Enhanced Transglucosylation/Hydrolysis Ratio of Mutants of *Pyrococcus furiosus* β-Glucosidase: Effects of Donor Concentration, Water Content, and Temperature on Activity and Selectivity in Hexanol

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Abstract: The transglucosylation reaction catalyzed by wild-type β-glucosidase CelB from hyperthermophilic *Pyrococcus furiosus* and active site mutants (M424K, F426Y, M424K/F426Y) was studied. The conversion of pentyl-β-glucoside to hexyl-β-glucoside in hexanol was used as a model transglucosylation reaction. Hydrolysis to glucose was a side reaction. The selectivity towards transglucosylation was quantified by the S value defined as follows:

$$S = \frac{r_S \cdot a_w}{r_H \cdot a_{hex}}$$

where r_S and r_H are the initial rates of transglucosylation and hydrolysis and a_w and a_{hex} are the thermodynamic activities of water and hexanol. The activity (rates of hydrolysis and transglucosylation) and the selectivity (S value) were measured as a function of pentyl-β-glucoside concentration (5–240 mM), water content (1–100% v/v), and temperature (50–95°C). All mutants had lower activity than the wild-type enzyme, but they had higher selectivity, which means that they provided a higher ratio of transglucosylation product to hydrolysis product. The largest increase in S-value (2.6 fold) was obtained by the F426Y mutant, which resulted in increased hexyl-β-glucoside yield from 56% to 69%. In addition, the F426Y enzyme had higher selectivity over the wide range of temperatures tested. The activity of CelB wild-type and CelB F426Y increased as a function of water activity (a_w), and complete activation by the water was obtained in a two-phase system with 20% water phase. In contrast to CelB wild-type, the F426Y mutant had transferase activity as low as a_w = 0.29. Surprisingly, the S value increased with increasing water activity up to a_w = 0.92. At still higher water content the S value decreased. *Biotechnol Bioeng* **75**: 656–665, 2001.
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INTRODUCTION

Hydrolases, which in nature catalyze hydrolysis reactions, have also proven to be quite useful catalysts for transferase-type reactions (transglycosylation, transesterfication, etc.), with important applications in the synthesis of peptides (Schellenberger and Jakubke, 1991), lipids (Adlercreutz, 1994), glycosides (van Rantwijk et al., 1999), and oligosaccharides (Bucke, 1996). Enzymes catalyzing only these kinds of reactions are called transferases, and the ones transferring a glycosyl group are glycosyl transferases. Glycosyl transferases need expensive nucleotide sugars as substrates, while glycosyl hydrolases use inexpensive substrates such as monosaccharides and disaccharides. Because of this, glycosyl hydrolases are attractive alternatives compared to glycosyl transferases in industrial synthetic processes. The disadvantage is their low selectivity. In recent years applied researchers have become increasingly interested in enhancing the transferase activity and suppressing the hydrolytic activity of glycosyl hydrolases.

In kinetically controlled reactions, it is the kinetics of the desired reaction together with other possible reactions that determine the yield, and thus the properties of the enzyme are of prime importance (Kashe, 1986). Important properties of the enzyme in this respect are the ability to stabilize the transition state of the desired reaction and to exclude the water, which causes hydrolysis. To obtain high synthesis yields, hydrolases with high ratios of transferase to hydrolase activity are needed. An important method in studies aiming at understanding factors governing this ratio of hydrolases is site-directed mutagenesis.

Many investigations have recently turned to the role of aromatic residues in determining the transferase/hydrolase ratio of glycosyl hydrolases, e.g., glycosidases (Hansson et al., 2001; Kuriki et al., 1996; Matsui et al.,

1991; Matsui et al., 1994; Nakamura et al., 1994; Saab-Rincon et al., 1999; Sin et al., 1994). The importance of aromatic residues on the transglycosylation to hydrolysis ratio has been observed in natural transferases. In the active center of *Bacillus sp*. 1011 cyclodextrin glycosyltransferase (CGTase) the researchers observed that Tyr 195 had an important role in binding of substrate; Phe183 and Phe259 were cooperatively involved in acceptor binding, and Phe283 was probably involved in transition-state stabilization (Nakamura et al., 1994). Comparing natural glycosyl transferases (like CGTases) with glycosyl hydrolases (some bacterial α-amylases), the transferases have Tyr and Phe residues, while the hydrolases have small residues like Ala, Val, and Ser in homologue positions in the active site (Saab-Rincon et al., 1999). Saab-Rincon et al. (1999) replaced Ala-289 in the active site of *Bacillus stearothermophilus* α-amylase by Phe and Tyr. The mutants produced methylmaltosides during alcoholysis with methanol from starch, a function not present in the wild-type enzyme, and produced oligosaccharides of higher molecular weight than the substrate (maltoheptose) in higher amounts than the wild-type enzyme.

Even though β-glycosidases have been studied frequently, not much has been published in related protein engineering studies; however, a few studies have contributed results of relevance to this article. Protein engineering of *Pyrococcus furiosus* β-glucosidase (Hansson et al., 2001) and *Cellulomonas fimi* β-l,4-glycosidase (Nikolova et al., 1996) made the improvement of the oligosaccharides yield, which indicates that the ratio of transglycosylation to hydrolysis was increased for the two enzymes. In other cases, site-directed mutagenesis of a few enzymes (Mackenzie et al., 1998; Malet and Planas, 1998; Moracci et al., 1998) led to an increase in oligosaccharide yield. Secondary hydrolysis was avoided by removal of the catalytic nucleophile of the enzyme. To restore the synthetic activity (but not the hydrolytic one) the modified enzymes were used in the presence of an external nucleophile, such as azide.

