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α-Amylase-catalysed synthesis of alkyl glycosides

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Abstract

Alkyl glycosides were synthesised from starch and alcohols using *Aspergillus oryzae* α-amylase as catalyst. In the degradation of starch by α-amylase, the alcohols competed with water as glycosyl acceptors. In the reaction with methanol, methyl maltoside and methyl maltotrioside were the main alcoholysis products. Conversion of 45 g/l starch in 30% methanol resulted in a product mixture containing 26 mM maltooligosaccharides and 3.6 mM methyl glycosides. With ethanol, propanol and butanol, alkyl maltosides and alkyl maltotetraosides were detected, and with benzyl alcohol, benzyl glycosides having two, three or five glucose units were formed. No alcoholysis reaction occurred with hexanol or octanol. In conclusion, α-amylase is promising for the one-step synthesis of alkyl glycosides having more than one monosaccharide unit, which are difficult to synthesise in other ways.

Keywords: Alkyl glycoside; α-Amylase; Alcoholysis; Biosurfactant; Biodegradable surfactant

1. Introduction

In addition to hydrolysis reactions, some glycosyl hydrolases also catalyse various types of transglycosylation reactions. In general, these reactions proceed via a glycosyl-enzyme intermediate, which is deglycosylated either by water (hydrolysis) or another glycosyl acceptor (transglycosylation). In most cases studied to date the acceptors in transglycosylation reactions are carbohydrates, but simple alcohols and other nucleophiles can also react in this way. The transglycosylation reactions with simple alcohols produce alkyl glycosides. Some alkyl glycosides (e.g. octyl glucopyranoside and dodecyl maltoside) are widely used as surfactants, but many potentially interesting alkyl glycosides have not been evaluated as surfactants because of difficulties in synthesising them. Enzymatic methods constitute an attractive synthesis route to alkyl glycosides which has been explored only to a minor extent. Most investigations on enzymatic synthesis of alkyl glycosides have used exo-acting glycosidases either for reversed hydrolysis or transglycosylation [1–3]. The alkyl glycosides produced normally only have one monosaccharide residue. Endo-acting enzymes are of special interest for the synthesis of alkyl glycosides having more than one monosaccharide residue. α-Amylases are endo-acting enzymes that are widely used for the degradation of starch. It has been reported that α-amylases can produce methyl glycosides in addition to maltooligosaccharides when degrading starch in the presence of methanol [4]. In the reaction catalysed by *Aspergillus niger* α-amylase, all alkyl glycosides from methyl glucoside to methyl maltopentaoside were detected. Furthermore, benzyl glucoside and benzyl maltoside have been detected in the degradation of starch in the presence of benzyl alcohol and α-amylase from *Aspergillus oryzae*, barley malt or porcine pancreas [5]. In the present study, several alcohols were evaluated as acceptors in the alcoholysis of starch catalysed by *A. oryzae* α-amylase, and the reaction with methanol was studied in detail.

2. Materials and methods

2.1. Materials

α-Amylase from *A. oryzae* (prod. no. A6211), amyloglucosidase from *A. niger* (prod. no. 10113) and soluble starch (prod. no. S9765) were purchased from the Sigma–Aldrich. Other chemicals were of p.a. quality.

2.2. α-Amylase-catalysed reactions

The reaction mixtures contained soluble starch (45 g/l), different alcohols (methanol: 15%–40% (v/v), ethanol: 20%–30%

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(v/v), *n*-propanol: 20%–30% (v/v), *n*-butanol: 5%–15% (v/v), *n*-hexanol: 50 g/l, *n*-octanol: 50 g/l or benzyl alcohol: 50 g/l) and *A. oryzae* α-amylase: (50 U/ml) in aqueous buffer (pH 4.0–5.0: 50 mM potassium acetate or pH 6.0–7.0: 50 mM sodium phosphate). Separate alcohol phases were formed in the experiments with butanol (when 15% was used), hexanol, octanol and benzyl alcohol. Reactions were carried out at 50 °C with a total reaction volume of 2.5 ml. Samples were analysed using high-performance thin layer chromatography (HPTLC), either directly or after degradation with glucoamylase.

