Application of a very high-throughput digital imaging screen to evolve the enzyme galactose oxidase

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Directed evolution has become an important enabling technology for the development of new enzymes in the chemical and pharmaceutical industries. Some of the most interesting substrates for these enzymes, such as polymers, have poor solubility or form highly viscous solutions and are therefore refractory to traditional high-throughput screens used in directed evolution. We combined digital imaging spectroscopy and a new solid-phase screening method to screen enzyme variants on problematic substrates highly efficiently and show here that the specific activity of the enzyme galactose oxidase can be improved using this technology. One of the variants we isolated, containing the mutation C383S, showed a 16-fold increase in activity, due in part to a 3-fold improvement in $K_m$. The present methodology should be applicable to the evolution of numerous other enzymes, including polysaccharide-modifying enzymes that could be used for the large-scale synthesis of modified polymers with novel chemical properties.

Keywords: alcohol oxidation/digital imaging spectroscopy/directed evolution/galactose oxidase/high-throughput screening

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid; 4CN, 4-chloronaphthol; EPP, error-prone PCR; GO, galactose oxidase

Introduction

Galactose oxidase (GO) is of great potential utility to synthetic chemistry because it oxidizes primary alcohols, such as the C6 hydroxyl group of galactose, with concomitant reduction of molecular oxygen to yield aldehydes and hydrogen peroxide (Equation 1). Conveniently, no nucleotide cofactors are required by this enzyme.

$$\text{RCH}_2\text{OH} + \text{O}_2 \rightarrow \text{RCHO} + \text{H}_2\text{O}_2$$ (1)

Although standard chemical methods exist for oxidizing alcohols, these traditional methods rely on heavy metal-containing compounds such as chromium(VI) reagents and are performed in organic solvents (ten Brink et al., 2000). In addition, industrial chemical oxidation reactions give low yields of the desired aldehyde owing to their tendency to proceed to the carboxylic acid. In contrast, the use of enzymes provides a clear benefit, in terms of both environmental impact and yield of desired product.

Guar, a natural polymer isolated from seeds of the guar plant and other sources, can be oxidized enzymatically to yield an aldehyde-bearing polymer called oxidized guar (Figure 1). This compound can be used as an additive in paper manufacturing to confer mechanical strength to paper products (Chiu et al., 1996; Dasgupta, 1996; Brady and Leibfried, 2000). For our purposes, the activity of GO towards guar is of particular interest. The enzyme oxidizes the galactose side chains of guar, under ambient conditions of temperature and pressure, in aqueous solution. However, when isolated from its native host, GO is insufficiently active to catalyze economically the desired conversion on an industrial scale. We therefore undertook to improve its specific activity by in vitro evolution.

GO has been the subject of intense study for many years (Ito et al., 1991, 1994; McPherson et al., 1992; Baron et al., 1994; Wang et al., 1998). GO variants have been described that clarify the roles of certain residues in and around its active site (McPherson et al., 1993; Reynolds et al., 1995), but to our knowledge we describe here the first GO variants showing improved activity ($V_{\text{max}}$ and $K_m$).

A frequently observed property of polymers is that they form viscous solutions or are difficult to dissolve. Guar is no exception. When dissolved at a concentration of 1% in water, it has a viscosity of 1000 cP. Because viscous solutions or insoluble materials are difficult to handle using typical high-throughput screening systems, polymers can be problematic substrates for directed evolution. To realize fully the promise of enzymes in the chemical and pharmaceutical industries (Marrs et al., 1999; Charrain et al., 2000), a general solution to this situation is desirable.