The overall objective of this study was to favor the transglycosylation activity and suppress the hydrolytic activity of the most thermostable β-glycosidase isolated so far, *Pyrococcus furiosus* β-glucosidase CelB. CelB is a family 1 glycosyl hydrolase and a retaining enzyme. It has been thoroughly characterized (Bauer et al., 1996; Kaper et al., 2000; Kengen et al., 1993; Kengen and Stams, 1994; Lebbink et al., 2000; Petzelbauer et al., 1999; Voorhorst et al., 1995), but investigations of its use in transglycosylation reactions are limited (Boon et al., 1998; Fischer et al., 1996; Hansson et al., 2001; Petzelbauer et al., 2000). In the only previous study focusing on protein engineering of CelB and its transferase property (Hansson et al., 2001), an increase in oligosaccharide yield from lactose was observed by changing Phe426 to a Tyr residue (F426Y). In addition, a mutation of Met424 to a Lys residue (M424K) increased the pH optimum of CelB, and the double M424K/F426Y mutant showed much better transglucosylation properties at low substrate concentrations compared to the wild-type enzyme. On the basis of protein engineering studies of CelB and other enzymes discussed above, aromatic residues in the active site were expected to be of prime importance to increase the selectivity of the enzyme. In the present study, factors affecting the activity and selectivity of CelB wild-type and an active site mutant thereof (F426Y) were explored in a model reaction under different reaction conditions (donor concentrations, water contents, and temperatures). In addition, two active site mutants of CelB (M424K and M424K/F426Y) which have shown interesting effects in oligosaccharide synthesis (described above) were studied.

MATERIALS AND METHODS

Materials

The enzyme solution of wild-type β-glucosidase from *Pyrococcus furiosus* (CelB) (EC 3.2.1.21) (derived from *celB* gene) and mutants thereof were gifts from the Laboratory of Microbiology, Wageningen Agricultural University, The Netherlands. Acetonitrile was obtained from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma Chemicals (St. Louis, MO).

Enzyme Preparation and Purification

The wild-type β-glucosidase CelB and variants (EC 3.2.1.21) from *P. furiosus* was purified from *Escherichia coli* BL21 (DE3), as described by Kaper et al. (2000). All enzyme solutions were pure from other proteins, as judged by SDS-PAGE analysis, by applying heat-incubated cell-free extract (30 min at 80°C) on a Q-sepharose ion exchange column followed by gel filtration. The enzyme solutions contained 0.02% azide to prevent bacterial contamination. Characterization of the mutant catalysts has been published earlier (Hansson et al., 2001; Kaper et al., 2000).

Enzyme Structure

In the studies of the enzyme structure of CelB, a model that was calculated on the basis of homology modeling and energy minimization was used, as reported earlier by Hansson et al. (2001). The model (SWISS-PROT AC code Q51723) was obtained from the database SWISS-MODELL Repository.

Enzyme Activity Assay

The β-glucosidase activities of CelB wild-type and the mutant enzymes were determined using *p*-nitrophenyl-β-D-glucoside as the substrate in a spectrophotometric assay (405 nm, 80°C), as described previously (Hansson et al., 2001).

Protein Concentration Assay

Total protein concentration was determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as a standard.

Enzymatic Reactions

Determination of Kinetic Constants

Dry 1-hexanol (dried with 3 Å molecule sieves) was added to dry lyophilized powder of pentyl-β-D-glucoside (substrate concentrations between 5 to 240 m*M*) and 120 μL 50 m*M* citrate buffer solution, pH 5.0, containing 41–152 μg/mL enzyme (a final enzyme concentration of 30 U/mL). The reaction volume was 2 mL. This yielded water content of 6% and a water activity of 0.92 at 95°C. The enzymatic reaction was run in closed glass vials in an oven at 95°C with shaking for some hours' (< 24 h). At time intervals 20–40 μL samples were taken from the reaction and placed in the freezer for at least 30 min. The samples were diluted with methanol before being injected on the HPLC.

The kinetic parameters (K_m, V_{max}, k_{cat}, k_{cat}/K_m) for CelB wild-type and variants were obtained from initial reaction rates (μmole/min/mg). The K_m and V_{max} constants were calculated by nonlinear regression in GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA). To determine the catalytic constant, k_{cat}, a molecular weight of 54665 Da for the wild-type enzyme was used.

Effects of Pentyl-β-Glucoside Concentration on Selectivity

In the studies of effects of pentyl-β-glucoside concentration on selectivity, a similar reaction system as in the determination of the kinetic constants was used.

Effects of Water Content on Activity and Selectivity

In the studies of effects of water content on activity and selectivity, a similar reaction system as in the determination of the kinetic constants was used containing 100 m*M* pentyl-β-glucoside and water contents of 1–8% (v/v). This range of water content corresponded to water activities of 0.29–1.03 at 95°C (calculated by UNIFAC, see below). A two-phase system (20% water phase/80% hexanol phase), aqueous solution containing 1% hexanol and purely aqueous solution were investigated as well with an enzyme concentration of 10 U/mL (14–51 μg/mL enzyme) and 100 m*M* pentyl-β-glucoside.

