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Preparation of two glycoside hydrolases for use in micro-aqueous media

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ABSTRACT

Enzymatic synthesis of alkyl glycosides using glycoside hydrolases is well studied, but has yet to reach industrial scale, primarily due to limited yields. Reduced water content should increase yields by limiting the unwanted hydrolytic side reaction. However, previous studies have shown that a reduction in water content surprisingly favors hydrolysis over transglycosylation. In addition, glycoside hydrolases normally require a high degree of hydration to function efficiently. This study compares six enzyme preparation methods to improve resilience and activity of two glycoside hydrolases from *Thermotoga neapolitana* (*Tn*Bgl3B and *Tn*Bgl1A) in micro-aqueous hexanol. Indeed, when adsorbed onto Accurel MP-1000 both enzymes increasingly favored transglycosylation over hydrolysis at low hydration, in contrast to freeze-dried or untreated enzyme. Additionally, they displayed 17–70× higher reaction rates compared to freeze-dried enzyme at low water activity, while displaying comparable or lower activity for fully hydrated systems. These results provide valuable information for use of enzymes under micro-aqueous conditions and build toward utilizing the full synthetic potential of glycoside hydrolases.

1. Introduction

Alkyl glycosides are a group of attractive surfactants. They exhibit antimicrobial activity, biodegradability and low toxicity [1], and find their use in cosmetics, biochemistry and pharmaceutical industry [2–4]. Currently, they are produced using conventional chemistry, which lead to a mixture of anomers [4], and require complicated separation techniques for purification. Enzymatic synthesis using glycoside hydrolases (β-glycosidases) is an attractive alternative, as it provides an anomerically pure product and reduced waste, thereby constituting a more environmentally sustainable option [5]. There are two possible enzymatic strategies: the thermodynamically controlled reverse hydrolysis or the kinetically controlled transglycosylation reaction [6].

Currently, the alkyl glycoside yields from enzymatic synthesis are too low for an economically feasible industrial process. This issue is intimately linked to the presence of water. In reverse hydrolysis the amount of water directly influences the equilibrium yield, while enzyme properties are a significant factor for transglycosylation. The catalytic mechanism involves a glycosyl-enzyme intermediate, which can be deglycosylated either by water or by alcohol, yielding hydrolysis or alkyl glycoside respectively. Therefore, the yield is determined by the acceptor specificity of the enzyme, often quantified as the ratio of transferase over hydrolase activity (r_s/r_h). Several previous studies have been aimed at increasing alkyl glycoside yield by improving r_s/r_h through protein engineering [7–10].

Another way to impair the hydrolytic side reaction, and increase the alkyl glycoside yield, is to reduce the water content in the reaction media. However, previous reports of transglycosylation, catalyzed by a wide range of β-glycosidases has, counter-intuitively, shown reduced selectivity (r_s/r_h) at low a_w [9,11,12]. In addition, most enzymes are not well suited for anhydrous conditions [13]. β-glycosidases in particular have been reported to require a water activity (a_w) as high as 0.6 [11,14], in contrast e.g. lipases such as CALB, which has been shown to retain activity at a_w as low as 0.02 [15].

In this paper, we attempt to increase the synthetic usefulness of two β-glycosidases by improving their selectivity and activity in micro-aqueous media. Six enzyme preparation methods are compared for synthesis of hexyl-β-D-glucoside (HG) from *p*-nitrophenol-β-D-glucopyranoside (*p*NPG) in hexanol. As model enzyme the β-glucosidase with the highest reported r_s/r_h, from

Abbreviations: a_w, water activity; r_s, transglycosylation rate; r_h, hydrolysis rate; HG, hexyl-β-D-glucoside; *p*NPG, *p*-nitrophenol-β-D-glucopyranoside; *p*NP, *p*-nitrophenol; AOT, dioctyl sodium sulfosuccinate.

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Thermotoga neapolitana (*Tn*Bgl3B), is used. It belongs to the glycoside hydrolase family 3 and has been reported to have very low activity in micro-aqueous media, when no enzyme preparation method was used [16]. In contrast, β-glycosidase from *P. furiosus*, belonging to glycoside hydrolase family 1, has been shown to be most active in absence of a separate aqueous phase [7]. To avoid bias from this potential discrepancy between the two glycoside hydrolase families, *Thermotoga neapolitana* enzymes from both families are studied in parallel. For lipases, up to 400-fold activation has been demonstrated by selecting a proper enzyme preparation method [17], but to the best of our knowledge, no such attempt has previously been made for β-glycosidases.

2. Materials and methods

2.1. Material

Hexyl-β-D-glucoside (HG), *p*-nitrophenol (*p*NP) and *p*-nitrophenol-β-D-glucoside (*p*NPG) were obtained from Sigma–Aldrich (St Louis, Missouri, USA) and all other chemicals from VWR International (Stockholm, Sweden).

2.2. Mutagenesis

The genes encoding *Tn*Bgl1A and *Tn*Bgl3B were previously cloned into PET22b(+) (Novagen, Madison, WI, USA) [16,18]. Mutagenesis for construction of the N220F mutant was performed in a previous study, using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), with the sequence with GenBank accession number AF039487 as the template and the primer 5′-GGAAAGATAGGGATTGTTTTCTTCAACGGATACTTCGAACCTGC-3′ [10]. The resulting plasmid was transformed into *Escherichia coli* Nova Blue cells for storage and into *E. coli* BL21 (Novagen) for expression. The complete gene was sequenced by GATC Biotech AG (Konstanz, Germany) to confirm the mutations.