It has previously been demonstrated that digital imaging can be interfaced with mutagenesis to isolate mutants having a desired phenotype with high efficiency (Arkin et al., 1990; Delagrave et al., 1995; Youvan et al., 1995). Bylina et al. (Bylina et al., 1999, 2000) described an instrument and methods for screening enzyme variants that is more efficient than robotics-based high-throughput systems. This system, known commercially as ‘Kcat’, combines single-pixel imaging spectroscopy with a solid-phase screening format; it can be applied to a broad range of problems because it relies on simple, widely known colorimetric activity assays. As we will show, this technology enables screens on solid substrates or highly viscous substrate solutions that are refractory to traditional high-throughput screening methods.

Materials and methods

Materials

Plasmid pGAO11 comprising the entire gaoA open-reading-frame (orf) (GenBank No. M86819) (McPherson et al., 1992), plasmid pPICZαA encoding GO fused to the yeast alpha factor under control of the AOX1 promoter and a rat anti-GO polyclonal antibody were kindly provided by Professor Michael McPherson of Leeds University, UK. Enzymes were obtained from Roche Molecular Biochemicals (Indianapolis, IN),
New England Biolabs (Beverly, MA), Epicenter (Madison, WI) and Sigma (St. Louis, MO). Oligonucleotides were synthesized by Operon (Alameda, CA). Kits from Qiagen (Valencia, CA) were used for plasmid preparation and extraction. DH10B competent cells were obtained from Life Technologies (Grand Island, NY).

DNA manipulations

Molecular biology techniques described by Sambrook et al. (Sambrook et al., 1989) were generally followed. The pBAD-myc/his vectors (Invitrogen, Carlsbad, CA) were used for recombinant expression of GO in Escherichia coli. The entire GO orf was subcloned into pBADmyc/his by digesting an overlap PCR product with SphI and HindIII and ligating this fragment to similarly digested vector. A silent XhoI restriction site at the 5' end of the GO orf and a 3' HindIII site immediately after two engineered stop codons were introduced by the oligos used for overlap PCR. The resulting construct, in which the GO orf was not in frame with the C-terminal myc/his tag provided by the vector, was designated pBADG06. A silent KpnI site was engineered into pBADG06 to yield clone pBADG03. This latter construct was used as a wild-type (WT) control in subsequent experiments. The GO orfs of both plasmids were sequenced completely.

Error-prone PCR was performed as described (Leung et al., 1989). PCR products were cloned using XhoI and HindIII sites to make mutant libraries, except for the libraries generated using clones GO.1-3 or 8-1 as templates, which were cloned using a PsI site internal to the GO orf and HindIII.

The expression ‘manual recombination’ simply refers to the construction of double and triple mutants from the single mutants obtained by error-prone PCR using standard molecular cloning techniques. Double mutant (C383S/Y436H) was constructed by subcloning a Smal–KpnI fragment from the C383S-containing clone into the Y436H-containing vector. The triple mutant was constructed by subcloning a Smal–BstXI C383S/Y436H-containing fragment into the Y494A-containing vector.

pPICZαA8-1 was constructed by PCR amplification of the GO orf of clone 8-1 using a 3' primer that introduces an XbaI restriction site downstream of the stop codon. The resulting PCR product was digested with PsI and XbaI and cloned into pPICZαK3, a Pichia expression vector encoding the wild-type GO protein fused to mating factor alpha. The construction of this vector and its use in Pichia pastoris will be described elsewhere (M. McPherson et al., in preparation.)

All mutants were sequenced completely in their orf. The 310 Genetic Analyzer (PE Biosystems, Foster City, CA) and sequencing reagents were used according to the manufacturer’s instructions. Sequencing data were analyzed using Sequencher (Gene Codes, Ann Arbor, MI).