Effects of Temperature Variations on Activity and Selectivity

The temperature effects on the rates and selectivity were investigated using 100 m*M* pentyl-β-glucoside in the temperature range between 50–95°C. The hexanol was water-saturated at each temperature. The water content (v/v) in water-saturated hexanol at the different temperatures was: 50°C, 6.2%; 60°C, 6.6%; 75°C, 7.2%; 85°C, 7.6%; 95°C, 8.0% measured by Karl-Fischer titrator.

HPLC Analysis

The reaction mixtures were analyzed by HPLC. The HPLC system used was a LaChrom system with an L-7100 pump, an L-7250 autosampler, and a D-7000 Interface system (all from Merck, Hitachi, Darmstadt, Germany). The detector was an 500 ELSD evaporative light-scattering detector (Alltech Associates, Inc., Deerfield, MI) under the following operating conditions: nebulizer gas flow, 3.1 SLPM (standard liters per minute); drift tube temperature 100°C. The other analytical conditions were: column, LiChrospher 100 RP-18 (4 × 250 mm, 5 μm) (Merck, Sweden); column temperature, room temperature; mobile phase, 25% acetonitrile/75% water; flow rate = 0.8 mL/min. Glucose (ret. time: 2.2 min), pentyl-β-D-glucoside (ret. time: 4.5 min), hexyl-β-D-glucoside (ret. time: 8.1 min) were used as reference standards for qualitative and quantitative analyses of the enzyme treated samples.

Calculation of the Thermodynamic Activity Coefficients

The water (a_w) and hexanol (a_{hex}) activities in the different reaction systems were calculated using UNIFAC Activity Coefficient Calculator 3.0 (University of Sydney, Australia and Louisiana State University, LA). The water activities were compared with those obtained from water determination by Karl Fischer titration and standard curves obtained by equilibration with saturated salt solutions and pure water.

Calculation of the Selectivity (*S*) Parameter

The ratio of hexyl-β-glucoside formation rate (r_S) and glucose formation rate (r_H) was calculated to measure the competition between the nucleophiles, water and hexanol, for the glucosyl-enzyme complex. The medium composition (expressed in thermodynamic activities) was taken into account to this ratio and a selectivity parameter (S) was calculated using following equation (van Rantwijk et al., 1999):

$$\frac{r_S}{r_H} = S \cdot \frac{a_{hex}}{a_w} \tag{1}$$

where a_{hex} is the hexanol activity and a_w is the water activity. The S value was used to express the nucleophile specificity (hexanol vs. water) of wild-type enzyme and mutants under a wide range of reaction conditions.

Calculation of Expected Hexyl-β-Glucoside Yield

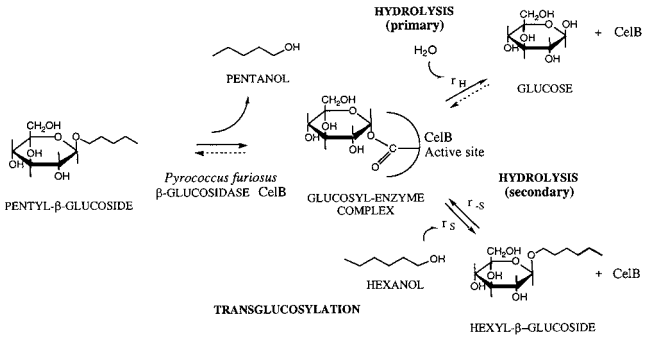
The experimental maximum yield (mole %) was compared with an expected yield (η) calculated from initial reaction rates and assuming the absence of secondary hydrolysis (van Rantwijk etal., 1999):

$$\eta = \frac{r_S}{r_S + r_H} \quad (2)$$

where r_S is the rate of synthesis (hexyl-β-glucoside formation) and r_H is the rate of hydrolysis (glucose formation).

RESULTS AND DISCUSSION

The conversion of pentyl-β-glucoside to hexyl-β-glucoside (transglucosylation) and glucose (hydrolysis) was used as a model reaction for studies of the transferase/hydrolase properties of CelB wild-type and active site mutants thereof (Scheme 1). The transglucosylation product, hexyl-β-glucoside, can be used as a biosurfactant in laundry detergents, cosmetics etc. The reaction can be seen as two steps: a glycosylation step and a deglycosylation step. In the glycosylation step the enzyme cleaves the pentyl-β-glucoside and forms a glucosyl-enzyme complex with the liberation of pentanol. The total initial reaction rate (sum of transglucosylation and hydrolysis) has been used to describe the interaction of the donor substrate and the free enzyme. In the deglycosylation step, glucose is formed as the hydrolysis product and hexyl-β-glucoside as the transglucosylation product. The competition between water and hexanol for the glucosyl-enzyme complex has been characterized by the *S* value [see Materials and Methods, Eq. (1)]. The yield of hexyl-β-glucoside increases until its rate of synthesis (r_S) and dealkylation (r_{-S}) become equal. Thermodynamic control becomes gradually more important and the hexyl-β-glucoside is hydrolyzed by the enzyme (secondary hydrolysis), which results in a decrease of the yield. The yield in these kinds of reactions depends on (1) the ratio of the synthetic (r_S) and the hydrolytic (r_H) rate, and (2) product hydrolysis.



Scheme 1. Reaction scheme of the model reaction. Conversion of pentyl-β-glucoside to hexyl-β-glucoside in hexanol (transglucosylation reaction) by *Pyrococcus furiosus* β-glucosidase CelB. The conversion to glucose with water as the nucleophile (hydrolysis reaction) was a side reaction. The hexyl-β-glucoside can also be hydrolyzed by the enzyme (secondary hydrolysis).

