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Glycine Oxidase Based High-Throughput Solid-Phase Assay for Substrate Profiling and Directed Evolution of (*R*)- and (*S*)-Selective Amine Transaminases

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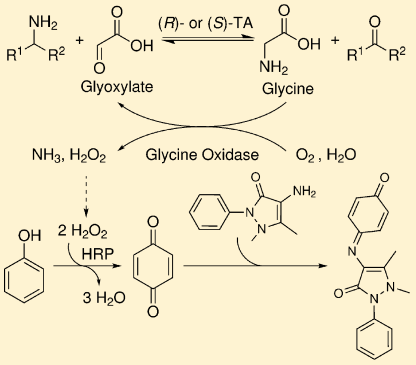
Supporting Information

ABSTRACT: Transaminases represent one of the most important enzymes of the biocatalytic toolbox for chiral amine synthesis as they allow asymmetric synthesis with quantitative yields and high enantioselectivity. In order to enable substrate profiling of transaminases for acceptance of different amines, a glycine oxidase and horseradish peroxidase coupled assay was developed. Transaminase activity is detected upon transfer of an amine group from an amino donor substrate to glyoxylate, generating glycine, which is subsequently oxidized by glycine oxidase, releasing hydrogen peroxide in turn. Horseradish peroxidase uses the hydrogen peroxide to produce benzoquinone, which forms a red quinone imine dye by a subsequent condensation reaction. As glycine does not carry a chiral center, both (*R*)- and (*S*)-selective transaminases accepting glyoxylate as amino acceptor are amenable to screening. The principle has been transferred to establish a high-throughput solid-phase assay which dramatically decreases the screening effort in directed evolution of transaminases, as only active variants are selected for further analysis.



Transaminases belong to the largest group of pyridoxal-5′-phosphate (PLP)-dependent enzymes[1] and catalyze the transfer of an amino group of a donor amine to the carbonyl carbon atom of an α-keto acid, a ketone, or an aldehyde. In the past decade, amine transaminases came into strategic focus to be applied as efficient biocatalysts for the preparation of optically pure amines, which represent highly valuable key intermediates or products in the pharmaceutical, chemical, and agricultural industries.[2,3] However, for application as efficient biocatalysts, numerous challenges have to be overcome, such as a limited substrate scope, issues of thermostability, or tolerance to high concentrations of organic solvent, achieving high levels of enantioselectivity and unfavorable equilibrium in asymmetric synthesis mode, as well as substrate and product inhibition. Besides rational protein engineering approaches, directed evolution still represents the key to engineer proteins to fit the manufacturing process, inevitably causing huge library sizes predominantly containing inactive or less active variants.[4] To speed up a directed evolution process, the availability of fast, sensitive, and efficient assays is crucial to avoid time-consuming and technically demanding analysis of each variant by HPLC or GC analysis as successfully applied by Savile et al.[5] Several assays for amine transaminases have been developed, mostly working in kinetic resolution mode due to the unfavorable equilibrium of asymmetric synthesis, and all of them are based on screening in microtiter plate format only.[6] Among those, the acetophenone assay represents the most facile screening method for transaminases, as transamination of α-phenylethylamine can be followed directly by spectrophotometric detection of the corresponding ketone.[7] However, the assay is restricted to amines carrying a phenyl group in the α position. Hence, Hopwood et al. developed a coupled microtiter plate assay for the detection of alanine by application of an L-amino acid oxidase (L-AAO) for (*S*)-selective transaminases and a D-amino acid oxidase for (*R*)-selective transaminases, respectively.[8] This way, a range of amine substrates and pyruvate can be applied as substrate pairs for screening, generating hydrogen peroxide which is detected by horseradish peroxidase in turn. However, the drawback of this assay is that L-amino acid oxidases are difficult to access for larger screening projects, as few L-AAOs have been successfully expressed as recombinant proteins in reasonable amounts.[9,10] Similar assays are also available for amino acid aminotransferases.[11,12]

Solid-phase assays have been successfully applied for directed evolution of many different proteins to increase the throughput by directly screening colonies expressing variants of the gene of interest.[13−18] The first solid-phase assay in which the protein of interest is coexpressed together with an additional assay enzyme has been developed for directed evolution of an alanine racemase by Willies and co-workers.[15] While preparing our manuscript, O'Reilly and co-workers published the application of ortho-xylenediamine dihydrochloride as amino donor substrate that forms a colored precipitate via spontaneous polymerization of the aromatic isoindole formed