Solid-phase assay

Solid phase assays and their use with the Kcat system have been described previously (Bylina et al., 2000) but additional details specific to this work are provided below. Freshly transformed cells were applied to a polyester 0.2 µm pore size membrane (Osmonics, Minnetonka, MN) that was placed on the surface of a growth plate (LB-agar containing 25 µg/ml carbenicillin, 100 µg/ml ampicillin). After overnight growth, the membrane was lifted from the growth plate and transferred to an induction plate (LB-agar containing 25 µg/ml carbenicillin, 100 µg/ml ampicillin, 0.2% l-arabinose and 0.5 mM CuSO4). This induction step was performed for at least 4 h at 26°C. To lyse the microcolonies on the membrane prior to assay, the membrane was placed in a chloroform vapor chamber for 45 s. Note that in each of these steps, microcolonies were always ‘facing up’ so that no colony lifts were performed. Also, microcolonies were generally grown and induced such that their final radii were uniform and <0.4 mm.

Methylgalactose (5 mM) assay plates were made by pouring the following into polystyrene petri dishes: 1% agarose (Biorad, Hercules, CA) in 50 mM potassium phosphate buffer (pH 7), 5 mM methyl-α-D-galactopyranoside (methylgalactose) (Sigma), 0.5 mM CuSO4, 1.5 mM 4-chloronaphthol (4-CN) (Pierce Chemical, Rockford, IL), 0.1 ml soybean or horseradish peroxidase dilution. Membranes bearing lysed microcolonies were transferred to assay plates and immediately introduced into a Kcat instrument (Kairos Scientific, Santa Clara, CA). This instrument was described in detail by Bylina and colleagues (Bylina et al., 1999, 2000).

Guar assay plates had a similar composition to methylgalactose assay plates except that the following modifications were necessary. Hot (>50°C) solutions of 2% guar (cationic guar, 80H1F; Hercules, Wilmington, DE) and 2% agarose, both prepared in 50 mM potassium phosphate buffer (pH 7), were first mixed in hot centrifuge tubes. CuSO4 and 4-CN were added and mixed thoroughly using a ‘vortex’. The resulting solution was centrifuged briefly to remove bubbles and poured into hot petri dishes. After solidification, a peroxidase solution was spread homogeneously on to the surface of the guar/agarose gel and allowed to diffuse into the gel at 4°C overnight.

Liquid-phase assay

Km and Vmax were measured to compare the variant enzymes to the wild-type. The velocity of each reaction (ΔA405/min) was determined by a linear fit to the increase in absorbance for the first 2 min of reaction. The velocity versus concentration of methyl-α-D-galactose was then fitted to the Michaelis–Menten equation to determine Km and Vmax.

A single colony was used to inoculate 3.0 ml of LB containing 60 µg/ml of carbenicillin, 0.002% l-arabinose and 0.32 mM CuSO4. The culture was grown for 24 h at 26°C with shaking to yield a saturated culture. Cleared cell lysate was used in the assays without further purification and could be stored at 4°C for several days. All components except the lysate were dissolved in buffer containing 50 mM potassium phosphate, 1 mM CuSO4, pH 7.0. The 250 µl assay mixture

Fig. 1. Structure of the guar monomer. Galactose oxidase (GO) catalyzes the oxidation of the circled hydroxyl group to yield an aldehyde-bearing polysaccharide. The resulting oxidized guar is a valuable new papermaking additive that increases paper strength.
Fig. 2. A library of mutants expressing GO variants was assayed using the Kcat system. Bacterial microcolonies expressing mutant GO genes were grown on a membrane and subsequently transferred to an assay medium. Expression of active GO enzyme caused microcolonies on the membrane to absorb light at 550 nm. The inset at the lower left shows the entire membrane as it appeared under illumination at 550 nm after the assay was completed. About 80% of all microcolonies on the membrane were not visible in this image because they expressed inactive GO mutants. The boxed area was expanded to provide the background image of this figure. Pixels in the image showing high OD 550 at 360 s (inset OD vs time plot) were color-coded to identify highly active mutants on the membrane. Red pixels in the image correspond to red curves in the OD vs time plot and identify a mutant designated GO.1-7 (see Table I). A second mutant (GO.1-8) was also found on this membrane but is omitted for clarity.