Quantification of Water and Hexanol Activities

The water and hexanol activities were calculated by UNIFAC (see Materials and Methods) (Table I). The water activities calculated by this method were similar to the ones measured by Karl Fischer titration and standard curves obtained by equilibration with saturated salt solutions (Table I). At a water content of 8% in hexanol the UNIFAC calculations resulted in a water activity slightly above 1.0. This might indicate that a two-phase system was formed (this is very close to the solubility limit of water in hexanol) or was due to small errors in the UNIFAC calculations.

Effects of Pentyl-β-Glucoside Concentration

The total activity (sum of transglucosylation and hydrolysis) of CelB and variants as a function of pentyl-β-glucoside concentration followed Michaelis-Menten kinetics. For the M424K/F426Y double mutant, some substrate inhibition was observed at the highest donor concentration tested (240 mM). Substrate inhibition at high substrate concentrations by this mutant has also

Table I. Water (a_w) and hexanol activity (a_{hex}) at 95°C calculated using UNIFAC[a] or Karl-Fischer titration[b] in systems containing different water content.

Water content (%, v/v)	Water activity (a_w) UNIFAC[a]	Water activity (a_w) Karl-Fischer[b]	Hexanol activity (a_{hex}) UNIFAC[a]
1	0.29	0.22	0.94
3	0.64	0.57	0.85
5	0.85	0.82	0.78
6	0.92	0.91	0.76
8	1.03	0.99	0.72
20	1.00	-	0.72
99	1.00	-	0.34

[a]UNIFAC = UNIFAC Activity Coefficient Calculator 3.0 (University of Sydney, Australia and Louisiana State University, LA).
[b]Water activities determined by Karl Fischer titration and standard curves obtained by equilibration with saturated salt solutions and pure water.

Table II. Kinetic parameters of the total enzymatic activity and of the separate reaction pathways (hydrolysis and transglucosylation) by CelB wild-type (wt) and mutants thereof (M424K, F426Y, M424K/F426Y).

Enzyme[a]	Activity	Kinetic parameters				
		K_m (m*M*)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	$(k_{cat}/K_{m\ trans}\sigma)/(k_{cat}/K_{m\ hyd})$[c]
CelB wt	Total[b]	46 ± 5	19.3 ± 0.6	17.6 ± 0.5	0.38	1.6
	Transglucosylation	46 ± 7	11.7 ± 0.1	10.7 ± 0.1	0.23	
	Hydrolysis	49 ± 7	7.8 ± 0.3	7.1 ± 0.3	0.14	
CelB M424K	Total	28 ± 1	14.8 ± 2.2	13.5 ± 2.0	0.48	2.8
	Transglucosylation	24 ± 4	9.4 ± 2.2	8.6 ± 2.0	0.36	
	Hydrolysis	39 ± 5	5.5 ± 0.1	5.0 ± 0.1	0.13	
CelB F426Y	Total	19 ± 1	4.8 ± 0.7	4.4 ± 0.7	0.23	4.2
	Transglucosylation	19 ± 3	3.7 ± 0.2	3.4 ± 0.2	0.18	
	Hydrolysis	21 ± 1	1.0 ± 0.1	0.9 ± 0.1	0.04	
CelB M424K/F426Y	Total	32 ± 1	4.3 ± 0.6	3.9 ± 0.6	0.12	3.0
	Transglucosylation	34 ± 5	3.4 ± 0.2	3.1 ± 0.2	0.09	
	Hydrolysis	27 ± 2	0.9 ± 0.1	0.8 ± 0.1	0.03	

[a]41 μg/mL of CelB wt, 52 μg/mL of CelB M424K, 86 μg/mL of CelB F426Y, and 152 μg/mL of CelB M424K/F426Y was used in the reactions.
[b]Total activity = sum of hydrolytic and transglucolytic activity.
[c]Ratio of specificity constants for transglucosylation and hydrolysis.

been seen with *p*-nitrophenyl-β-glucoside (Hansson et al., 2001).

The kinetic constants are listed in Table II together with apparent kinetic constants of the separate transglucosylation and hydrolysis reactions. The F426Y mutation caused a larger decrease in enzyme activity than M424K (75% and 23% decrease in V_{max}, respectively, Table II). The M424K/F426Y double mutant had an activity similar to that of the F426Y single mutant. The F426Y mutation showed larger decrease in V_{max} with pentyl-β-glucoside substrate (75% reduction) compared to the reaction with *p*-nitrophenyl-β-glucoside (52% reduction) (data not shown). This was not observed for the M424K mutation, which showed a 23% decrease in V_{max} in a reaction with pentyl-β-glucoside compared to 38% with *p*-nitrophenyl-β-glucoside. For all the catalysts at $a_w = 0.92$, V_{max} of the transferase reaction was higher than V_{max} of the hydrolase reaction (Table II). For the wild-type enzyme and M426Y it was 1.5 and 1.7 times higher, respectively, and for F426Y and M424K/F426Y even more (3.7 and 3.8 times higher). The K_m values were lower for all the mutants than for CelB wild-type. The largest decrease was seen for the F426Y mutant.