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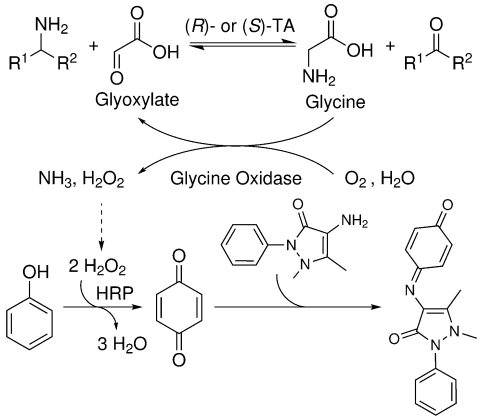
upon transamination.[19] Hence, direct high-throughput screening of transaminase libraries for asymmetric synthesis by application of a range of ketones is feasible owing to a shift of the equilibrium by *in situ* coproduct removal. However, it is yet unclear if this artificial amine donor is a widely accepted substrate for transaminases.

In this study, we report on a solid-phase assay that is amenable to high-throughput screening of both (*R*)- and (*S*)-selective transaminases for activity toward different amine substrates. In order to do so, we took advantage of the fact that many transaminases accept glyoxylate as the amino acceptor at similar rates compared to pyruvate.[20−22] Glyoxylate is converted into achiral glycine upon transamination of any chiral amine. Formation of glycine is detected by application of a thermostable glycine oxidase from *Geobacillus kaustophilus* that has been characterized by Martínez-Martínez et al.,[23] leading to the formation of hydrogen peroxide. For detection of hydrogen peroxide formation at alkaline pH values up to pH 9.5, the 4-aminoantipyrine assay involving horseradish peroxidase catalyzed oxidation of phenol to benzoquinone and formation of a red quinone imine dye by a subsequent condensation reaction was adapted (Scheme 1).[23] The

Scheme 1. Application of Glyoxylate as Amino Acceptor Substrate Allows Screening for Activity Towards Different Amines by Either (*R*)- or (*S*)-Selective Transaminases (TA)[a]



[a]Production of achiral glycine is then followed by oxidation and hydrogen peroxide formation leading to the formation of a red quinone imine dye. Toxic phenol can be substituted by vanillic acid as described by Holt and Palcic (Supporting Information 2.1).[24,25]

principle of this versatile assay is easily transferable to a microtiter plate assay (liquid-phase assay), by supplementing purified glycine oxidase externally to the reaction mixture.

EXPERIMENTAL PROCEDURES

Materials and Software. The following chemicals and enzymes were ordered from Sigma-Aldrich: Horseradish peroxidase, glyoxylic acid monohydrate, 4-aminoantipyrine, phenol, and agarose. Nitrocellulose membranes (HP44.1, 0.2 μm pore size, >200 μg/cm^2 binding capacity for proteins) were obtained from Carl Roth.

Data analysis and graphs were made using R.[26] Where applicable, the ggplot2 package was applied.[27]