2.3. Glucoamylase-catalysed degradation of reaction products

In order to quantify the total amount of alkyl glycosides produced, the products were degraded with glucoamylase, which cleaves all glycosidic bonds except those between the alcohol and glucose. All alcoholysis products were, thus, converted to alkyl glucosides (such as α-methyl-glucopyranoside) and were quantified using HPTLC. Samples from the reaction mixtures (100 μl) were diluted with 900 μl buffer (pH 4.0–5.0: 50 mM potassium acetate, pH 6.0–7.0: 50 mM sodium phosphate). One hundred microliters of this solution was mixed with 200 μl of a solution of glucoamylase (100 U/ml). After incubation at 40 °C for 100 min, glucoamylase was inactivated by heating at 100 °C for 10 min, after which the samples were analysed with HPTLC.

2.4. HPTLC analysis

HPTLC equipment from CAMAG was used. Samples were applied to HPTLC plates (Kieselgel 60), using a CAMAG automatic TLC Sampler III. The samples were then eluted three times with a mobile phase containing butanol, ethanol and water (3:5:2). After drying, the plates were dipped in a derivatisation solution (1 g vanillin, 25 ml ethanol, 25 ml water and 35 ml 85% phosphoric acid), followed by heating at 190 °C for 6 min. The bands were quantified densitometrically using a TLC scanner from CAMAG in the absorbance/reflection mode. The slit size and wavelength were set to 4 mm × 0.3 mm and to 500 nm, respectively. Standard curves for glucose, maltose, maltotriose, maltotetraose, maltopentaose and α-methyl-glucopyranoside were used for quantification of these substances.

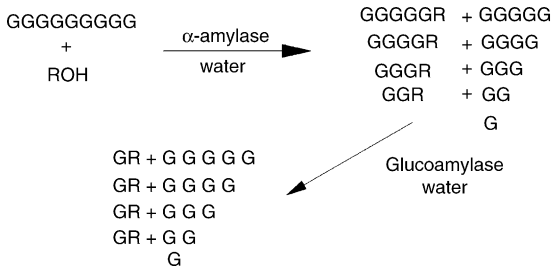
2.5. Calculation of thermodynamic activities

The thermodynamic activities of water and alcohols were needed for the determination of selectivity factors. These were calculated using UNIFAC Activity Coefficient Calculator 3.0 (University of Sydney, Australia and Louisiana State University, LA, USA).

3. Results and discussion

3.1. Identification of reaction products

A. oryzae α-amylase was incubated with aqueous solutions of starch in the presence of methanol, and the reaction mixtures were analysed with HPTLC. The dominating products were maltose, maltotriose and other oligosaccharides, but also other bands appeared, and these were suspected of being alkyl glycosides. In order to confirm the identity of the bands and to obtain a measure of the total amount of alkyl glycosides, the reaction products were treated with amyloglucosidase (Scheme 1). Samples were then analysed with HPTLC and the identity of α-methyl-glucopyranoside was confirmed by comparison with a standard. However, α-methyl-glucopyranoside was not formed in detectable amounts directly from the α-amylase-catalysed reaction. This correlates with the fact that very little glucose was formed in starch hydrolysis, and indicates that very little glucosyl enzyme is formed when α-amylase reacts with the starch substrate (glucose and α-methyl-glucopyranoside are formed from glucosyl enzyme and water or alcohol, respectively). These observations demonstrate that α-amylase is indeed an endo-acting enzyme also in methanol–water mixtures. Two new compounds with retention factors (R_f) slightly lower and higher than glucose were detected in the reaction mixtures. The distances between the bands were similar to the distances between the bands of glucose, maltose and maltotriose, and they were tentatively identified as methyl maltoside and methyl maltotrioside. Thus, maltosyl- and maltotriosyl-enzymes seem to be important intermediates in the degradation of starch by *A. oryzae* α-amylase. The product distribution of a typical reaction is shown in Fig. 1.