2.3. Expression and purification

The enzymes were synthesized in 0.5 L cultivations of *E. coli* BL21 (Novagen) in Erlenmeyer flasks at 37 °C, pH 7 in Luria-Bertania (LB) media containing 100 μg/ml Ampicillin, inoculated with 1% over night precultures. After reaching an OD_{620} of 0.6 *Tn*Bgl1A and *Tn*Bgl3B gene expression was induced by addition of 0.5 ml 100 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and production was continued for 20 h. Cells were harvested by centrifugation for 10 min (4 °C, 5500 × *g*), re-suspended in binding buffer (20 mM imidazole, 20 mM Tris–HCl, 0.75 M NaCl, pH 7.5) and lysed by sonication 6 × 3 min at 60% amplitude and a cycle of 0.5 using a 14 mm titanium probe (UP400 S, Dr. Hielscher). Heat treatment (70 °C, 30 min) and centrifugation (30 min, 4 °C, 15,000 × *g*) was used to remove most of the native *E. coli* proteins before purification by immobilized metal affinity chromatography using an ÄKTA prime system (Amersham Biosciences, Uppsala, Sweden). The protein slurry was applied to a Histrap FF crude column (GE Healthcare) pretreated with 0.1 M Copper (II) sulphate. Bound proteins were eluted using elution buffer (250 mM imidazole, 20 mM Tris–HCl, 0.75 M NaCl, pH 7.5). Fractions containing protein were pooled and dialyzed against 50 mM citrate phosphate buffer, pH 5.6, over night using a 3500 Da molecular weight cut-off dialysis membrane (Spectrum laboratories, Rancho Dominguez, CA, USA) and stored at −20 °C until use. Purity of the expressed proteins was estimated using SDS-PAGE according to Laemmli [19].

2.4. Lyophilization

The glycosidases were diluted up to 1 ml in 0.1 M citrate phosphate buffer, pH 5.6 to roughly 0.25–0.30 mg/ml and centrifuged to remove insoluble residues. The supernatants were immediately frozen at −80 °C and then freeze-dried for 18 h.

2.5. Surfactant modification

A reverse micellar system was created according to a previously described method [20]. 150 μl suspensions of 0.6–0.85 mg/ml glycosidase in 0.1 M citrate phosphate buffer, pH 5.6 was added to 5 ml 100 mM dioctyl sodium sulfosuccinate (AOT) in 2,2,4-trimethylpentane and shaken vigorously. The trimethylpentane was removed by rotary evaporation and the residue was further dried in a vacuum desiccator.

2.6. Factorial immobilization test

The influence of buffer strength, pH and incubation time for adsorption and covalent immobilization of *Tn*Bgl1A and *Tn*Bgl3B on the supports listed in sections 2.7 and 2.8 was tested using a 2^3 factorial design. The software Minitab® (Release 14.1) was used to evaluate the data. Three factors were studied (buffer strength 0.05, 0.15 and 0.25 mM; buffer pH 4, 5.5 and 7 and incubation time 1, 7 and 24 h). Two replicates and 4 central points was used giving a total number of 20 runs per enzyme.

2.7. Adsorption

Both glycosidases were immobilized by adsorption to a hydrophobic support (Accurel MP-1000) and an anion-exchange resin (IRA-400). 7 ml 0.09–0.11 mg/ml enzyme in 0.1 mM citrate phosphate buffer, pH 5.6 was added to 400 mg Accurel MP-1000, which was pre-wetted with 3 ml ethanol/g support, and to 400 mg IRA-400, which was pre-washed with 0.1 mM citrate phosphate buffer, pH 5.6. The enzyme and support was incubated on a nutating mixer overnight and thereafter filtered and washed with buffer. Finally, the preparations were dried in a vacuum desiccator. For the MP-1000 support, a milder drying technique previously described by Moore et al. was also evaluated [21]. After removing the aqueous enzyme solution, the support was washed three times with n-propanol, the same volume as the original aqueous solution, set to the desired water activity. This was followed by two washes with the same volume of hexanol, set to the desired water activity. The hexanol was removed immediately prior to addition of substrate.

2.8. Covalent immobilization

400 mg epoxy-activated matrix, Eupergit® C250L, was washed with 0.1 mM citrate phosphate buffer, pH 5.6. 7 ml 0.09–0.11 mg/ml enzyme in 0.1 mM citrate phosphate buffer, pH 5.6 was added, incubated on a nutating mixer overnight and thereafter filtered, washed with buffer and dried in a vacuum desiccator.

2.9. Protein determination

Total protein concentration was estimated at 595 nm by the Bradford method [22] using bovine serum albumin as standard.

2.10. Water activity

Substrate solutions (34 mM *p*NPG in hexanol) were incubated over saturated salt solutions to defined water activities. The salts used for equilibration were KCH_3CO_2 (a_w = 0.23), $MgCl_2$ (a_w = 0.33), $Mg(NO_3)_2$ (a_w = 0.53), NaCl (a_w = 0.75), KCl (a_w = 0.84) and K_2SO_4