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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.

2.4. Enzyme assays and detection of hydrolysis and synthesis products

The purified β-glucosidase was dialysed extensively towards 20 mM citrate phosphate buffer, pH 5.6, in a Spectra/Por Membrane MWCO 3500 (Spectrum Laboratories, Rancho Dominguez, CA) before the enzyme assays.

The protein content in the purified protein sample was estimated by the BCA-copper method with BSA as standard (Sigma, Steinheim, Germany). SDS-PAGE according to Laemmli (1970) was used to analyse the purity of the enzyme.

In the standard assay for measuring the β-glucosidase activity, *p*-nitrophenol was released from *p*-nitrophenyl-β-D-glucopyranoside (pNPG). To 170 μl of 2.94 mM pNPG dissolved in 20 mM citrate phosphate buffer, pH 5.6, 30 μl of enzyme solution was added and incubated for 5 min in a Biometra T Gradient thermal cycler (Nordic BioSite, Taby, Sweden) at appropriate temperature (90 °C normally). After the completed incubation, the temperature was decreased to 4 °C and the samples were transferred to a microtitre plate, adjusted to room temperature and the absorbance was read at 405 nm in an ASYS Hitech DigiScan plate reader (ASYS Hitech, Eugendorf, Austria). One unit corresponds to the amount of enzyme that will release 1 μmol of *p*-nitrophenol per minute under the described conditions.

Kinetic parameters were determined for *p*-nitrophenyl-β-D-glucopyranoside by incubating 20 μl purified *Tn*Bgl3B (0.05 mg ml^{-1}) with 980 μl pre-warmed pNPG of varying concentrations dissolved in 20 mM citrate phosphate buffer, pH 5.6. The release of *p*-nitrophenol was observed at 90 °C and 405 nm in a UV–vis spectrophotometer UV-1650PC (Shimadzu, Kyoto, Japan). A standard curve was similarly created by incubating 20 μl *p*-nitrophenol (2–15 mM in pH 5.6) with 980 μl buffer. The Michaelis–Menten constant K_m and the apparent V_{max} are obtained from Eadie–Hofstee plots using the program UV-Probe 2.01 (Shimadzu).

To determine the synthesis ability of *Tn*Bgl3B, transglycosylation reactions, with varying water content, temperature, and pH, were performed using alcohols and *p*-nitrophenyl-β-D-glucopyranoside as substrates to produce alkyl glucosides. The influence of water was determined by adding 5–30% (v/v) of 20 mM citrate phosphate buffer, pH 5.8, containing 0.002 mg ml^{-1} *Tn*Bgl3B, to 3 ml of 34 mM pNPG in hexanol in a 4.5 ml vial provided with a septum and lid and then incubated at 60 °C on a thermo shaker (Heiz-Thermo Mixer HTMR-131, Haep Labor Consult, Bovenden, Germany). The samples were analysed by GC (see below).

The effects of temperature (60–90 °C) and pH (4–7.2) on the synthesis of alkyl glucoside were investigated by mixing 3 ml of 34 mM pNPG dissolved in hexanol with 458 μl 20 mM citrate phosphate buffer. The reaction was started with 100 μl of enzyme dissolved in 20 mM citrate phosphate buffer to give a final enzyme concentration of 0.003 mg ml^{-1} and a total water content of 16% (v/v). The samples were analysed by GC.

The effect of alcohol length was determined by dissolving pNPG (34 mM) in hexanol or octanol. The reactions were run as described above (16% (v/v) total water and 0.003 mg ml^{-1} total enzyme concentration in 3 ml pNPG/alcohol solution) with the distinction that the reactions had to be performed at 80 °C due to the low solubility of pNPG in octanol. The samples were analysed by GC.

2.5. Apparent unfolding temperature

The apparent unfolding temperature was determined by differential scanning calorimetry (DSC) by incubating varying concentrations (0.25–2 mg ml^{-1}) purified *Tn*Bgl3B in a VP-DSC (MicroCal, Northampton, MA). The temperature was increased by 1 °C min^{-1} from 25 to 100 °C and the excess heat capacity was measured.

