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A novel variant of *Thermotoga neapolitana* β-glucosidase B is an efficient catalyst for the synthesis of alkyl glucosides by transglycosylation

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Abstract

Alkyl glycosides are surfactants with good biodegradability and low toxicity, attractive to produce by an enzymatic method to get a well-defined product. In this paper, we report a novel thermostable variant of a family 3 β-glucosidase to be an efficient catalyst in alkyl-glucoside forming reactions using transglycosylation with hexanol or octanol as the acceptor molecule. The enzyme has an apparent optimum for hydrolysis at 90 °C, which coincides with its unfolding temperature. The total activity is lower at lower temperature (60 °C), but the ratio of alcoholysis/hydrolysis is slightly more favourable. This ratio is however more heavily influenced by the water content and the pH. Optimal reaction conditions for hexyl glucoside synthesis from *p*-nitrophenyl-β-glucopyranoside were a water/hexanol two-phase system containing 16% (v/v) water, pH 5.8, and a temperature of 60 °C. Under these conditions, the total initial reaction rate was 153 μmol min^{-1} mg^{-1} and the alcoholysis/hydrolysis ratio was 5.1. Comparing with alcoholysis/hydrolysis ratios of other β-glycosidases, *Tn*Bgl3B can be considered to be a very promising catalyst for alkyl glucoside production.

Keywords: β-Glucosidase; Alkyl glycosides; Glycoside hydrolase family 3; Thermostable enzyme; Biosynthesis

1. Introduction

Alkyl glycosides are attractive surfactants because of their high surface activity, good biodegradability and low toxicity. Large scale chemical synthesis methods yield products containing a variety of related compounds, differing in number of monosaccharide units, linkages between them and type of linkage (α or β) to the alcohol. On the other hand, well-defined alkyl glycosides of high purity can be prepared using either chemical synthesis with protection/deprotection techniques and leaving group activation or preferably enzymatic synthesis (von Rybinski and Hill, 1998). The most useful enzymes for this purpose are glycosidases. Normally, these enzymes catalyse the hydrolysis of glycosides, but in an environment containing high amounts of alcohols and relatively low amounts of water, many of those enzymes can use the alcohols as acceptors (nucleophiles), resulting in the formation of alkyl glycosides. When monosaccharides are used as glycosyl donors, the alkyl glycoside forming reaction is a reversed hydrolysis reaction and thus under thermodynamic control. Higher reaction rates and higher yields can often be obtained by using activated substrates, such as disaccharides or *p*-nitrophenyl glycosides, as glycosyl donors. These transglycosylation reactions are under kinetic control, which implies that the properties of the enzyme has a large influence on the maximal yield of alkyl glycoside.

In the present study, we have focused on the formation of β-alkyl glucosides, and in order to enzymatically form β-linkages, β-glucosidases are the enzymes of choice. β-Glucosidases play a role in the carbohydrate metabolism in many organisms by acting on β-glycosidic linkages of cello-oligosaccharides containing β-D-1,4 glycosidic bonds. These enzymes are classified under 3 glycoside hydrolase (GH) families: GH1, 3 and 9, according to the classification by Coutinho and Henrissat (1999). Both GH1 and 3 are families with a retaining mechanism, dominated by enzymes acting on oligosaccharide substrates, while family 9 has an inverting mechanism and mostly contain endoglucanases. The oligosaccharide-utilising enzymes include representatives acting on a broad range of oligosaccharides such as β-D-xylosides, β-D-galactosides, β-D-fucosides and

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α-L-arabinosides (http://afmb.cnrs-mrs.fr/CAZY/). For the usage in alkyl glucoside synthesis applications, most effort has so far been put on GH1 enzymes, and several β-glucosidases from this family have previously been investigated. The most widely used and characterised representative is the commercially available almond-β-glucosidase (Andersson and Adlercreutz, 2001; Basso et al., 2002; Kobayashi et al., 2000; Kouptsova et al., 2001; Ljunger et al., 1994; Thanukrishnan et al., 2004; Vic and Crout, 1995). There are also numerous examples of thermostable β-glucosidases from the family used in synthesis reactions, e.g. β-glucosidase B from *Thermotoga maritima* (Goyal et al., 2001) and β-glucosidase from *Pyrococcus furiosus* (Hansson et al., 2001). The benefit of using family 1 enzymes is that they are well characterised and that a number of three-dimensional structures have been determined by X-ray crystallography, e.g. β-glucosidases from *Bacillus circulans* (Hakulinen et al., 2000), *Bacillus polymyxa* (Sanz-Aparicio et al., 1998), *Sulfolobus solfataricus* (Aguilar et al., 1997) and *T. maritima* (Zechel et al., 2003). There is however still a great need for finding better glycosidases than those currently tested, in order to compete with traditional chemical methods to produce alkyl glycosides.