Scheme 1. Reactions studied: starch was degraded by α-amylase in the presence of an alcohol. The reaction products (maltooligosaccharides and alkyl glycosides) were analysed using HPTLC. In order to quantify the total amount of alkyl glycosides, the reaction products were degraded by glucoamylase to glucose and alkyl glucoside. The latter was quantified with HPTLC. G: glucose residue.

Other alcohols were evaluated as acceptors in the α-amylase-catalysed reaction where several alkyl glycosides were formed. For each alcohol, a regular pattern was observed on the HPTLC plates, which made it possible to identify the products with reasonable certainty (Fig. 2). When ethanol, propanol or butanol was used as acceptor, alkyl glycosides with two or four glucose residues were detected, and in the presence of benzyl alcohol, alkyl glycosides with two, three or five glucose residues were formed. It is possible that other alkyl glycosides were also formed in the reactions, but these were probably masked by hydrolysis products formed in large amounts with similar R_f values. With our methodology it would, for example, not be possible to detect methyl maltotetraoside since it would coincide with maltose. Furthermore, no detectable amount of alkyl glucoside was formed in any of the α-amylase-catalysed reactions,

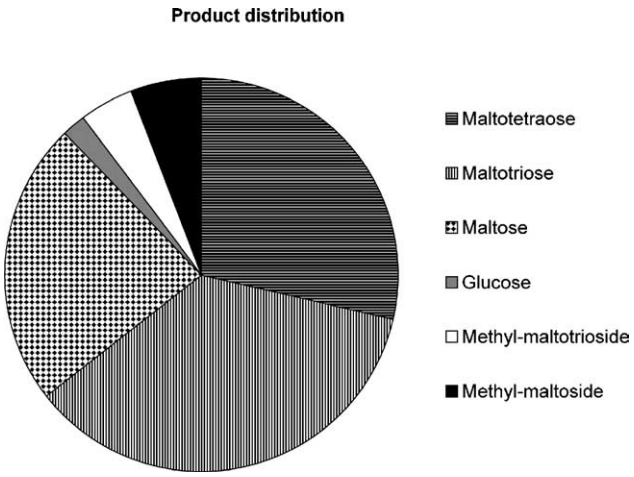


Fig. 1. Low molecular weight products after α-amylase-catalysed degradation of starch in the presence of 25% (v/v) methanol; pH 6.0 and reaction time = 18 h. Quantification was based on area% in the HPTLC analysis. After this reaction time about 70% of the starch substrate remained as high molecular weight material.

but it was, however, formed when the reaction products were degraded with glucoamylase.

No alkyl glycosides were detected in the presence of hexanol or octanol, which might be due to the low water solubility of the alcohols or to the selectivity of the enzyme.

3.2. Effects of pH and methanol concentration

The reactions involving methanol were subjected to more detailed study. It has previously been shown that pH can change the product distribution in α-amylase-catalysed alcoholysis reactions: at pH 5.0 only benzyl glucoside was formed, but at pH 7.0 benzyl maltoside was the dominating product [5]. In the present study, no significant difference in product pattern was observed at different pH values. Both the hydrolytic reaction and the formation of alkyl glycosides were fastest at pH 5–6 (results not shown). The highest ratio of alcoholysis to hydrolysis was observed at pH 6, which was used in further experiments. The rate of starch hydrolysis decreased with increasing concentration of methanol in the reaction mixture (Fig. 3a). This might be due to partial inactivation of the enzyme or to partial precipitation of the starch by the alcohol. On the other hand, the concentration of alcoholysis products was maximal at a methanol concentration of 30% (Fig. 3b).