2.6. GC analysis

Samples taken during the synthesis ability tests of *Tn*Bg13B were analysed by GC (GC-14A, Shimadzu, Kyoto, Japan) provided with a capillary column (SBP-5, 15 m × 0.32 mm, 0.25 μm Film, Supelco, Bellafonte, PA) with a temperature programme (initial temperature: 80 °C; initial time: 1 min; temperature gradient: 15 °C min^{-1}; end temperature: 330 °C; hold time: 0 min; injector temperature: 350 °C; detector temperature: 350 °C). Samples (30 μl) were withdrawn from the organic phase (alcohol phase) and collected in a 1.5 ml vial, to which 54 μl of pyridine was added. Thirty microliters of the mixture was transferred to a 1.1 ml drop vial, provided with a septum and lid. Sixty microliters of MSHFBA (*N*-methyl-*N*-trimethylsilyl heptaflourobutyramide) derivatisation reagent (Macherey-Nagel, Düren, Germany) was added and the mixture was incubated for 30 min at ambient temperature and thereafter diluted to 500 μl with pyridine. Standard curves were created for *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenol (pNP), hexyl-β-D-glucopyranoside and octyl-β-D-glucopyranoside dissolved in hexanol and octanol.

3. Results and discussion

3.1. Sequence analysis, expression and purification

Sequence analysis of the *bgl3B*-gene from the *T. neapolitana* strain DSM 4359 revealed that its open reading frame encoded a protein of 721 amino acids (with a theoretically calculated molecular weight of 81.1 kDa, excluding the 8 residue C-terminal tag added in the cloning design) that was 96% identical to the previously characterised BglB isolated from *T. neapolitana* strain Z2706-MC24 (Zverlov et al., 1997). Sequence alignment showed the main difference to be located at a 24-residue stretch close to the N-terminus in the respective primary sequence [residue 17–40 in Bgl3B from the DSM-strain (Fig. 1)]. Moreover, the two *T. neapolitana* enzyme homologues showed low sequence conservation with structure-determined representatives from the family, the highest having 19% overall sequence identity with the exoglucanase from *Hordeum vulgare* (barley). The low sequence conservation included the area surrounding the catalytic acid–base, hindering predictions of this residue. Better conservation was found in the area sur-

