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TITLE

Development and application of a screening assay for glycoside phosphorylases

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SHORT TITLE

Screening glycoside phosphorylases

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ABSTRACT

Glycoside phosphorylases (GPs) are interesting enzymes for the glycosylation of chemical molecules. They only require a glycosyl phosphate as sugar donor and an acceptor molecule with a free hydroxyl group. Their narrow substrate specificity, however, limits the application of GPs for general glycoside synthesis. Although an enzyme's substrate specificity can be altered and broadened by protein engineering and directed evolution, this requires a suitable screening assay. Such a screening assay has not yet been described for GPs. Here, we report a screening procedure for GPs based on the measurement of released inorganic phosphate in the direction of glycoside synthesis. It appeared necessary to inhibit endogenous phosphatase activity in crude *Escherichia coli* cell extracts with molybdate, and inorganic phosphate was measured with a modified phosphomolybdate method. The screening system is general and can be used to screen GP enzyme libraries for novel donor and acceptor specificities. It was successfully applied to screen an E649 saturation mutagenesis library of *Cellulomonas uda* cellobiose phosphorylase (CP) for novel acceptor specificity. An E649C enzyme variant was found with novel acceptor specificity towards alkyl β-glucosides and phenyl β-glucoside. This is the first report of a CP enzyme variant with modified acceptor specificity.

KEYWORDS

Cellobiosides; Glycoside phosphorylase; High-throughput screening; Inorganic phosphate determination; Mutagenesis

INTRODUCTION

Glycosylation can significantly influence the properties of a molecule [1]. In the case of pharmaceuticals, glycosylation can improve the pharmacokinetic properties of a drug and induce drug targeting to specific organs, resulting in less side effects and smaller doses [2]. Glycoside phosphorylases (GPs) such as sucrose phosphorylase (SP) and cellobiose phosphorylase (CP) are promising enzymes for glycoside synthesis since they do not require expensive nucleotide-activated sugars as glycosyl donor but instead use a glycosyl phosphate (e.g. α-glucose 1-phosphate) [3]:

R-OH (acceptor) + glycosyl phosphate (donor) ⇆ R-glycoside + P_i

Furthermore, since GPs catalyze reversible reactions, the glycosyl phosphate donor can be easily obtained by enzymatic phosphorolysis of a simple sugar such as sucrose or lactose [4; 5]. Several unnatural trisaccharides and disaccharides with potential prebiotic effect could be synthesized with cellobiose phosphorylase (CP) [6; 7], and natural sucrose phosphorylase (SP) from *Bifidobacterium longum* has been used for the glycosylation of vitamin C, resulting in the more stable 2-*O*-α-D-glucopyranosyl-L-ascorbic acid [8].

The relatively narrow substrate specificity of GPs, however, limits their use for a general glycosylation technology. A possible solution is to optimize the enzymes by protein engineering, and more specifically *via* directed evolution. In this technique, enzyme libraries are created by mutagenesis of the DNA encoding the enzyme of interest, followed by recombinant expression in e.g. *Escherichia coli* and screening for improved variants with a high-throughput screening (HTS) system. Typically hundreds to thousands of enzymes variants need to be screened in order to find improved mutants [9]. Reports of directed evolution on GPs are very limited, possibly due to the lack of a suitable HTS system. We have previously created *Cellulomonas uda* CP enzyme variants with modified donor specificity that were found using a selection and screening system based on the release of glucose from

lactose phosphorolysis [10]. However, this screening system is not suitable for identifying enzyme variants with modified acceptor specificity and is not generally applicable to other GPs. Therefore, the availability of a general high-throughput screening systems for GPs is highly desirable since it would accelerate the development of enzyme variants with modified substrate specificity towards various aglycons.

Independent of their substrate specificity, GPs have the common feature that they release inorganic phosphate in the direction of glycoside synthesis using glycosyl phosphate as glycosyl donor. The amount of released phosphate is directly related to enzyme activity. Many methods have been described for the measurement of inorganic phosphate, and most of them are based on the method of Fiske and Subbarow [11]. In this method, a phosphomolybdate complex is reduced under acidic conditions resulting in the formation of a molybdenum blue compound which can be measured spectrophotometrically. The original method could be improved using ascorbic acid as a reducing agent instead of ferrous sulfate and by adding stop solution to stop color development which is useful in the presence of acid-labile organic phosphates [12]. The assay is cheap, fast, sensitive and robust, and thus convenient for high-throughput screening.

