy efficiency and other of the principles of green chemistry. To make a fair comparison, the CGTase catalysed step presented here should be considered in combination with the synthesis of the type of acceptors used in our method. As mentioned above, a direct condensation between glucose and an alcohol can be achieved using a glucosidase as catalyst[10] and thus producing alkyl glucosides, which are good acceptors for CGTase. Further research is needed to make both these enzymatic steps useful for large scale synthesis, but it seems clear that they constitute a promising "green" alternative to existing chemical methods. The use of robust glycosyl hydrolases from extremophilic organisms[18,19] can aid in improving the operational stability of the enzymatic processes and thus making them more economically viable.

Experimental section

Materials

Bacillus macerans CGTase (EC. 2.4.1.19) was purchased from Amano Enzyme Europe Ltd. (Milton Keynes, U.K.). Dodecyl maltoside (DDM) (Anatrace Inc., Maumee Ohio, USA) was kindly provided by AstraZeneca AB (Lund, Sweden), α-cyclodextrin (α–CD) was purchased from Wacker Chemie AG (Stuttgart, Germany) and other chemicals were of pro-analysi grade from VWR International.

Synthesis

Synthesis of **2b**: **2a** (205.3 mg, 402.1 μmol) and **1** (3.129 g, 3.216 mmol) were dissolved in 8 ml 10 mM Na-citrate buffer pH 5.2 (containing 2 mM $CaCl_2$) at 60 °C, conditions known to be suitable for this reaction.[20] The reaction was started by adding 40 μl *B. macerans* CGTase enzyme (Amano, 21.9μg protein/ml). During the reaction 30 μl samples were diluted to 1000 μl with acetonitrile:H_2O 50:50 and heat deactivated at 100 °C (1 min) and analysed by HPLC. No product peaks appeared after mixing the reactants in the absence of enzyme, which shows that the peaks do not represent simple inclusion complexes expected to be formed to some extent between **1** and the hydrocarbon chain of alkyl glycosides. At satisfactory conversion, the reaction was terminated by heat treatment at 100 °C for 2 minutes and diluted with 60 ml of 20:80 MeOH:H_2O solution. The mixture was fractionated using C-18 RP "flash chromatography" by gradient elution (20–75%v/v methanol-water solution) to obtain pure **2b** (298.1 mg, 50%).

Experiments using **2a**, **3a** and **4a**: **2a** (13 mg, 25.5 μmol), **3a** (7.2 mg, 20.7 μmol), **4a** (14.6 mg, 21.7 μmol) and **1** (391.3 mg, 408 μmol) were dissolved in 1 ml 10 mM Na-citrate buffer pH 5.2 (containing 2 mM $CaCl_2$) at 60 °C. The reaction was performed and analysed by HPLC as above.

Experiments using **2a**, **5a** and **6a**: **2a** (13 mg, 25.5 μmol), **5a** (12.2 mg, 25.3 μmol), **6a** (12.4 mg, 23.0 μmol) and **1** (396.9 mg, 402 μmol) were dissolved in 1 ml 10 mM Na-citrate buffer pH 5.2 (containing 2 mM $CaCl_2$) at 60 °C. The reaction was performed and samples taken as above.

HPLC analysis

HPLC analysis was carried out on a Merck Hitachi instrument (Lachrom(pump L-7100, interface L-7000, Autosampler L-7250 with a 20 μl injection loop), Hitachi, Ltd. Tokyo, Japan) equipped with an evaporative light scattering detector (Alltech 500 ELSD, Alltech Associates, Inc., Deerfield, USA). Column: Kromasil 100–5C18, (25 cm x 4.6 mm; Kromasil, Eka Chemicals AB, Separation Products, Bohus, Sweden). Elution (1 ml/min) involved a linear gradient of 35:65 to 60:40 acetonitrile:water (with 0.1% v/v acetic acid) during 23 min with a final hold time of 1 minute at 60:40 acetonitrile:water. For analysis of samples from the reaction starting with **2a**, **5a** and **6a** a gradient from

25:75 to 75:25 acetonitrile:H_2O in 42 min was used. **2a**, **2b**, **3a** and **4a** were quantified using calibration curves obtained using the pure compounds. A rough quantification of the other products was obtained from the standard curve of the most similar compound and the assumption that the detector response was proportional to the mass of the substance.