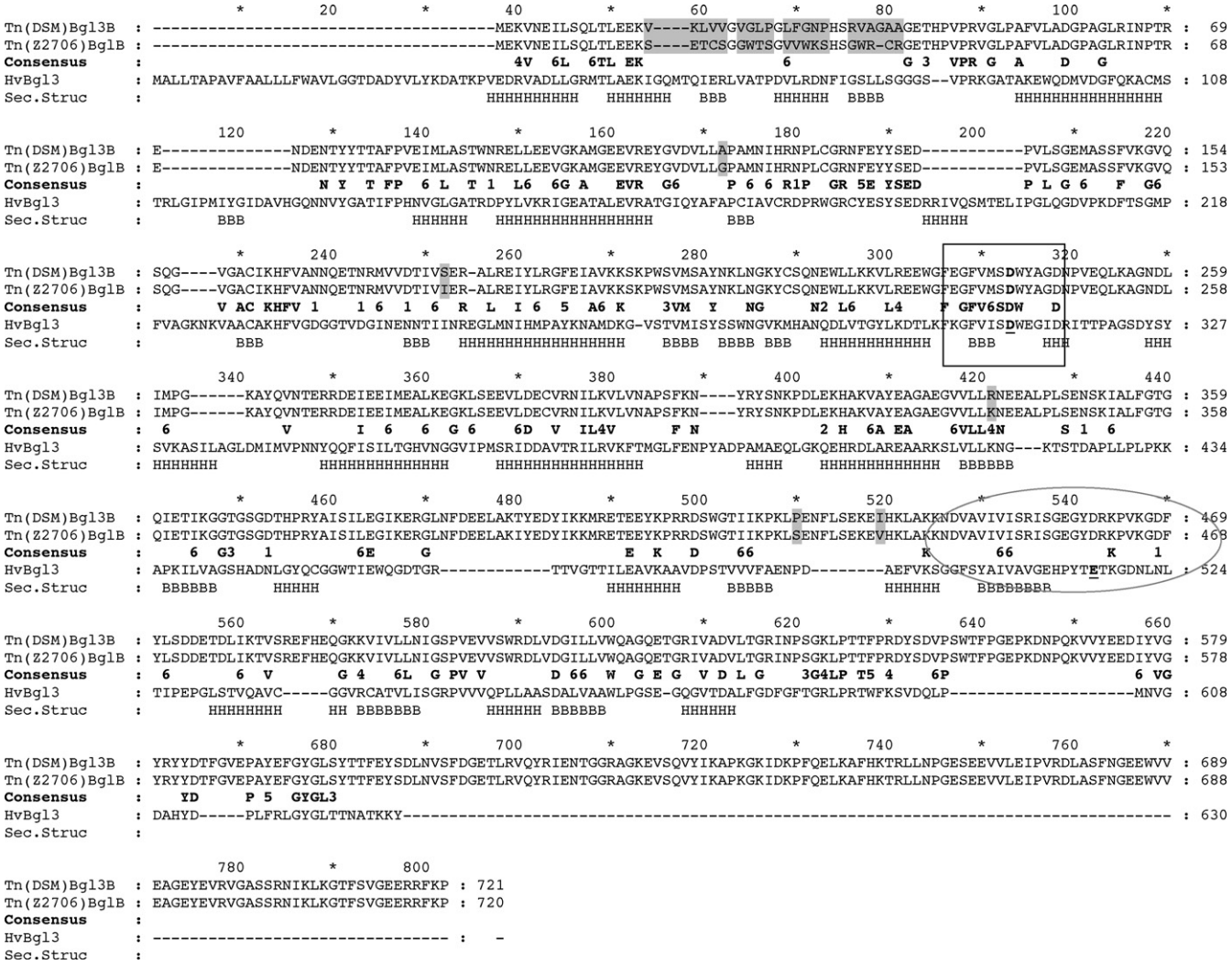

Fig. 1. Alignment of Bgl3B from *Thermotoga neapolitana* DSM 4359 [Tn(DSM)Bgl3B], with its homologue from a *T. neapolitana* strain Z2706-MC24 [Tn(Z2706)BglB], and the most similar structure-determined member of GH 3 (exoglucanase from *Hordeum vulgare* [HvBgl3]). The alignment was created using ClustalW at the EBI-server using default settings. Residues differing between the two *Thermotoga* homologues are shaded. The conserved area surrounding the nucleophile is boxed, while the area of low conservation including the acid–base of the *H. vulgare* enzyme is circled. The respective catalytic residue is underlined for the *H. vulgare* exoglucanase and its secondary structure elements are shown below the sequence for clarity (H = helix, B = β-strand). The consensus sequence shows residues conserved in all three enzymes, and similarity groups are indicated by numbers (1 = N, D; 2 = E, Q; 3 = T, S; 4 = K, R; 5 = F, Y, W; 6 = I, L, V, M).

rounding the nucleophile, which was predicted to be residue D242.

The recombinant *Tn*Bgl3B was over-expressed in *E. coli* Tuner (DE3) mainly as soluble and active protein (Fig. 2). The IPTG concentration used for induction was decreased from the standard 1 mM to 0.1 mM to be more optimal for efficient folding of the protein, and followed a protocol developed for production of a GH13 enzyme, where reduction of the IPTG concentration for gene expression under control of the T7/lac promoter was proven to be a good method for production of the active soluble enzyme (Turner et al., 2005b). Indeed, also in this case, when a higher IPTG concentration was used, more insoluble protein was formed (data not shown). Heat treatment removed a large part of the *E. coli* proteins (Fig. 2, lane 6), and parallel analysis of concentrated extracts of the same *E. coli* strain lacking the Bgl3B-encoding gene showed that this step alone was sufficient to remove all contaminating host-specific activity. The subsequent affinity chromatography by immobilised copper ions on a Sepharose column further removed remaining proteins to yield a purity of over 90% (Fig. 2, lane 7). A protein with a high purity was hence used in all characterisation and synthesis trials.