Here, we report on the development of a high-throughput screening system for GPs, based on the measurement of released inorganic phosphate when an acceptor molecule is glycosylated. It appeared necessary to inhibit endogenous *E. coli* phosphatase activity in crude cell extracts because it causes high background values and consumes the glycosyl phosphate substrate. For the measurement of inorganic phosphate, a modified phosphomolybdate method was developed based on the method of Gawronski and Benson [12] and the screening procedure was evaluated using *C. uda* CP as a model. CP uses α-glucose 1-phosphate (αGlc1*P*) as glycosyl donor and glucose as acceptor. The acceptor specificity of *C. uda* CP is relatively narrow in comparison to other GPs and furthermore,

there is a strict specificity for a free anomeric hydroxyl group with glucose as acceptor [3; 13]. Since this is a serious limitation for general glycosylation applications, our first aim was to eliminate this requirement in CP. Residue E649 is located near the acceptor binding site and was hypothesized to be important for substrate specificity. Screening of an E649 saturation library with a substrate mix composed of several acceptors identified an E649C enzyme variant with modified acceptor specificity towards alkyl β-glucosides and phenyl β-glucoside.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The pXCP expression vector encoding *C. uda* cellobiose phosphorylase has been described earlier [10]. *Escherichia coli* XL10-Gold cells (Stratagene, USA) were used for protein expression. *E. coli* containing plasmid was routinely grown overnight at 37 °C and 200 rpm in LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0) supplemented with 100 mg/L ampicillin. All chemicals were from Sigma, except for ethyl β-glucoside and octyl β-glucoside which were purchased from Carbosynth (UK).

Protein expression

E. coli XL10-Gold was transformed with the expression vector and a colony was picked and grown overnight. The overnight culture was used to inoculate fresh medium and expression of the recombinant enzyme was induced by adding IPTG to a final concentration of 0.1 mM when the optical density at 600 nm (OD600) of the culture reached 0.6. After 6 hours of induction, 1 mL samples of the culture (OD600~4.0) were centrifuged for 10 minutes at 15,000 x *g* and the pellets were frozen at -20°C.

Inhibition of E. coli phosphatase activity

Crude cell extracts were prepared by enzymatic lysis of frozen *E. coli* XL10-Gold/pXCP pellets using the EasyLyse Bacterial Protein Extraction Solution (Epicentre, USA). These cell extracts were then used to optimize phosphatase inhibitor concentrations. Enzyme reactions were carried out at 37 °C with 5% of crude cell extract and 30 mM of αGlc1*P* in 50 mM Mes-buffer, pH 6.6. As a test for phosphatase inhibition, different concentrations of potassium fluoride (KF; 0-50 mM) and sodium molybdate (Na_2MoO_4; 0-500 mM) were added. Assays were carried out in triplicate and phosphatase activity was measured by determining the amount of released glucose per unit of time with the Biochemistry Analyzer 2700 Select (YSI).

Determination of inorganic phosphate

The determination of inorganic phosphate in the presence of acid-labile organic phosphate was based on the method of Gawronski and Benson [12], but had to be modified because molybdate was already present as phosphatase inhibitor in the reaction mixture. In the original method, a color solution is prepared by mixing 2 parts of 12% (w/v) ascorbic acid in 1 N HCl with 1 part of 2% (w/v) ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$). One hundred fifty microliter of this color solution is added to 50 µL of sample and incubated for 5 minutes. If phosphate is present, a blue color develops. Afterwards, 150 µL of stop solution (2% (v/v) acetic acid, 2% (w/v) sodium citrate tribasic dihydrate) is added and the absorbance is read at 655 nm with a Bio-Rad 680XR microtiter plate reader. The method is linear up to 2 mM phosphate present in the sample.