The family 3 enzymes have not frequently been used in synthesis applications, although they, like GH1, have a retaining mechanism and several members with substantial transglycosylation activity (Crombie et al., 1998; Goyal et al., 2001; Kawai et al., 2004; Saloheimo et al., 2002; Seidle and Huber, 2005; Watt et al., 1998). GH3 glucosidases are reported to have a broad substrate specificity and are frequently active towards different kinds of glycosides, such as xylosides and aryl glucosides (Faure, 2002), but otherwise this enzyme family is not as well characterised as family 1 and there are up to now only two crystal structures known, one β-D-glucan exohydrolase from barley (Varghese et al., 1999) and a β-*N*-acetylhexosaminidase from *Vibrio cholerae* (pdb 1TR9). To get better insight into the alkyl glucoside synthesis possibilities we selected a representative from GH3, in order to evaluate its potential as a catalyst.

In this paper, we report on the cloning and production of a novel variant of a thermostable family 3 β-glucosidase from *Thermotoga neapolitana* which is highly active and efficient in catalysing the transglycosylation of *p*-nitrophenyl-β-glucoside to hexyl-β-glucoside and octyl-β-glucoside.

2. Materials and methods

2.1. Chemicals

All chemicals were pro-analysi from Merck Eurolabs (Darmstadt, Germany) unless otherwise stated.

2.2. Cloning of bglB

Genomic DNA from *T. neapolitana* (DSM strain 4359) was used as template. Primers were designed based on the coding sequence of *bglB* from *T. neapolitana* strain Z2706-MC24 (Zverlov et al., 1997) obtained from the NCBI server under the accession number Z77856. Primers were forward 5′-TAT TCT TAT *CAT ATG* GAA AAG GTGAAT GAA ATC CTG and reverse 5′-TAT TCT TTA *CTC GAG* CGG TTT GAA TCT TCT CTC C with the restriction-sites for cloning, *Nde* I and *Xho* I, respectively, in italics. The complete gene was PCR-amplified under standard conditions (94 °C 5 min; 25 cycles: 94 °C 30 s, 55 °C 30 s, 68 °C 2 min; 68 °C 7 min) on a Biometra T Gradient thermal cycler (Nordic BioSite, Täby, Sweden) using the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany) for insertion into the expression vector pET-22b(+) (Novagen, Madison, WI) incorporating the C-terminal hexa-histidine tag. The PCR products were purified with QIAEXII Gel Extraction kit (QIAGEN, Hilden, Germany) after gel separation. Both the PCR product and the vector were digested with appropriate restriction enzymes (New England Biolabs, Beverly, MA) and the vector was treated with bacterial alkaline phosphatase before being ligated to the insert using T4 DNA ligase (Invitrogen Life Technologies, Frederick, MD). The resulting plasmids were transformed into *Escherichia coli* Nova Blue cells (Novagen) and screened by colony PCR using the T7 forward and T7 reverse primers and *Taq* DNA polymerase under standard conditions. Positive clones were transformed into the *E. coli* expression host Tuner (DE3).

Similarity searches by BLAST were performed on the NCBI server (http://www.ncbi.nlm.nih.gov). The ClustalW tool on the EBI server (http://www.ebi.ac.uk/clustalw) was used to create multiple sequence alignments. The sequence encoding Bgl3B from *T. neapolitana* strain DSM 4359 is deposited under GenBank accession number DQ873691.

2.3. Expression and purification

The gene was expressed at 37 °C in a 2.5 l bioreactor during a probed temperature limited fed-batch cultivation as described previously (de Maré et al., 2005). The expression was initiated by the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, IPTG, and the production was continued for 6 h before the cells were harvested by centrifugation at 5000 × *g*, 4 °C, 5 min. The cell pellet was dissolved in binding buffer (20 mM Tris–HCl, 0.75 M NaCl, 20 mM imidazole, pH 7.5) and lysed in a Gaulin 60 high pressure homogeniser (APV-Schröder, Lübeck, Germany) at 600 bar (three cycles). The crude extract was obtained by centrifugation at 27,000 × *g*, 4 °C, 30 min. After heat treatment at 70 °C for 40 min, the protein fraction was centrifuged twice at 27,000 × *g*, 4 °C, 30 min. The his-tagged recombinant *Tn*Bgl3B was purified by immobilised metal ion affinity chromatography (IMAC). Onto a column containing 30 ml IDA-linked DEAE Sepharose CL-6B (Amersham Biosciences, Uppsala, Sweden), 60 ml 5 mg ml^{-1} copper sulphate was applied and the matrix was subsequently washed with 120 ml ultra-filtrated deionised water. Next, the column was equilibrated with 150 ml binding buffer before the heat-treated protein solution (300 ml) was loaded by gravitational flow. Unbound proteins were washed off by 90 ml binding buffer. The his-tagged β-glucosidase was eluted by passing through 80 ml 20 mM Tris–HCl, 250 mM imidazole, 0.75 M NaCl, pH 7.5, and collected in 10 ml fractions.