3.2. Temperature effect on activity and stability

To investigate the temperature effect on activity the purified protein reacted with *p*-nitrophenyl-β-D-glucopyranoside for 5 min at varying temperatures using a programmed temperature gradient in a thermal cycler. The optimum temperature for activity was 90 °C (Fig. 3). By differential scanning calorimetry, it was shown that the protein aggregated upon unfolding, and that the apparent unfolding temperature increased with

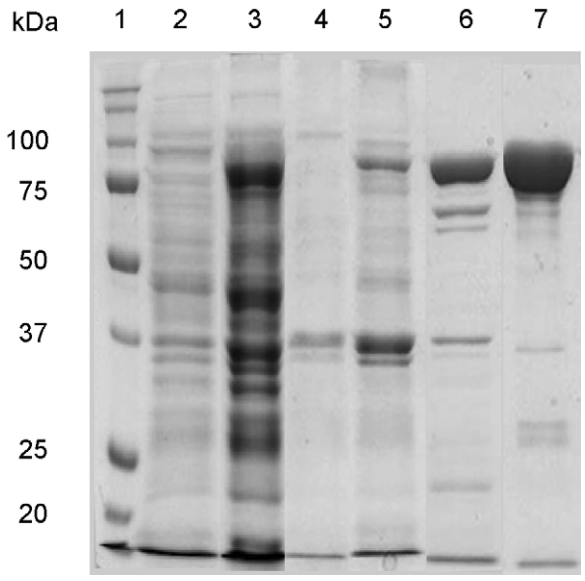

Fig. 2. SDS-PAGE analysis of expression and purification of *Tn*Bgl3B. The samples in the lanes are—lane 1: molecular weight standard Precision Plus (Bio-Rad); lane 2: soluble fraction before induction; lane 3: soluble fraction after 2 h induction; lane 4: insoluble fraction before induction; lane 5: insoluble fraction after 2 h induction; lane 6: heat-treated fraction; lane 7: protein purified by IMAC.

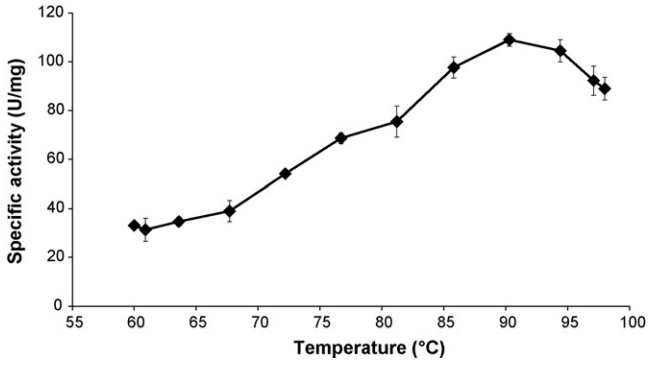

Fig. 3. Temperature effect on *Tn*Bgl3B-catalysed hydrolysis activity of *p*-nitrophenyl-β-D-glucopyranoside (2.5 mM) measuring the release of *p*-nitrophenol after 5 min incubation with 0.45 μg enzyme.

decreasing protein concentration. It was shown that the unfolding temperature increased from 86.6 to 89.7 °C when the protein concentration was decreased from 2 to 0.25 mg ml^{-1} (Table 1). The aggregation also occurred more slowly at the lower protein concentration, which can explain the fact that the apparent temperature optimum for activity was 90 °C (estimated using a concentration of 0.45 μg ml^{-1}), several degrees higher than the unfolding temperature obtained for the higher enzyme concentration (necessary for detection by DSC).

Table 1
Apparent unfolding temperatures of *Tn*Bgl3B obtained by differential scanning calorimetry at different enzyme concentrations

Enzyme concentration (mg ml^{-1})	Apparent unfolding temperature (°C)
2	86.6
1	87.8
0.5	88.0
0.25	89.7

3.3. Hydrolysis

The kinetic parameters were determined for *p*-nitrophenyl-β-D-glucopyranoside at 90 °C. One microgram of purified protein (>90% purity) was incubated with 0.01–0.75 mM pNPG and time course graphs of absorbance were obtained. The resulting values for K_m and V_{max} were 0.11 ± 0.03 mM and 93 ± 13 U mg^{-1}, respectively. This can be compared with the values reported for BglB isolated from *T. neapolitana* strain Z2706-MC24 in the earlier work by Zverlov et al. (1997), where the reported K_m of 0.1 mM is identical but with an almost three times higher V_{max} of 255 U mg^{-1}.