The original method was modified to be compatible with the screening protocol by omitting molybdate from the color solution and by varying the concentration of ascorbic acid (12%-5%-0.24%) and HCl (0-0.1-0.5-1 N) in the color solution. A phosphate standard curve

ranging from 0-3 mM in 50 mM Mes buffer (pH 6.6) containing 200 mM sodium molybdate was used for optimization and three replicates were performed for each standard curve point. The optimized color solution in the modified method was composed of 0.24% (w/v) ascorbic acid in 0.1 N HCl solution, and 150 µL is added to a 50 µL sample containing 200 mM sodium molybdate. The subsequent steps are the same as in the original method.

Site-saturation mutagenesis

Site-saturation mutagenesis of residue E649 in the *C. uda* cellobiose phosphorylase gene was performed with the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene, USA). The pXCP expression vector was used as template (150 ng) and 100 ng of primer was used. The sequence of the degenerate primer was as follows:

5'-CCAGGTGCACATGGGCNNSGTCTCCACGTACCCGC-3'.

It contains the NNS codon encoding all possible 20 amino acids and one stop codon. Thermal cycling was performed in a 25 µL reaction volume containing 4% of QuikSolution, with the following cycling conditions: 95 °C (3 min); 30 cycles of 95 °C (1 min), 55 °C (1 min) and 65 °C (14 min). After the reaction, 10 units of *Dpn*I restriction enzyme were added to the reaction mixture and incubated for 6 hours at 37 °C to completely digest parental template DNA. The PCR mixture was transformed into *E. coli* XL10-Gold and the transformation mixture was plated on a Qtray (Genetix, UK) containing LB-agar medium supplemented with ampicillin.

Screening procedure

Colonies were picked with a QPix2 robotic colony picker (Genetix, UK) and inoculated into 96-well flat-bottomed microtiter plates containing 175 µL LB medium per well, supplemented with ampicillin. The microtiter plates were incubated for 16 hours at 37°C and 250 rpm.

Recombinant enzyme expression was then induced by inoculation of the grown mini-cultures into new microtiter plates containing 175 µL LB medium per well, supplemented with ampicillin and 0.1 mM IPTG. After incubation for 16 hours at 37°C and 250 rpm, the microtiter plates were centrifuged at 1,500 x *g* for 10 minutes, and the pellets were frozen at -20°C. The following steps were all carried out on a Tecan Freedom EVO 200 liquid handling robot. The pellets were lysed with 100 µL of lysis buffer composed of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5% Triton X-100, 4 mM $MgCl_2$, 50 mM NaCl and 1 mg/mL lysozyme. Lysis was carried out for 30 minutes at 37°C. After lysis, the plates were centrifuged at 3,000 x *g* for 10 minutes and the supernatants (crude cell extracts) were used for screening. Enzyme reactions were carried out at 37°C in microtiter plates by mixing 30 µL crude cell extract with 170 µL substrate solution. For screening of the E649 saturation library the reaction mixture contained 50 mM Mes-buffer (pH 6.6), 200 mM sodium molybdate, 30 mM αGlc1*P*, and an acceptor mix containing 100 mM each of methyl α-glucoside, methyl β-glucoside, sucrose and fructose. After 3 hours of incubation, 50 µL samples were taken from the reaction mixtures and transferred to a new microtiter plate to determine the amount of released phosphate. One hundred fifty microliter of a 0.24% ascorbic acid in 0.1 N HCl solution was added to the sample and incubated for 5 minutes at room temperature. Afterwards, 150 µL of stop solution (2% acetic acid, 2% citric acid) was added and absorbance at 655 nm was measured with a microplate reader. Positive clones were grown for plasmid extraction and sequenced to identify their mutations.

Characterization of enzyme variants

For an initial characterization of wild-type CP and the improved variant, the corresponding expression vector was used to transform *E. coli* XL10-Gold and the enzyme was expressed as described before. Crude cell extracts were prepared using the EasyLyse kit (Epicentre, USA).

To determine acceptor specificity, enzyme reactions were carried out at 37 °C with 5% (v/v) crude cell extract and substrate solution composed of 50 mM Mes-buffer (pH 6.6), 200 mM sodium molybdate, 30 mM αGlc1*P* and 100 mM of acceptor (either methyl β-glucoside, ethyl β-glucoside, octyl β-glucoside, methyl α-glucoside, sucrose or fructose). Samples were taken at regular time intervals and inactivated at 95 °C for 5 minutes. Inorganic phosphate release was determined with the modified phosphomolybdate method (see earlier).