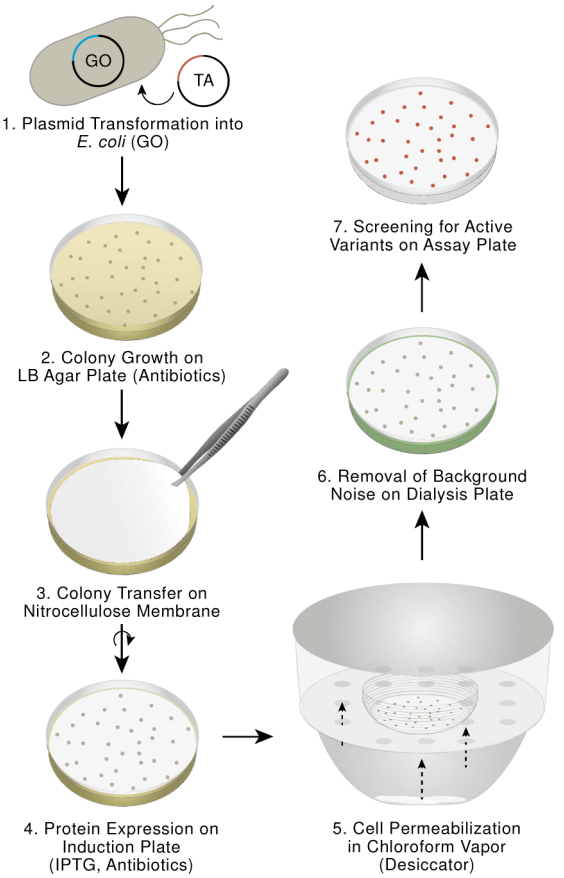
Cloning, Expression, and Purification of *Geobacillus kaustophilus* Glycine Oxidase. Locus tag GK0623 of *Geobacillus kaustophilus* encoding for glycine oxidase was ordered as a codon optimized synthetic gene in the pET28a(+) vector using *Eco*RI and *Not*I, downstream of the T7 polymerase promotor (GenScript). Subcloning in the compatible pCDF-1b vector was carried out by *Bam*HI and *Not*I restriction and ligation. For addition of the N-terminal His-Tag, a frame shift was carried out using the following oligonucleotides (5′-CGT AGC GAT GCG TCA TTG AAT TCG GAT CC-3′ and 5′-GGA TCC GAA TTC AAT GAC GCA TCG CTA CG-3′) in a 50 μL QuikChange-PCR (2 μL of each oligonucleotide (10 μM), 13 ng of template vector, 5 μL of 10× PfuPlus buffer, 1 μL of dNTPs (10 mM), 0.5 μL of PfuPlus! DNA polymerase (1 U/μL)). The reaction was performed using the following thermocycling conditions: (1) 95 °C for 2 min, (2) 24 cycles: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 6 min 30 s, and (3) 72 °C for 12 min. Afterward, 10 U of DpnI was added; the reaction mixture was incubated for 2 h at 37 °C to digest the parental DNA and finally was used to transform *E. coli* TOP10 cells.

For production of His-tagged glycine oxidase, transformed *E. coli* BL21(DE3) cells were grown at 37 °C in LB medium supplemented with 50 μg/mL spectinomycine. When the OD_{600} reached 0.8, expression was induced by addition of 1 mM IPTG and cells were incubated for 20 h at 30 °C at 180 rpm. Harvested cells were resuspended in sodium pyrophosphate buffer (75 mM) pH 8.5 containing 0.5 M NaCl, 0.2 μM FAD, and 5 mM imidazole and were lysed twice with a French press at 1700 psi. Two mg of DNaseI was added, and the suspension was centrifuged at 8000*g* for 30 min. The insoluble fraction was washed by resuspending in sodium pyrophosphate buffer (75 mM) pH 8.5 containing 0.5 M NaCl, 0.2 μM FAD, 5 mM imidazole, and 0.5% Triton X-100 and incubated at 37 °C, 180 rpm for 20 min. After another centrifugation, both the supernatant and the lysate were passed through a 0.45 μm filter and applied for IMAC purification using an ÄKTA-FPLC system at room temperature. A 5 mL His-Trap FF chelating affinity column was equilibrated with the resuspension buffer. After the sample injection, the column was washed with resuspension buffer until the UV-absorption reached baseline level again. Elution was carried out by application of a gradient from 0% to 55% of elution buffer (resuspension buffer supplemented with 0.5 M imidazole) over 24 column volumes. Finally, fractions at around 30% of elution buffer were collected and dialyzed against pyrophosphate buffer (25 mM) pH 8.5 containing 0.2 μM FAD and 0.1% Triton X-100 overnight at 4 °C. For long-term storage, 30% glycerol was added and samples were stored at −80 °C.

Liquid-Phase Screening. Detailed experimental procedures for expression, preparation of crude lysate of the different transaminases, and screening are given in Supporting Information 2.1. For screening using the GO assay or the acetophenone assay,[7] 20 μL of crude lysate dilution was pipetted in a microtiter plate and the reaction was started by adding the corresponding master mix solutions. Final substrate concentrations amounted to 2 mM α-phenylethylamine and 2 mM glyoxylate in CHES buffer (50 mM) pH 9.0 in total reaction volumes of 200 μL (acetophenone assay) or 150 μL (GO assay). In the glycine oxidase assay, the following final assay enzyme concentrations and reagents were additionally included: 0.14 mg/mL horseradish peroxidase (52 U/mg lyophilizate), 0.12 mg/mL glycine oxidase, 3 mM 4-aminoantipyrine, and 4.7 mM vanillic acid. In previous experiments, 7 mM phenol was applied instead of vanillic acid (data not shown). An increase of absorbance was followed at 498 nm (GO assay) and at 245 nm in the acetophenone assay at 37 °C.