3.4. Synthesis of alkyl glycosides

The *Tn*Bgl3B-catalysed hexyl glucoside forming reactions were influenced to a large extent by the amount of water in the reaction mixture. Since the solubility of water in hexanol is about 6.7% (v/v) at 60 °C (the temperature chosen as most favourable, see below), total water contents below this value did not give a distinct aqueous phase and under these conditions, very low reaction rates were observed (results not shown). When enough water was present to form a distinct aqueous phase, high reaction rates were observed in a broad range of water contents with a maximum at 16% (v/v) water (Fig. 4). At even higher water content, the reaction rate decreased, possibly because mass transfer of pNPG from the organic to the aqueous phase became rate limiting. The results agree with previous observations that glycosidases generally require relatively high water activity compared to other hydrolytic enzymes. While many lipases are active down to a water activity close to 0, it has been reported that β-glucosidase from almond (Ljunger et al., 1994) and several other glycosidases (Hansson et al., 2001) require

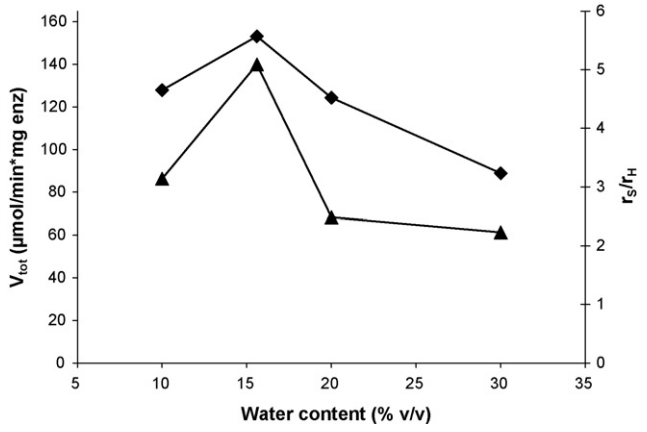

Fig. 4. The effect of water on the total initial reaction rate (squares) and on the selectivity (triangles) of *Tn*Bgl3B in the conversion of *p*-nitrophenyl-β-D-glucopyranoside to hexyl glucopyranoside and glucose at pH 5.8 and 60 °C in water/hexanol two-phase systems. The selectivity is given as the initial quotient of the rate of synthesis of hexyl glucopyranoside and the rate of hydrolysis (r_s/r_H).

a water activity of at least 0.6 to express significant catalytic activity.

When using glycosidases in transglycosylation reactions for the synthesis of alkyl glycosides, the enzyme selectivity is of prime importance. The reactions proceed via a glycosyl–enzyme intermediate, which can be deglycosylated either by water, in the normal hydrolytic reaction, or by other nucleophiles, such as alcohols, yielding alkyl glycosides. To quantify this competition between nucleophiles, the ratio between the alcoholysis and hydrolysis reactions was used in this study. In the range of water contents between 10 and 30% (v/v), this ratio varied considerably with a maximum at 16% (v/v) water (Fig. 4). This indicates that the microenvironment of the enzyme is different in these reaction mixtures although they all contain a distinct aqueous phase. This might be due to variations in local alcohol concentrations due to mass transfer limitations or to interactions between the enzyme and the interface or other components in the reaction mixtures. The optimal water content both concerning total reaction rate and alcoholysis/hydrolysis ratio was thus 16% (v/v), which corresponds to a hexanol/water two-phase system with a small aqueous phase. When the family 1 β-glucosidase from *P. furiosus* was studied in various hexanol/water mixtures, it was found that alcoholysis was maximally favoured at water contents just below water saturation, and quite low alcoholysis/hydrolysis ratios were found in hexanol/water two-phase systems (Hansson and Adlercreutz, 2001).