Wild-type *C. uda* CP and the E649C enzyme variant were purified as described earlier [14], and their kinetic parameters were determined. Michaelis-Menten parameters on various acceptors were determined by incubating purified enzyme with a fixed concentration of αGlc1*P* (30 mM) and various concentrations of acceptor in 50 mM Mes-buffer (pH 6.6) at 37 °C. Initial reaction velocities were determined by measuring the amount of released inorganic phosphate using the original phospomolybdate method [12]. The k_{cat} value was calculated assuming a molecular mass of 91.4 kDa for the catalytic unit (one monomer). Protein concentrations were determined using the BCA Protein Assay kit (Thermo Scientific).

RESULTS

Inhibition of phosphatase activity in E. coli cell extracts

When crude *E. coli*/pXCP cell extracts were incubated with αGlc1*P*, significant glucose and phosphate release was observed. This is due to hydrolysis of the phosphate-ester bond by *E. coli* phosphatases present in the crude cell extract. Phosphatase activity interferes with phosphorylase activity because it consumes the glycosyl donor molecule and produces phosphate which causes high background levels in the inorganic phosphate assay. Therefore it was necessary to inhibit phosphatase activity. Although several phosphatase inhibitors have been described in literature, most of them are not useful here because they can also inhibit

phosphorylase enzymes, or because they are incompatible with the phosphate determination method. For example, vanadate was shown to be an inhibitor of phosphatases [15] but also inhibits CP activity [13]. Fluoride and molybdate, both shown to be potent phosphatase inhibitors [15; 16; 17], were tested at different concentrations for inhibition of phosphatase activity in *E. coli*/pXCP cell extracts. In the case of fluoride, a maximum inhibition of 70% could be achieved at 15 mM (Fig. 1), while for molybdate, more than 90% inhibition was observed at concentrations higher than 100 mM (Fig. 2). Cellobiose phosphorylase activity was not influenced by these inhibitor concentrations (data not shown). A molybdate concentration of 200 mM gave approximately 95% inhibition and was used in further tests.

Determination of inorganic phosphate

The original Gawronski and Benson method for phosphate determination was successfully applied here for phosphate standards ranging from 0 to 2 mM. A standard curve with high linearity (A655 = 1.545 x [mM P_i] + 0.051; R^2>0.99) was obtained. Ascorbic acid (AA) is used for reduction of the phosphomolybdate complex under acidic conditions. Stop solution, composed of 2% acetic acid and citrate, is added to prevent further color development caused by the release of acid-labile phosphate. The citrate present in the stop solution complexes residual molybdate, so that no new phosphomolybdate complexes are formed and no more blue color can be produced.

In the original Gawronski and Benson method, a final concentration of 37.8 mM molybdate is present after the addition of color solution (150 µL) to the sample (50 µL). However, when molybdate is used as an inhibitor (at 200 mM) in the sample, a total amount of 87.8 mM is present. Since this is more than twice as much as in the original method, it was expected that this would influence the test. Therefore, a standard curve of phosphate (0-3 mM) was made with 200 mM sodium molybdate in every standard. In this case, the original

protocol gave unmeasurably high absorbances (A655 >3.5) at phosphate concentrations above 0.05 mM. Even a blank sample containing no phosphate gave an absorbance higher than 1.0. Because it is important to have low background values in screening, and to prevent dilution steps in the screening protocol, the method was modified for samples containing 200 mM molybdate.