Results and discussion

Very high-throughput assay

To detect GO activity, we employ a coupled assay that generates a colored product. In this assay, hydrogen peroxide, a byproduct of GO activity (see Equation 1), is used by a peroxidase to transform 4-chloronaphthol (4CN) or ABTS into colored compounds. When the assay is performed in the liquid phase, ABTS is used to generate a soluble green compound. In the solid phase, 4CN is used, yielding an insoluble compound absorbing visible light maximally at 550 nm. The solid-phase assay, a general description of which was provided by Bylina and colleagues (Bylina et al., 2000), can be used to visualize the activity of GO in bacterial colonies, as described below.

About 4000–5000 bacterial microcolonies (each <0.4 mm in size) can be grown and induced on a single microporous membrane and subsequently exposed to chloroform vapor to lyse the bacterial cells. Colony lifts are conveniently avoided by growing the microcolonies directly on the surface of a membrane that lies on the surface of a growth medium such as LB-agar. After chloroform lysis, the membrane is transferred to the surface of an agarose gel (solid phase) containing a GO substrate such as methylgalactose or guar, as well as CuSO4, 4CN and horseradish peroxidase. The resulting assay plate is immediately inserted into a Kcat instrument, as described in detail by Bylina and colleagues (Bylina et al., 1999).

The instrument periodically captures a digital image of the membrane illuminated at a specified wavelength (in this case, 550 nm). Color develops in the microcolonies as a result of GO activity and the stored digital images are used to compute an absorbance versus time plot for each pixel in the image. The kinetic data associated with each pixel can be displayed and compared to determine rapidly which mutant colony on the assay plate is most active. By computing and comparing the activities of individual pixels, we avoid the complex problem of resolving contiguous microcolonies on the membrane without losing any useful information (Yang et al., 2000).

Figure 2 illustrates a typical output of the imaging and analysis software. Pixels in the image were color-coded according to their relative activity: red pixels corresponded to the most active colony on the entire assay plate. The red colony
corresponded to a mutant that was subsequently shown to have increased activity, as discussed below.

As seen in the inset in Figure 2, the reaction rate of GO in bacterial microcolonies is roughly linear in the first 7 min. As a result, at least four membranes (15 000–20 000 mutants) can be screened in 1 h using one instrument while still allowing adequate time for analysis of the data. At this rate, 80 000 mutants can easily be screened in a single day, which is an order of magnitude more than typical robotic high-throughput screens. Moreover, there is no need for co-expression of a peroxidase (Joo et al., 1999a,b) because it is simply added to the assay medium. Since the assay and instrumentation rely on a simple colorimetric reaction, we expect that the present methodology or variations thereof will be applicable to a wide range of different enzymes and chemistries.

In vitro evolution of GO using methylgalactose

The structure of guar (Figure 1) suggested that methylgalactose could be used as a proxy for this compound. We investigated the usefulness of this proxy substrate in directed evolution by comparing mutants isolated using methylgalactose with mutants isolated using guar.

The pedigree shown in Figure 3 summarizes how several mutants of the present study were obtained. The figure also clearly illustrates how the different mutations identified in the first round of mutagenesis and screening were used to generate a highly active GO variant (clone 8-1) and other mutants described below.

Error-prone PCR (EPP) encompassing most of the GO orf (~2 kb) was performed to yield two libraries in which ~58 and 79% of mutants were inactive. The latter library had a mutation rate of about 0.3% (six mutations detected in 2184 bp of sequence from randomly picked clones). Mutants were screened for improved activity at 37°C relative to the WT clones present as a background on each assay plate. Two mutants were picked from the assay plate shown in Figure 2: mutants GO.1-7 (red) and GO.1-8 (not shown, see also Table I). Twenty-one more plates like this one were assayed to give a total of seven improved mutants in this first EPP experiment. Each mutant, once purified by streaking, was compared with WT using the KCat instrument to ensure that it was more active (data not shown).