All mutants showed an increase in specificity for hexanol compared to that of water (Table II). The ratio of the specificity constants (k_{cat}/K_m) for transglucosylation and hydrolysis was increased 1.8-, 2.6-, and 1.9- fold for M424K, F426Y, and M424K/F426Y, respectively. For M424K the increase was due to a larger decrease in K_m for transglucosylation relative to hydrolysis by the mutant compared to the wild-type. For F426Y and M424K/F426Y the increase was mainly due to a larger decrease in k_{cat} for the hydrolytic reaction than for the transglucolytic reaction by the mutations. For these mutants, the k_{cat} of the hydrolytic pathway was only 20% of the total rate compared to 40% for the wild-type enzyme.

The selectivity was increased for all the mutants compared to CelB wild-type (Fig. 1). For the M426K single mutant the *S* value was only slightly higher than for CelB wild-type (Fig. 1A), but for the F426Y enzyme at 100 m*M* pentyl-β-glucoside a 2.6-fold increase in *S* value was seen (from 1.9 to 4.9) (Fig. 1B). The large increase in selectivity of the F426Y mutation seems to influence also in the double M424K/F426Y mutant (Fig. 1C). As expected, the selectivity of CelB was independent of the donor concentration. This was seen for the mutants as well. The only exception was a decrease in *S* value at low concentrations (5–30 m*M*) for the F426Y mutant. Interestingly, and in agreement with earlier results in oligosaccharide synthesis from lactose (Hansson et al., 2001), the M424K/F426Y double mutant showed a high synthesis to hydrolysis (r_S/r_H) ratio at a low substrate concentration. At the lowest pentyl-β-glucoside concentration tested for this mutant (20 m*M*), the ratio was 3.71 compared to 1.65 for CelB wild-type.

In the results presented below the effects of water content and temperature on activity and selectivity of CelB wild-type was compared with the F426Y mutant, which showed the highest increase in selectivity.

Effects of Water Content

The enzymatic activity of CelB increased with increasing water activity (Fig. 2A). As has been reported earlier (Chahid et al., 1992; Ljunger et al., 1994), glycosidases need high water content to be active. For glycosidase-catalyzed reactions in hexanol, transglycolytic activity is usually not observed at lower water activity than 0.6 (Chahid et al., 1992). However, the F426Y mutant

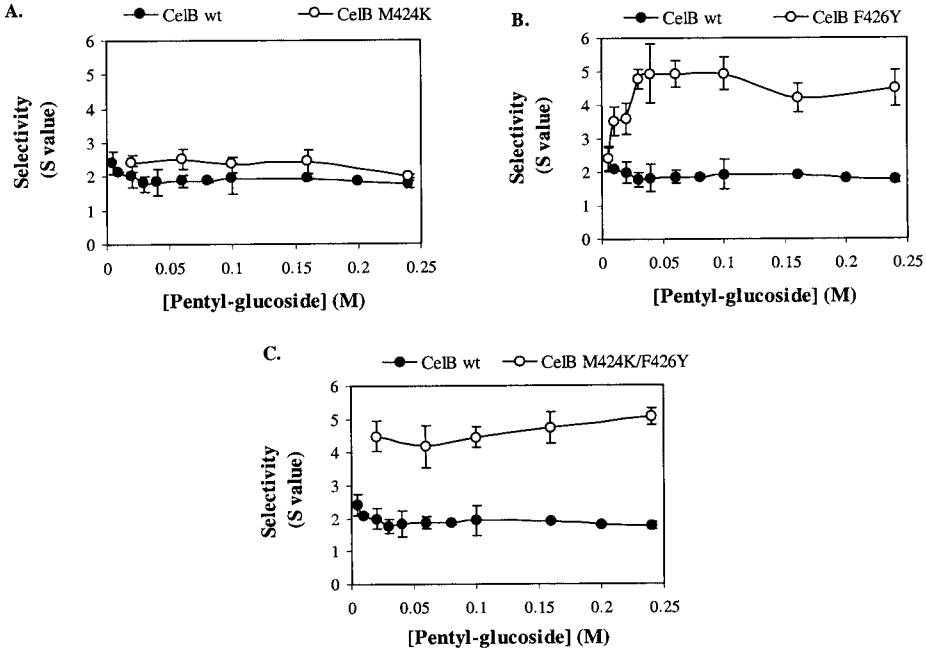


Figure 1. The selectivity (*S* value) as a function of pentyl-β-glucoside concentration for CelB wild-type (wt) (A–C), M424K (A), F426Y (B), and M424K + F426Y (C) mutants of CelB.