A first step was to omit molybdate from the color solution. A color solution of 12% AA in 1 N HCl resulted in measurable absorbances between 0 and 1.5 mM phosphate. However, the standard curve was not linear under these conditions (Fig. 3). Therefore, we decided to vary the concentration of AA (12%; 5%; 0.24%) and HCl (1.0 N; 0.1 N; 0 N) in the color solution, using standard solutions of phosphate (0-3 mM) prepared in 200 mM molybdate and 50 mM Mes (pH 6.6). The results are shown in Fig. 3. It appeared that higher AA and HCl concentrations in the color solution result in a higher sensitivity of the test. However, a too sensitive test is not always desirable because it can require dilution steps which slow down the screening process. The 12% AA solutions (1.0 N and 0.1 N) and the 5% AA/1 N HCl solution were unable to measure a 3 mM phosphate standard. On the other hand, the 0.24% AA/1 N HCl solution had an identical slope as that of the 5% AA/0.1 N HCl solution, although with a 20 times lower AA concentration. The 0.24% AA/0.1 N HCl solution also performed well, with good linearity. In general, all solutions with 0.1 N HCl showed greater linearity than the 1.0 N solutions, especially at the higher AA concentrations (12% AA and 5% AA).

Therefore, we decided to use a color solution composed of 0.24% AA in 0.1 N HCl for screening and enzyme tests where molybdate is used as an inhibitor at 200 mM. This color solution gives low background values, good linearity, good reproducibility. The stop solution, which is necessary to stop color development before release of acid-labile phosphate, did not

need to be modified. As in the original method, the developed color was stable for several hours.

Validation of the screening procedure

The optimized methods for phosphatase inhibition and inorganic phosphate determination were combined in a robotic screening procedure as described in the Materials and Methods section. The screening procedure was first validated by screening an *E. coli* XL10-Gold/pXCP microtiter plate for CP activity. Since every well contains an identical wild-type enzyme, the variation should be as low as possible. The reaction mixture contained 50 mM Mes, 200 mM molybdate, 30 mM αGlc1*P* and 30 mM glucose and the enzyme reaction was carried out for 15 minutes. In this case, the coefficient of variance (CV) of the whole screening procedure was 10.1% and is thus sufficiently low for use in directed evolution experiments [18].

Screening of a cellobiose phosphorylase E649 saturation library

The crystal structure of *C. gilvus* CP has been solved earlier (PDB 2cqt and 2cqs) and contains the natural acceptor glucose as a ligand at the acceptor site in the catalytic center [19]. Because the CP enzymes of *C. gilvus* and *C. uda* show high identity on the amino acid level (89%), the available 3D structure was used as a model for the *C. uda* enzyme. From the structure it can be seen that glucose is in the β-configuration and forms a hydrogen bond with residue E649, which is located closely to the anomeric hydroxyl group (Figure 4). It was already shown that CP enzymes are very specific for the anomeric hydroxyl group and that glucose molecules containing a substitution on C-1 are no or very poor acceptors [3; 13]. This might be due to the lack of space around the anomeric hydroxyl group because of the presence of the glutamate side chain. We hypothesized that mutagenesis of E649 could result

in acceptance of C-1 substituted molecules and broaden CP acceptor specificity. Therefore saturation mutagenesis was applied on site E649 with the degenerate NNS codon. This results in 32 possible genotypes encoding all amino acids plus one stop codon, and requires screening of about 94 clones (~3 times oversampling) to have a 95% coverage of the theoretical library [20].

An acceptor mix composed of several C-1-substituted glucose molecules was used for screening of the E649 library. Methyl α-glucoside, methyl β-glucoside, sucrose and fructose were simultaneously present in the substrate solution, each at 100 mM. None of them have been reported as acceptor for wild-type *C. uda* CP. The use of an acceptor mix allows the simultaneous and thus fast screening of several substrates, especially in cases where the precise effect of an amino acid site on specificity is not known. Ninety-six clones were screened for novel acceptor specificity and the result is shown in Fig. 5. Three clones were found with significantly improved activity as they gave high absorbance values at 655 nm. Sequencing revealed that they all contain the same E649C amino acid substitution, caused by a GAG to TGC codon change. Interestingly, no other hits could be observed, suggesting that only a limited number of amino acids is suitable on this site for high activity on the acceptor mix. Since mutation of glutamate (E) into cysteine (C) requires three consecutive DNA mutations, the E649C mutation would be very difficult to obtain by error-prone PCR. This illustrates the intrinsic power of saturation mutagenesis combined with semi-rational design.