Solid-Phase Assaying. *E. coli* BL21(DE3) cells carrying the pCDF-1b His-tagged glycine oxidase gene construct were made electro-competent and were used for the transformation of different transaminases of interest or for transformation of error-prone PCR (epPCR) libraries of transaminases. These transaminases were available in pET22 vectors subcloned as described previously.[28] Transformed libraries were directly plated out on LB agar supplemented with 50 μg/mL of spectinomycine (spec) and 100 μg/mL of ampicillin (amp) and were applied for solid-phase assaying. In the case of wild-type transaminases, transformation was carried out only once and the resulting colonies on LB agar plates (amp, spec) were stored at 4 °C. To plate out cells with different wild-type transaminases, a 5 mL LB-medium tube containing 50 μg/mL spec and 100 μg/mL amp was inoculated with the cells carrying both plasmids coding for glycine oxidase and the plasmids for the transaminases of interest. After 75 min of incubation at 37 °C at 180 rpm, the OD_{600} was determined. Finally, 20 μL of a dilution corresponding to an OD_{600} of 3.3×10^{-4} was plated out on LB agar plates containing 100 μg/mL amp and 50 μg/mL spec. After 11 h of incubation of the plates at 37 °C, colonies have grown to a reasonable size and a nitrocellulose membrane was placed on top of the agar plate. When the membrane is taken off, colonies stick to it and the membrane can be placed, colonies facing up, on an induction plate containing 50 μg/mL spec, 100 μg/mL amp, and 1 mM IPTG. For coexpression of glycine oxidase and the transaminase of interest, the induction plates with the membranes are incubated for 8 h at 30 °C. After expression, the membranes were placed in a desiccator containing chloroform for 45 s at room temperature for cell permeabilization. Afterward, membranes were placed on dialysis plates (0.4% agarose in TRIS buffer (30 mM) pH 8.5, 40 μM PLP, and 5 μM FAD) and incubated overnight at 4 °C. Assaying transaminase activity was then carried out by incubation of the membranes on assay plates (1% agarose, 50 mM CHES pH 9.0 or 9.5, 1.2 mM 4-aminoantipyrine, 3.86 mM vanillic acid, 2−10 mM glyoxylate, and variable concentrations of amine donor) at 37 °C. Before incubation, 1.3 mg of horseradish peroxidase lyophilizate (52 units/mg solid) dissolved in 150 μL of Milli-Q water was spread on the assay plate applied for screening. In Scheme 2, the solid-phase assay procedure is depicted for screening of an epPCR library of any transaminase of interest.

Scheme 2. General Procedure of the Solid-Phase Assay[a]



[a]*E. coli* BL21(DE3) cells are transformed with both plasmids coding for glycine oxidase and the transaminase library of interest (1) and colonies are grown on dual selection LB agar plates (2). Afterwards, colonies are transferred to nitrocellulose membranes (3) that are placed, colonies facing up, on induction plates containing IPTG for expression of the proteins (4). Then, cells are permeabilized by chloroform treatment (5). To eliminate false positive background color formation, permeabilized cell colonies are dialyzed overnight by placing the membranes on dialysis plates (6). Finally, screening is conducted by incubation of the membranes on assay plates (7).

RESULTS AND DISCUSSION

Expression, Purification, and Cloning of Glycine Oxidase. The gene coding for *Geobacillus kaustophilus* glycine oxidase has been subcloned in a pCDF-1b vector carrying a CloDF13 origin of replication, which is compatible to ColE1 (other pET vectors), P15A, and RSF1030 replicon carrying vectors.[29] As reported by Martínez-Martínez et al. and Mortl et al. for *B. subtilis* glycine oxidase, no expression of the *Geobacillus kaustophilus* glycine oxidase was detectable without the N-terminal His-tag.[30,31] Interestingly, the comparison of the original pET28a construct with the new pCDF-1b_HisTag_GO construct with regard to expression level revealed that the ratio of soluble to insoluble glycine oxidase increased significantly (Supporting Information 2.2). *Geobacillus kaustophilus* glycine oxidase was purified by IMAC purification using an ÄKTA-FPLC system. Desalting was carried out in a convenient one-step dialysis without any detectable protein precipitation. Expression yields of the pCDF-1b glycine oxidase construct with the His-tag amounted to 47.5 mg of purified and desalted protein per liter of LB fermentation broth.