The orf of each mutant was sequenced completely. As can be seen in Table I, three types of substitutions (C383S, Y436N/H and V494A) could be unambiguously assigned to the increased activity of these mutants. Two of these (C383S and Y436N/H) occurred in several mutants and all three occurred alone in at least one clone. Silent mutations are omitted from the table.

Based on previously reported observations, (Wells, 1990; Chen and Arnold, 1991) it is apparent that the underlying mechanism for the stepwise improvements of phenotype seen in many directed evolution experiments involves mostly the accumulation of mutations whose effects on the phenotype (e.g. free energy of catalysis or thermostability) are roughly additive. It is therefore expected that many mutations can be combined in a single clone to yield a variant that is severalfold better than WT. We manually recombined (MR) the three mutations discussed above into a double mutant (6-1) and a triple mutant (8-1). The latter showed an ~16-fold higher activity than WT towards methylgalactose. A further round of EPP and screening was carried out using clone 8-1 as template or parent molecule. Among seven mutants isolated (Table I), clone 7.5.1 showed a slight improvement (20%) in activity towards methylgalactose compared with its parent. Compared with WT, clone 7.5.1 was 19-fold more active on methylgalactose. The data presented so far suggest that a proxy substrate is adequate to identify variants, such as C383S, that have improved activity towards a problematic substrate such as guar.

Screening GO mutants using guar

We wished to determine whether KCat technology could also be used to identify mutants with improved activity directly on highly viscous polysaccharides. Guar assay plates (see Materials and methods) were used to screen the WT-derived EPP library described above. A key observation was that the substitution C383S, as well as a new substitution of the same residue, C383G, were identified in two of five mutants isolated (mutant GO.05A, not shown in Table I because it is essentially identical with GO.1–10, encoding C383S as its only substitution and mutant GG51R, Table I). Another variant that we identified, GG41R encoding substitution N535D (Table I), showed that even slight improvements in GO activity could

![Fig. 3. Pedigree showing the relationships between different GO mutants and their activity compared with wild-type (WT). Mutants are identified by black dots (one amino acid substitution), ellipses (two substitutions), triangles (three substitutions) or squares (four or more substitutions). The position of a mutant along the vertical axis of this figure specifies its activity (Vmax/Km, methylgalactose) relative to WT. (EPP, error-prone PCR; MR, manual recombination.) For example, mutants encoding substitutions C383S and Y436H were isolated from an EPP library made using the wild-type sequence as a template; both mutants were manually recombined to generate the double mutant C383S/Y436H which was ~11-fold more active than WT.](image-url)
Table I. Amino acid substitutions of GO mutants and their kinetic properties

<table>
<thead>
<tr>
<th>Origin</th>
<th>Name</th>
<th>Recurring substitutions</th>
<th>Non-recurring substitutions</th>
<th>Km (mM)</th>
<th>Vmax (JOD405/min)</th>
<th>Vmax/Km</th>
<th>Activity 1% guar Rel. to WT</th>
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<td>Wild Type</td>
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<td>Y436H</td>
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<td>V494A</td>
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<td>N46D</td>
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<td>0.18</td>
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</table>

Kinetic parameters ($K_m$ and $V_{max}$) and activity towards a 1% guar solution were measured as described in the text. Rel. to WT: activity was measured as described in the text and is reported as a -fold improvement relative to WT. Recurring substitutions were isolated independently more than once (or a different mutation at the same residue).
be detected using this assay format, as verified by liquid-phase measurements.

**Mutants have improved intrinsic activity**

We measured and compared the activities in the liquid phase of several mutants, both on guar and on methylgalactose (Figure 4). This assay was similar to the solid-phase assay used to screen mutants, but required ABTS as a chromogenic substrate instead of 4CN. We measured the activity of cell lysates on 1% solutions of low molecular weight guar produced by acid hydrolysis and ultrafiltration. This guar had significant but reduced viscosity, permitting reproducible liquid-phase measurements on a limited number of samples. Size-exclusion chromatography and HPLC showed that the resulting hydrolyzed guar had a narrow distribution of molecular weights and that cleavage did not preferentially release galactose side-chains (Lei Qiao, personal communication.) $V_{\text{max}}$ and $K_m$ were measured for the same mutants using methylgalactose as a substrate in the liquid-phase assay (Table I). Figure 4 shows the correlation between the activity of mutants on guar ($\Delta\text{OD 405/min}$) versus methylgalactose ($V_{\text{max}}/K_m$).