Substrate specificity of the E649C enzyme variant

Since a substrate mix was used during screening, enzyme tests were performed to determine the modified acceptor specificity of the E649C enzyme variant. Enzyme tests were carried out with crude cell extracts of E649C and wild-type *C. uda* CP, using different acceptors in the direction of glycoside synthesis (Table 1). The E649C enzyme variant was found to be active

on methyl β-glucoside, but not on methyl α-glucoside, sucrose nor fructose. As expected from literature data, wild-type CP did not show detectable activity on any of the tested acceptors, except for glucose. For the E649C enzyme variant, the activity on glucose was decreased 10-fold compared to the wild-type enzyme. Because of its high activity on methyl β-glucoside, it was hypothesized that E649C would also be active on longer alkyl β-glycosides. The E649C mutant indeed showed activity towards ethyl β-glucoside, although it was only one-third of the activity on methyl β-glucoside. No activity was detected with octyl β-glucoside as acceptor. Remarkably, the E649C variant was active on phenyl β-glucoside as acceptor (Table 1).

The kinetic parameters of purified wild-type *C. uda* CP on glucose and of purified E649C enzyme on glucose, methyl β-glucoside and ethyl β-glucoside were determined (Table 2). It appeared that the E649C variant has similar k_{cat} values for all tested acceptors, but that the K_m values increase drastically with increasing alkyl chain length. For the E649C variant, the catalytic efficiency (k_{cat}/ K_m) was still highest with glucose as acceptor, although the K_m value for glucose increased more than 10-fold in comparison with wild-type. This is probably due to the loss of the hydrogen bond interaction between the anomeric hydroxyl group of glucose and the E649 residue, which was reported to be important for activity [13; 19]. Also the k_{cat} value for glucose decreased in comparison with wild-type, albeit to a much lesser extent.

DISCUSSION

Measuring inorganic phosphate released by GPs in the direction of glycoside synthesis is a convenient way to screen for enzyme variants with modified substrate specificity. The advantage is that no substrate-mimics are needed and that any substrate can be tested as donor or acceptor molecule without changing the screening protocol. A high-throughput screening method for phosphatases based on the identification of released inorganic phosphate in cells has been described earlier [21]. However, in some cases, it suffered from low signal-to-background ratio because of endogenous phosphatase activity, especially when glucose 1-phosphate was used as a substrate. In the present study, we report inhibition of phosphatase activity in crude *E. coli* cell extracts by the addition of fluoride and molybdate to the substrate solution. The high molybdate concentration (200 mM) used as inhibitor in enzyme reactions could conveniently be applied for the colorimetric determination of inorganic phosphate. Inorganic phosphate determination methods are usually based on the reduction of a phosphomolybdate complex under acidic conditions resulting in the formation of a molybdenum blue compound. Many variations of the original method described by Fiske and Subbarow [11] have been reported [12; 22; 23]. We decided to base our method on that of Gawronski and Benson [12] since it was specifically developed for inorganic phosphate measurement in the presence of acid-labile organic phosphate compounds (e.g. ATP and sugar phosphates). Furthermore, the produced color is stable over a long period of time and the absorbance of the molybdenum blue complex can be easily measured at 655 nm in a microtiter plate reader. The original method was modified to accommodate the high molybdate concentration already present in the sample as phosphatase inhibitor. Our modified method is different from others since it requires a relatively low ascorbic acid concentration and because molybdate is present in the sample instead of in the color solution. The modified inorganic phosphate assay is sensitive enough to detect low enzyme activity but does not

require dilution steps in the case of a 15 minute reaction with wild-type CP on its natural substrates. This is advantageous for high-throughput screening since dilution steps slow down the process and increase the variation.

The screening procedure was successfully applied for the detection of CP enzyme variants with modified substrate specificity. An E649C enzyme variant with novel acceptor specificity towards methyl β-glucoside, ethyl β-glucoside and phenyl β-glucoside, was identified by screening of a *C. uda* CP E649 saturation library. Replacement of the glutamate residue by the smaller cysteine residue allows alkyl and aryl β-glucosides to function as acceptor probably because more space is created around the anomeric hydroxyl group of glucose. If so, it is remarkable that we have found only the E649C beneficial mutation, while other small amino acids at site 649 could result in a similar effect. Although it cannot completely ruled out that some mutations were missing in our library, there might be also another explanation for the apparent preference of cysteine at site 649. Cysteine, commonly being classified as a hydrophilic amino acid, actually displays a strong hydrophobic character in proteins [24]. Consequently, it might create an optimal microenvironment to accommodate the hydrophobic substitutions of the tested β-glucosides, together with the closely located C491 residue (Figure 4). Possibly other small and hydrophobic amino acids are less appropriate than cysteine in this case.