The reaction rate in the *Tn*Bgl3B-catalysed conversion of pNPG in hexanol/water mixtures increased with increasing temperature up to 90 °C, as was the case for hydrolysis of the same substrate in aqueous buffer as mentioned above. The ratio between alcoholysis (r_s) and hydrolysis (r_H) reaction was highest at 60 °C (the r_s/r_H-ratio was 5.1 and the total rate of pNPG conversion 153 μmol min^{-1} mg^{-1}) and decreased only slightly at higher temperature (the r_s/r_H-ratio at 90 °C was 4.5 and the total reaction rate 831 μmol min^{-1} mg^{-1} enzyme), showing that temperature does not have a large influence on the competition between water and hexanol as nucleophiles in this reaction. This agrees with a study that reported virtually no effect of the temperature on a similar reaction in hexanol/water catalysed by the β-glucosidase from *P. furiosus* (Hansson and Adlercreutz, 2001).

The pH optimum for the *Tn*Bgl3B-catalysed conversion of pNPG in hexanol/water mixtures was 5.3 concerning total reaction rate and 5.8 concerning r_s/r_H ratio (Fig. 5). The same enzyme has a pH optimum of 5.8 for the hydrolysis of quercetin-glucoside (Turner et al., 2005a). At pH 4, no activity was observed and at pH 7.2, the activity was considerably reduced. Interestingly, the ratio between alcoholysis and hydrolysis was highly influenced by pH. Both an increase and a decrease of pH from the optimal value of 5.8 caused a large reduction of this ratio (Fig. 5), thus indicating that the ionization state of the enzyme has a large impact on its nucleophile specificity.

The time course of the reaction was then studied at 60 °C and at pH 5.8 to show the product (hexyl glucoside) formation profile in relation to substrate (pNPG) consumption (Fig. 6). Hexyl glucoside was the only detectable reaction product in the hexanol phase. After 180 min reaction time, all the substrate was

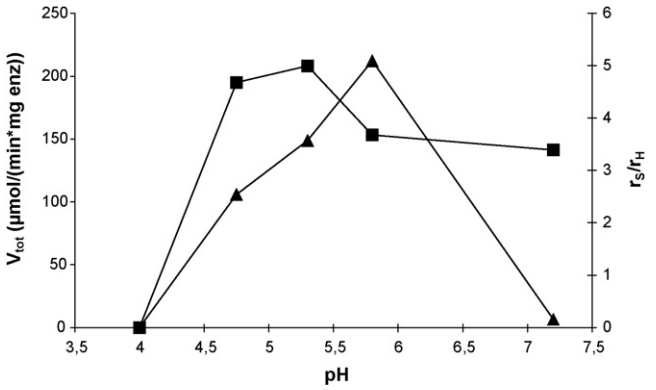

Fig. 5. The effect of pH on the total initial reaction rate (squares) and on the selectivity (triangles) of *Tn*Bgl3B in the conversion of *p*-nitrophenyl-β-D-glucopyranoside to hexyl glucopyranoside and glucose at 60 °C, pH 5–7.2 and 16% (v/v) water in water/hexanol two-phase systems. The selectivity is given as the initial quotient of the rate of synthesis of hexyl glucopyranoside and the rate of hydrolysis (r_s/r_H).

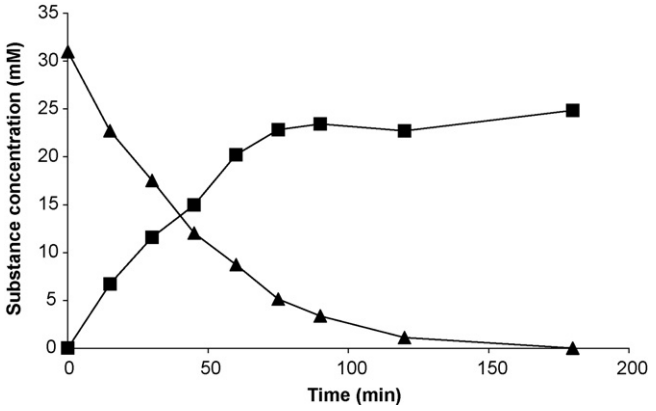

Fig. 6. The time course of the synthesis of hexyl glucopyranoside (squares) and the consumption of *p*-nitrophenyl-β-D-glucopyranoside (triangles), catalysed by *Tn*Bgl3B at pH 5.8, 60 °C and 16% (v/v) water in a water/hexanol two-phase system.