3.3. Quantification of acceptor selectivity

The relative rates of the competing alcoholysis and hydrolysis reactions depend on the thermodynamic activities or concentrations of water and alcohol in the reaction mixture and on the intrinsic acceptor specificity of the enzyme. The use of selectivity factor (S) has been proposed to quantify the selectivity of the enzyme [2]:

$$\frac{r_s}{r_h} = S\left(\frac{a_{alc}}{a_w}\right)$$

where r_s and r_h are the rates of alcoholysis and hydrolysis and a_{alc} and a_w are the activities of alcohol and water. Ideally, the selectivity factor should be independent of the alcohol concentration, but in reality, the selectivity of the enzyme is influenced by the composition of the reaction medium, and therefore, the selectivity factors of glycosyl hydrolases can vary with the concentration of the alcohol acceptor in the reaction mixture. In the present study, the maximal selectivity factor ($S = 0.66$) was obtained at a methanol concentration of 30% (Fig. 4). Relatively few values of selectivity factors are available for comparison. However, van Rantwijk et al. [2] have made a useful compilation of some data. The selectivity factor of invertase was 8.1 for methanol and decreased with increasing size of the alcohol [2]. Moreover, several β-glycosidases have been investigated using hexanol as acceptor, reporting selectivity factors between 0.2 and 9 [3]. Likewise, for β-mannosidase from *A. niger* and ethanol, a selectivity factor of 5 was obtained [6]. Reports on β-xylosidases with ethanol as acceptor having a selectivity factor of >100 for the *A. niger* enzyme [7] and 30 for the *T. reesei* enzyme [8], show that enzymes can express quite high selectivity for alcohols, which is promising for synthesis applications. It is clear that the selectivity factor of *A. oryzae* α-amylase is lower

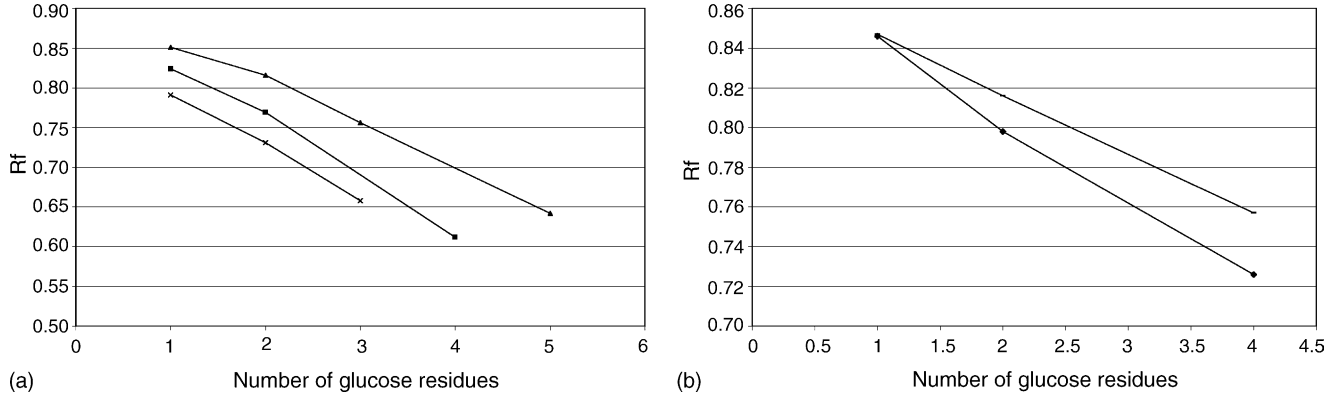


Fig. 2. R_f values from HPTLC of alkyl glycosides formed in the degradation of starch by α-amylase in the presence of alcohols, as a function of the number of glucose residues. Symbols indicate which products were detected. (a) Methyl glycosides (×), ethyl glycosides (■) and benzyl glycosides (▲); (b) propyl glycosides (♦) and butyl glycosides (–). Methyl glucoside was identified by comparison with a standard. Other glucosides were identified as major products in addition to glucose after glucoamylase degradation of reaction products. Other glycosides were tentatively identified based on their R_f values.