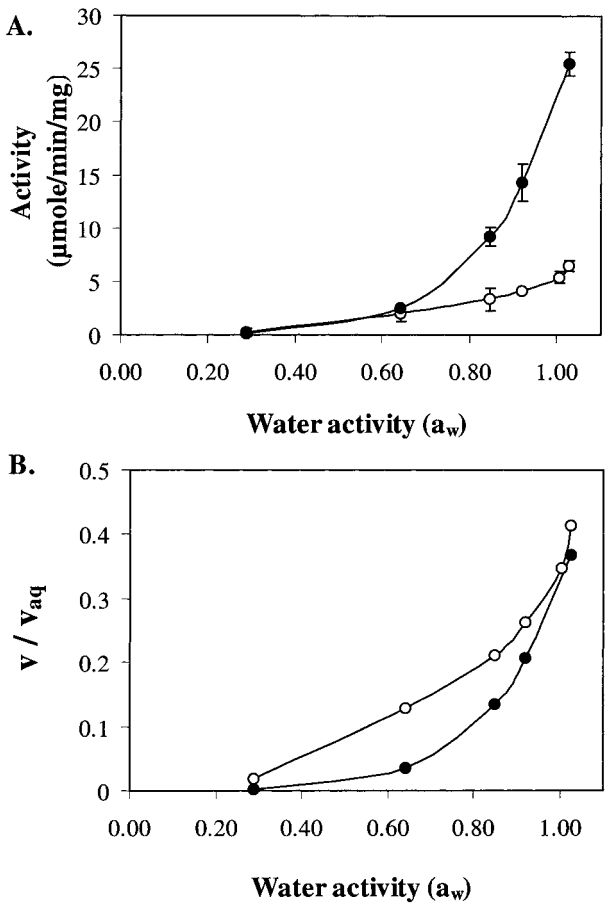


Figure 2. (A) Total enzymatic activity as a function of water activity, and (B) total enzymatic activity at different water activities (v) relative to their maximal activities in aqueous solution (v_{aq}) for CelB wild-type (●) and CelB F426Y (○).

showed good activity at low water content. The mutant had some transferase activity (0.21 μmole/min/mg) at a water activity as low as 0.29. Only a very small amount of hydrolytic activity (0.08 μmole/min/mg) could be detected for the wild-type enzyme at that low water activity. To evaluate the performance of the wild-type and the F426Y mutant at low water activity, the ratios of the rate at different water activities and in aqueous solution were calculated (Fig. 2B). It is thus clear that the mutant in relation to its maximal activity is considerably more effective at low water content. The mutant expressed 13% of its maximal activity at a_w = 0.64 compared to 3% for the wild-type enzyme. At higher water content than water saturated hexanol (water-hexanol two-phase system), the activity of the enzymes increased further (Table III). For both catalysts, the activity as a function of water content showed saturation kinetics, in which maximal activity was almost reached in a two-phase system with 20% water phase. It is worth pointing out that CelB (and F426Y) showed good activity in hexanol one-phase system. In water-saturated hexanol, 37% activity remained compared to aqueous solution (41% for F426Y) (Table III).

In aqueous solution containing 1% hexanol and in purely aqueous solution, two other transglucosylation products besides hexyl-β-glucoside were observed. These were not formed when using higher hexanol concentrations. The compounds were formed in the initial part of the reaction and then hydrolyzed by secondary hydrolysis. They were produced in much higher yields (3.5 times more) by the mutant compared to the wild-type.

Table III. Total activity, the ratio of transglucosylation rate over hydrolysis rate (r_S/r_H), and the selectivity (S value) in reaction systems with different water content catalyzed by CelB wild-type (wt) or CelB F426Y.

Reaction system	CelB wt			CelB F426Y		
	Activity (μmole/min/mg)	r_S/r_H	S	Activity (μmole/min/mg)	r_S/r_H	S
Hexanol, a_w = 0.64	2.4 ± 0.3	1.6 ± 0.2	1.2 ± 0.2	2.0 ± 0.7	3.7 ± 0.3	2.8 ± 0.2
Hexanol, water saturated	25.5 ± 1.1	0.52 ± 0.01	0.74 ± 0.01	6.4 ± 0.5	0.9 ± 0.1	1.3 ± 0.2
Hexanol/water, (80/20, v/v)	52.3 ± 4.5	0.33 ± 0.03	0.45 ± 0.05	13.5 ± 1.2	0.44 ± 0.07	0.6 ± 0.1
Water, 1% (v/v) hexanol	65.5 ± 3.3	0.3 ± 0.1	0.4 ± 0.1	12.8 ± 1.5	0.3 ± 0.1	0.5 ± 0.2
Water	69.2 ± 5.1	–	–	15.6 ± 2.1	–	–

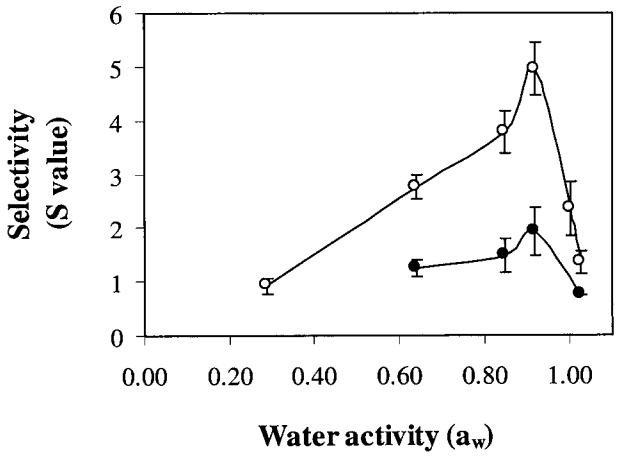


Figure 3. The selectivity (S value) as a function of water activity for CelB wild-type (●) and CelB F426Y (○).

One of the products was probably pentyl-β-cellobioside. Quantitative estimations of these products were not carried out.