Clearly, high activity towards methylgalactose correlates well with high activity towards guar. An interesting observation, however, is that one mutant (7.3.2, first identified on a methylgalactose plate) serendipitously has a reproducible preference for guar. When this mutant is included in a least-squares fit of the data, the correlation coefficient ($r^2$) is 0.72 but this value increases to 0.95 when the mutant is excluded from the fit. One might conclude, therefore, that mutants having a preference for guar over methylgalactose do exist and this argues in favor of using guar plates for screening libraries. We suspect that if additional generations of mutants had been screened on guar plates, clones like 7.3.2 would have been isolated more efficiently.

Although *E.coli* is a convenient organism for carrying out directed evolution, it is not always ideal for the expression of large amounts of protein. We are therefore interested in improving the intrinsic characteristics of an enzyme, such as kinetic parameters, rather than potentially host-specific characteristics relating, for example, to expression. The Michaelis constant, $K_m$, can be measured via liquid-phase assay of cell lysates without determining total enzyme concentration. This permits a rapid identification of GO variants that are more active due to an improvement in their catalytic properties rather than improved expression or solubility in *E.coli*. As shown in Table I, a decrease in $K_m$ for methylgalactose of approximately 3-fold is observed for GO mutants carrying a C383S substitution. An additional 1.7-fold increase in $V_{\text{max}}$ is also observed for this mutant.

As a further verification that mutants such as 8-1 are intrinsically more active, the genes for wild-type GO and clone 8-1 were introduced in expression vector pPICZα and expressed in two host strains of the yeast *Pichia pastoris*. Table II shows that the $K_m$ of wild-type and GO variant 8-1 were very similar to the *E.coli* data. The $K_m$ of 8-1 was again found to be decreased approximately by a factor of three relative to wild-type. Total activity ($V_{\text{max}}$) is higher in the mutant strains than wild-type.

Amino acid C383 resides in the internal cavity of domain 2 and is the only free cysteine in the protein, as discussed by Ito and colleagues (Ito *et al.*, 1994). Remarkably, our data show that changing a single sulfur atom in GO to an oxygen can decrease the enzyme’s $K_m$ significantly. The decreased volume occupied by serine compared with cysteine may allow a rearrangement of residues in the active site favoring tighter binding of substrate.

**Conclusion**

We have shown that a very high-throughput digital imaging screen can be applied to the isolation of enzyme variants with increased activity towards polymeric substrates. The screen can be performed on highly viscous substrates such as guar or on a small molecule proxy such as methylgalactose to isolate useful mutants. Our data also indicate that guar-specific phenotypes exist, supporting the notion that a guar-based screen would be preferable. This latter conclusion reinforces the significance of the screening method described here because it enables the evolution of enzymes to functionalize valuable substrates such as guar, cellulose, carboxymethylcellulose and other polymers that have problematic physical properties such as high viscosity.

**Acknowledgements**

Special thanks are due to Professor Michael McPherson for the goaA gene and GO antiserum and to Dr Lei Qiao for hydrolyzed guar samples. Thanks are also due to Beverly Banks-Stallings for technical assistance and to Dr Robert Grasso for assistance with molecular modeling. Finally, thanks are due to Drs Alfred Haandrikman and H.N.Cheng for helpful discussions and logistical support during this project.

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Digital imaging screen to evolve galactose oxidase


Received August 23, 2000; revised November 29, 2000; accepted December 29, 2000.