LC/EIS-QTOF-MS Analysis

To identify the different components in the product mixtures, samples were analysed on a LC/EIS-QTOF-MS system based on a HPLC system from PerkinElmer (two PerkinElmer Series 200 Micro pumps and a PerkinElmer Series 200 Autosampler, PerkinElmer Life and Analytical Sciences, Inc., Waltham, Massachusetts 02451 USA) connected *via* a C-18 RP column (see HPLC section) and a split (1/5 of the flow goes through the MS-unit) to a API QSTAR(tm) 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, USA) run in positive mode. The mass range was set to m/z 300–3000. The ESI-voltage was set at 4.9 kV and nitrogen was used both as a nebuliser gas and as drying gas (250 °C). The same injection volume, flow, mobile phase and gradient were used as in the HPLC analysis. In most cases, the substances were identified as ammonium adducts rather than sodium adducts.

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References

1 W. von Rybinsky and K. Hill, *Angew. Chem. Int. Ed.*, 1998, **37**, 1328–1345.
2 K. Katsuraya, T. Shibuya, K. Inazawa, H. Nakashima, N. Yamamoto and T. Uryu, *Macromolecules*, 1995, **28**, 6697–6700.
3 K. Katsuraya, H. Nakashima, N. Yamamoto and T. Uryu, *Carbohydr. Res.*, 1999, **315**, 234–242.
4 G. Ljunger, P. Adlercreutz and B. Mattiasson, *Biotechnol. Lett.*, 1994, **16**, 1167–1172.
5 L. Cao, A. Fischer, U. T. Bornscheuer and R. D. Schmid, *Biocatal. Biotransform.*, 1997, **14**, 269–283.
6 F. J. Plou, M. A. Cruces, M. Ferrer, G. Fuentes, E. Pastor, M. Bernabe, M. Christensen, F. Comelles, J. L. Parra and A. Ballesteros, *J. Biotechnol.*, 2002, **96**, 55–66.
7 F. Ganske and U. T. Bornscheuer, *Org. Lett.*, 2005, **7**, 3097–3098.
8 F. Van Rantwijk, M. Woudenberg-van Oosterom and R. A. Sheldon, *J. Mol. Cat. B: Enzym.*, 1999, **6**, 511–532.
9 C. Panintrarux, S. Adachi, Y. Araki, Y. Kimura and R. Matsuno, *Enzyme Microb. Technol.*, 1995, **17**, 32–40.
10 G. Ljunger, P. Adlercreutz and B. Mattiasson, *Enzyme Microb. Technol.*, 1994, **16**, 751–755.
11 T. Hansson, M. Andersson, E. Wehtje and P. Adlercreutz, *Enzyme Microb. Technol.*, 2001, **29**, 527–534.
12 G. Richard, S. Morel, R.-M. Willemot, P. Monsan and M. Remaud-Simeon, *Carbohydr. Res.*, 2003, **338**, 855–864.
13 B. Henrissat, *Biochem. J.*, 1991, **280**, 309–316.
14 B. A. Van der Veen, G. van Alebeek, J. C. M. Uitdehaag, B. W. Dijkstra and L. Dijkhuizen, *Eur. J. Biochem.*, 2000, **267**, 658–665.
15 D. Vetter and W. Thorn, *Starch/Stärke*, 1992, **44**, 229–233.
16 S. H. Yoon and J. F. Robyt, *Carbohydr. Res.*, 2006, **341**, 210–217.
17 H. Zhao, H. Naito, Y. Ueda, K. Okada, K. Sadagane, M. Izumi, S. Nakajima and N. Baba, *Biosci., Biotechnol., Biochem.*, 2008, **72**, 3006–3010.
18 T. Hansson and P. Adlercreutz, *Biotechnol. Bioeng.*, 2001, **75**, 656–665.
19 P. Turner, D. Svensson, P. Adlercreutz and E. N. Karlsson, *J. Biotechnol.*, 2007, **130**, 67–74.
20 D. Svensson, S. Ulvenlund and P. Adlercreutz, submitted.