Unexpectedly, the selectivity of CelB increased up to a water activity of 0.92 (Fig. 3). This was seen for the mutant as well. At even higher water content the selectivity decreased (Fig. 3 and Table III). The increase in selectivity with increasing water activity can be due to an increase in internal flexibility in the active site of the enzyme and consequently the glucosyl-enzyme might accept a large nucleophile as hexanol. It has been reported earlier (Chahid et al., 1992), that large alcohols need higher water activity to be active as glycosyl acceptors compared to small ones. Chahid et al. (1992) studied optimal water activity for alcohols with different chain length during biocatalyzed alkyl-glucoside synthesis (highest maximal alkyl-glucoside yield) from glucose using almond β-glucosidase. In the case of β-glucosidase, a water activity of 0.43 was optimal for propanol as the nucleophile, 0.71 for hexanol and 0.81 for octanol. In another study (Ljunger et al., 1994) where almond β-glucosidase immobilized on XAD-4 was used in octyl-β-glucoside synthesis from glucose in octanol, a water activity of at least 0.67 was required.

The increased selectivity by the F426Y mutant resulted in increased hexyl-β-glucoside yield. In water-saturated solvent the yield increased from 33% to 45%, and from 56% to 69% at a water activity of 0.85, (Table IV). Furthermore, previously published results by the authors have shown a 13% increase in synthetic oligosaccharide yield by this mutant relative to the yield obtained by the wild-type enzyme (Hansson et al., 2001). In Table IV, the experimental maximal hexyl-β-glucoside yield is compared with the expected yield (in the absence of secondary hydrolysis) [Eq. (2)] at different water content. The predicted yield and the observed yields were almost the same for CelB wild-type except for a reaction system containing 1% hexanol. This indicates that significant secondary hydrolysis of the products was only seen in 1% hexanol. However, the decrease in experimental yield compared with expected yield in this system can also be due to the formation of other transglucosylation products besides hexyl-β-glucosides (as mentioned above). For the F426Y mutant,

Table IV. Experimental maximal hexyl-β-glucoside yield in comparison with the expected yield (η) (using initial reaction rates of transglucosylation and hydrolysis) in reaction systems with different water content by CelB wild-type (wt) or CelB F426Y.

Reaction system	CelB wt		CelB F426Y	
	Hexyl-β-glucoside yield		Hexyl-β-glucoside yield	
	η (%)[a]	Exp. (%)[b]	η (%)	Exp. (%)
Hexanol, $a_w = 0.85$	58	56	78	69
Hexanol, $a_w = 0.92$	62	57	80	68
Hexanol, water saturated	33	33	47	45
Hexanol/water, (80/20, v/v)	23	24	29	30
Water, 1% (v/v) hexanol	23	10	23	16

[a]The expected yield expressed as mole %.
[b]The experimental yield expressed as mole %.

notable difference between the predicted and the observed yields was detected in aqueous solution containing 1% hexanol as well as in the reaction systems with water activities of 0.85 and 0.92. However, in the time courses of hexyl-β-glucoside formation in the one-phase hexanol reaction systems (a_w = 0.85, 0.92, and water-saturated systems) no decrease in yield was observed after maximum yield was reached. Thus, the lower experimental yields than predicted yields in these systems were thus probably not due to secondary hydrolysis. Instead, the difference could be attributed to the reduction of the substrate concentration. If so, the reduction of the substrate concentration could cause the decrease of the F426Y mutant selectivity (Fig. 1B) and result in lower experimental yield when compared with the yields expected from the selectivity in the initial phase. In the reaction system containing 1% hexanol, the decrease in the observed yield compared with the predicted yield was smaller for the mutant than to the wild-type (Table IV), even though the mutant formed other transglucosylation products to a larger extent than the wild-type enzyme (as mentioned above). This indicates that the role of secondary hydrolysis in this reaction system was probably smaller for the F426Y mutant when compared to the CelB wild-type.

Effects of Temperature Variations

It is known from the literature that the selectivity of alcohol dehydrogenase from *Thermoanaerobium brockii* (Yang et al., 1997) and α-chymotrypsine from bovine pancreas (Jönsson et al., 1995) can be effectively modulated by temperature changes of an enzymatic reaction.

Thus, the influence of temperature (50–95°C) on activity and selectivity by CelB β-glucosidase (wild-type and F426Y) was investigated. Because CelB comes from a hyperthermophilic organism and consequently is highly thermostable, a wide range of temperatures could be used for the measurements thus improving the possibility of getting reliable results. The enzymatic activity (both hydrolysis and transglucosylation rates) increased as a function of temperature for both catalysts (Fig. 4). In contrast to the influence of temperature on the selectivity of dehydrogenases and proteases (Jönsson et al., 1995; Yang et al., 1997), the reaction selectivity and synthesis to hydrolysis (r_S/r_H) ratio by CelB wild-type and the F426Y mutant was unchanged when the temperature was varied (Fig. 5). As has been mentioned before, the F426Y mutant showed higher selectivity than the wild-type enzyme.

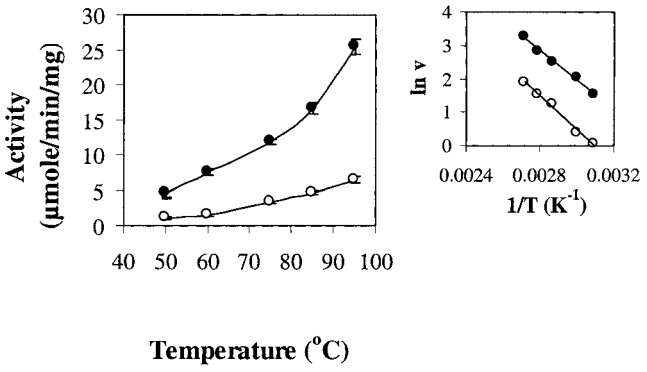


Figure 4. Total enzymatic activity as a function of temperature for CelB wild-type (●) and CelB F426Y (○). Arrhenius plot of the total activity (v) of CelB wild-type (●) and CelB F426Y (○) is shown in the small picture to the right.