consumed and a hexyl glucoside concentration of 24.9 mM was obtained, which corresponds to a yield of 80.3%. The byproduct glucose partitioned to the aqueous phase. Analysis of this phase by high performance thin layer chromatography did not show detectable levels of glucooligosaccharides (data not shown).

When the *Tn*Bgl3B-catalysed reactions were carried out in octanol instead of hexanol, the rate of pNPG conversion was decreased with a factor of about 5 (Table 2). The reaction occurs in the aqueous phase where the enzyme is, and this phase is saturated with the alcohol. The solubility of the alcohol in water is thus a key factor to consider, and the solubility of hexanol in

Table 2
Comparison of 1-hexanol and 1-octanol as nucleophiles in the transglycosylation of pNPG in two-phase systems containing the alcohols and aqueous buffer

Alcohol	Total initial reaction rate (μmol min^{-1} mg^{-1})	r_s/r_H	Alcohol] (M)	S_c
1-Hexanol	425	4.0	0.069	58
1-Octanol	85	0.64	0.0041	156

The concentrations refer to water saturated with the alcohol.

Table 3
Ratios of alcoholysis/hydrolysis in reactions catalysed by β-glycosidases in hexanol

Enzyme	r_s/r_H	Reference
Sulfolobus solfataricus β-galactosidase (GH1)	2.5	Hansson et al. (2001)
Pyrococcus furiosus β-glucosidase (GH1)	1.7	Hansson et al. (2001)
Caldocellulosiruptor saccharolyticus β-glucosidase (GH1)	1.2	Hansson et al. (2001)
Almond β-glucosidase (GH1)	0.8	Hansson et al. (2001)
Thermotoga neapolitana β-glucosidase B (GH3)	5.1	This study

In cases when more than one ratio was reported (at different reaction conditions), the highest value was taken.

water is about 17 times that of octanol. Although no detailed studies of the influence of alcohol concentration on reaction rate were carried out, it seems clear that octanol is not a poor substrate for the enzyme and the rate difference can be caused by the solubility of the alcohol in the aqueous phase. Another important factor to consider in the synthesis of alkyl glucosides by transglycosylation is the alcoholysis/hydrolysis ratio. This was 6.3 times higher for hexanol than for octanol. Again, the difference in solubility of the alcohols in water can account for this difference in alcoholysis/hydrolysis ratio.

In order to compensate for differences in concentrations of nucleophiles and attain information on the intrinsic specificity of enzymes, the use of selectivity factors (S_c) has been proposed (van Rantwijk et al., 1999):

$$\frac{r_s}{r_H} = S_c \frac{[\text{alcohol}]}{[\text{water}]} \quad (1)$$

The rates for alcoholysis and hydrolysis are given by r_s and r_H, respectively. *Tn*Bgl3B has a quite high S_c for both hexanol and octanol. In fact, the S_c-value for octanol is three times higher than that of hexanol, which indicates that the enzyme prefers octanol over hexanol as nucleophile, when they are present in equal concentrations. It is thus clear that the enzyme is well adapted to handle octanol and that it is the low octanol concentration in the aqueous phase that is the main problem, causing lower alkyl glucoside yields compared to reactions in hexanol.

Several other β-glycosidases (all classified under GH1) have been evaluated in transglycosylation reactions similar to the ones in the present study. The enzymes have slightly different optimal reaction conditions and in order to compare them in a practical way, we have listed the maximal alcoholysis/hydrolysis ratios reported (Table 3). The family 3 enzyme *Tn*Bgl3B provided the highest alcoholysis/hydrolysis ratio among the enzymes and can thus be considered a very promising catalyst for alkyl glucoside synthesis.

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