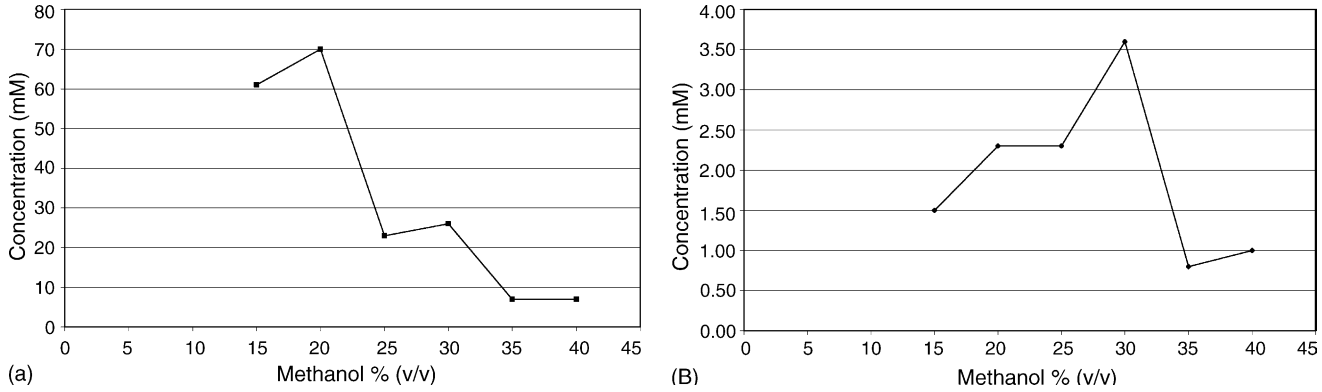


Fig. 3. Total concentrations of (a) hydrolysis and (b) alcoholysis products in the α-amylase-catalysed degradation of starch at pH 6.0, as a function of methanol concentration. Reaction time: 18 h.

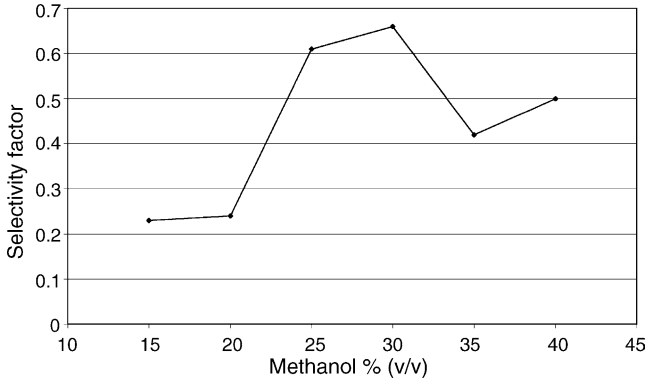


Fig. 4. Selectivity factor (*S*) in the α-amylase-catalysed degradation of starch at pH 6.0 as a function of methanol concentration. Reaction conditions as in Fig. 3.

than most other reported values. However, protein engineering work has shown that subtle changes in enzyme structure can have drastic effects on their ability to catalyse alcoholysis reactions. Furthermore, wild-type bacterial α-amylases does not catalyse the formation of methyl glycosides at all, but it is possible to introduce alcoholysis activity by site directed mutagenesis of a *Bacillus* α-amylase [9]. Likewise, a single mutation in *P. furiosus* β-glucosidase increased the selectivity factor for hexanol from 2 to 5 [10]. Based on the large amount of available structure data on α-amylases, there are probably good opportunities to design new variants useful for alcoholysis reactions.

4. Concluding remarks

A. oryzae α-amylase can use different alcohols as acceptors when starch is used as donor substrate, and various alkyl glycosides can, thus, be produced. This is of interest for the preparation of alkyl glycosides having more than one monosaccharide unit, which are difficult to prepare via other routes. Therefore, other enzymes in the α-amylase family should be investigated with regard to this kind of reactions in order to find those most suited for alcoholysis reactions. The methodology presented is useful for characterisation of the acceptor specificity of this kind of enzymes. Moreover, the enzymes' selectivity for alcohols might be improved by protein engineering.

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