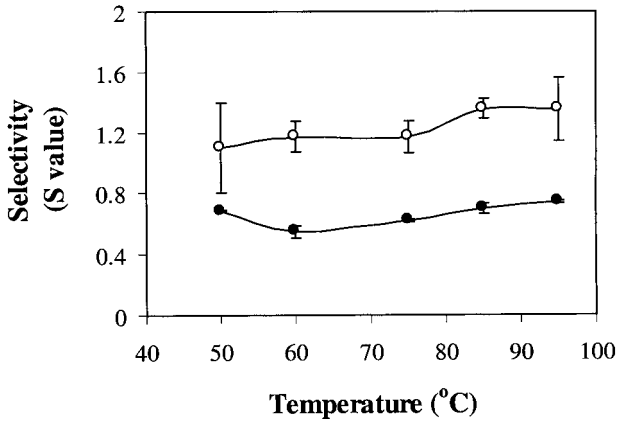


Figure 5. The selectivity (S value) as a function of temperature for CelB wild-type (●) and CelB F426Y (○).

Mechanistic Interpretations

Understanding of the mechanism behind the positive effect of the F426Y mutation on the transglucosylation/hydrolysis ratio of CelB was the fundamental problem of the conducted study. It has been known from other studies of related enzymes that tyrosine residues can be involved in substrate binding (Fothergill and Fersht, 1991; Nakamura et al., 1994). Tyr426 in the mutant is located in the active site so hydrogen bonding to the substrate is clearly possible although the model of CelB is not good enough to make any detailed statements. Further indications are obtained from the *Lactococcus lactis* 6-phospho-β-galactosidase (LacG), which is highly homologous with CelB. The residue in LacG corresponding to Phe426 of CelB is Tyr436 (Hansson et al., 2001; Kaper et al., 2000). Tyr436 in LacG has been reported to be involved in binding of the phosphate group as well as in hydrogen bonding with the oxygen at carbon 6 of the galactose residue of its substrate (Wiesmann et al., 1997). It is thus possible that Tyr426 in CelB can make the corresponding hydrogen bond with the oxygen at carbon 6 of the glucose residue of the pentyl-β-glucoside substrate. This interaction might also be of importance in the glucosyl-enzyme intermediate and in the transition states leading to transglucosylation and hydrolysis. An indication that the new hydroxyl

group in the F426Y mutant is indeed involved in substrate binding is the significant reduction in K_m value observed in the present study. The effect of the mutation is mainly to decrease the rates in the hydrolytic reaction, while the rates of the transglucosylation reaction are less influenced. Both reactions proceed via the same glucosyl-enzyme intermediate. The effect of the mutation can be explained in terms of activation energies of the transglucolytic and hydrolytic pathways. Because the mutation does not influence the transglucosylation rate, it seems that the energies of the glucosyl-enzyme and the transition state leading to transglycosylation are influenced by the mutation in a similar way so that the net effect on the activation energy is close to zero. On the other hand, the mutation reduces the rate of the hydrolysis reaction, which indicates an increase in activation energy for the mutant. This is probably due to energetically unfavorable interactions in the transition state leading to hydrolysis for the mutant enzyme.

An alternative explanation (but in our opinion less probable) of the effects of the F426Y mutation is that the introduced hydroxyl group traps water molecules and thereby prevents them from causing hydrolysis. Tyrosine has been shown to bind water by hydrogen bonding in several enzymes (Chen et al., 1993; Fischer et al., 1994; Luntz et al., 1989), and sometimes clusters of water molecules are formed. This possible water accumulation at the Tyr426 site in CelB is too far away from the catalytic glutamates to cause hydrolysis (> 10 Å from the catalytic acid/base Glu207), resulting in a larger decrease in hydrolysis efficiency relative to transglucosylation in a reaction medium with low water content.

CONCLUSIONS

Donor concentration and temperature did not influence the selectivity of the CelB catalysts. The only exception was the lower selectivity at low substrate concentrations for the F426Y mutant. More surprisingly, the selectivity increased with increasing water activity up to $a_w = 0.92$. Mutation of a phenylalanine residue in position 426 of CelB to a tyrosine residue significantly increased the ratio of transglucosylation over hydrolysis, resulting in increased hexyl-β-glucoside yield. In addition, relative to their maximal activities in aqueous solution, the mutant expressed higher activity at low water activity than the wild-type enzyme, and the mutant showed some transferase activity at a water activity as low as 0.29. The disadvantage of the mutation was the decrease in total enzymatic activity on pentyl-β-glucoside substrate at high water activity. However, the decrease in activity due to the mutation was less pronounced when *p*-nitrophenyl-β-glucoside was used as substrate. The effects of increased selectivity by the F426Y mutation were also observed in the M424K/F426Y double mutant.

A possible explanation of the positive effects of the F426Y mutation on the selectivity of CelB is that the new hydroxyl group is involved in hydrogen bonding with the glucose residue in a way that destabilizes the transition state leading to hydrolysis. Alternatively the hydroxyl group might trap incoming water molecules and thereby prevent them from causing hydrolysis. The results provide a base for further engineering of glycosidases and other hydrolytic enzymes with the aim to alter the enzymes towards becoming transferases rather than hydrolases.

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