Enzyme characterization revealed that the E649C mutation mainly affects binding of glucose and β-glucosides in the active site, rather than the chemical steps of catalysis. The kinetic parameters of the *C. uda* CP E649C variant on methyl β-glucoside are better than those of *Thermotoga maritima* CP, which is the only known CP active on methyl β-glucoside [25]. For this enzyme a K_m value of 135 mM and a k_{cat} value of 6.5 s^{-1} (k_{cat}/K_m=48 $M^{-1}s^{-1}$) were reported on the same acceptor [25]. The biggest difference is observed in the K_m value, which is 2.4-fold lower for the E649C mutant. There are no reports on the use of longer chain

alkyl glycosides as acceptor for *T. maritima* CP nor other phosphorolytic enzymes. The acceptor specificity of E649C towards various alkyl and phenyl β-glucosides allows the regiospecific enzymatic synthesis of the corresponding alkyl and phenyl β-cellobiosides, which are useful for enzyme studies with cellulases and cellobiases [26; 27].

The discovery of the E649C mutant shows that the screening procedure can be used to screen phosphorylase enzyme libraries for novel substrate specificities. The reported high-throughput screening system is general, and can be used to screen a whole range of different substrates, as was demonstrated here by using an acceptor mix composed of different molecules. Furthermore, it is also applicable to other GPs or phosphate releasing enzymes. The availability of a screening platform should now accelerate the creation of GP enzymes with modified substrate specificity towards aglycons such as vitamins and drugs.

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TABLES

Table 1. Overview of acceptor specificity of wild-type CP and the E649C mutant. One unit (U) of enzyme activity is defined as the amount of enzyme that converts 1 μmole of substrate in 1 min.

Acceptor[a]	Specific activity cell extracts (U/mg total protein)	
	Wild-type	E649C
Glucose	1.95	0.151
Methyl α-glucoside	-[b]	-
Methyl β-glucoside	-	0.215
Sucrose	-	-
Fructose	-	-
Ethyl β-glucoside	-	0.055
Octyl β-glucoside	-	-
Phenyl β-glucoside	-	0.159

[a] Acceptors were used at 100 mM each, except for glucose (30 mM) and phenyl β-glucoside (50 mM); [b] - = not detected

Table 2. Kinetic parameters of purified *C. uda* CP and the E649C enzyme variant on several acceptors

Enzyme	Acceptor	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
Wild-type CP	Glucose[a]	9.7 ± 0.8	2.1 ± 0.6	4660
E649C variant	Glucose[b]	6.3 ± 0.2	28.9 ± 2.3	220
	Methyl β-glucoside[c]	6.8 ± 0.1	56.6 ± 3.1	120
	Ethyl β-glucoside[d]	5.1 ± 0.1	155 ± 9.5	33

Values are obtained from non-linear fits of initial velocities at different substrate concentrations to the Michaelis–Menten equation and are presented as mean ± SE. Concentrations used: [a] 0.6-18.5 mM; [b] 4.6-148 mM; [c] 12-394 mM; [d] 62-493 mM

FIGURE LEGENDS

Figure 1. Influence of potassium fluoride concentration on phosphatase activity

Figure 2. Influence of sodium molybdate concentration on phosphatase activity

Figure 3. Effect of different compositions of color solution on the absorbances of a phosphate standard curve

Figure 4. View on the *C. gilvus* CP active site (PDB 2cqt), where E649 forms a hydrogen bond with the anomeric hydroxyl group of the glucose acceptor. The catalytic amino acid D490, C491, donor (glycerol mimic) and inorganic phosphate are shown as well.

Figure 5. Screening result of the E649 saturation library (●) and a wild-type CP plate as control (Δ) on non-native acceptors