Establishment of a Microtiter Plate Assay. In order to establish a liquid-phase assay for transaminases, the standard 4-AAP assay for detection of glycine oxidase activity was adopted for the detection of glycine produced upon the transamination reaction. Apart from horseradish peroxidase, purified glycine oxidase was added and, instead of glycine, the substrates for the transaminase of interest, glyoxylate and a range of amine donors, were added to the assay mixture. Calibration by application of glycine standards and end point measurements of absorbance at 498 nm revealed a linear range up to 0.67 mM (Supporting Information 2.1). In order to investigate the dynamic range of the assay involving continuous glycine formation, sequential dilutions of a purified (*R*)-selective transaminase were applied and screened against racemic α-phenylethylamine by following the increase of absorbance at 498 nm. Thus, an asymptotic relationship between the value of the observed initial rate and the amount of purified transaminase applied in the assay was ascertained (Figure 1).

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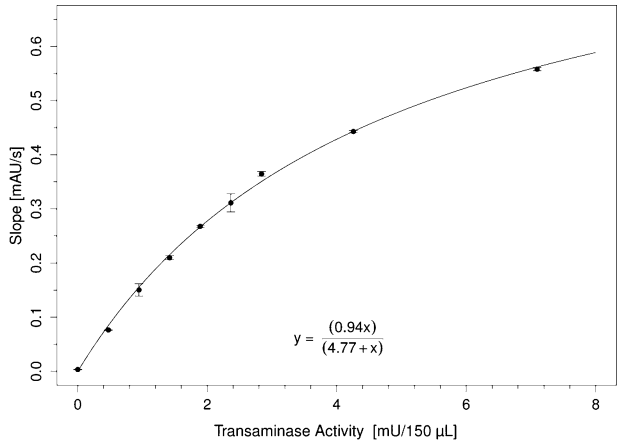

Figure 1. Relation between observed initial rate and transaminase activity. A purified (*R*)-selective transaminase was sequentially diluted and screened against racemic α-phenylethylamine.

For low transaminase activities up to 2 mU per well, a nearly linear relationship between the observed initial rate and the amount of transaminase activity in the assay has been found. The nonlinear relationship for higher transaminase activities can be explained by the fact that the transamination reaction is no longer the rate-determining step and the amount of assay enzymes has to be increased in turn to guarantee a faster downstream conversion of glycine to form the quinone imine.[12] These results are in accordance with the results of Barber et al., who also needed a >100-fold excess of assay enzymes for a linear relationship.[12] Increasing the amount of glycine oxidase in the assay could potentially lead to a larger dynamic range but would require the use of greater concentrations of purified GO. Controls without transaminase gave a negligible slope of $\sim 3 \times 10^{-3}$ mAU/s which may be explained by autocatalytic quinone imine dye formation. Hence, it was demonstrated that the assay distinguishes different transaminase activities, provided the amount of assay enzymes is adapted.

As a next step, it should be demonstrated that the glycine oxidase assay is suitable to screen different transaminases in crude lysate for acceptance of different amines. In order to do so, 25 different wild-type transaminase genes (Supporting Information 1.1.1) were expressed in *E. coli* BL21(DE3) in duplicates in a 96-deep-well block including a negative control of cells carrying an empty vector. The cells were lysed and two different dilutions of the resulting lysate were applied for a microtiter plate screening in both acetophenone assay and glycine oxidase assay against (*R*)- and (*S*)-α-phenylethylamine under identical conditions. On the basis of the data derived from the acetophenone assay, volumetric activities for each transaminase duplicate were calculated and compared with the slopes derived from the glycine oxidase assay.

All transaminases that have been found active against (*R*)- or (*S*)-α-phenylethylamine in the photometric assay were also found active in the glycine oxidase assay. As screening in crude lysate causes high deviations, only rough comparisons between the data were made and no quantitative correlation was made (Table 1). In our screening, a slope of more than 2-fold as high as the background signal was detected in the duplicates of the (*S*)-selective transaminase from *Vibrio fluvialis* in the glycine oxidase assay when screened against the (*R*)-enantiomer of α-phenylethylamine, while no increase of absorbance could be detected in the acetophenone assay. Particularly, for low

Table 1. Substrate Profiling Results of 15 Different Transaminases (Supporting Information 1.1.1) for Acceptance of (*R*)- and (*S*)-α-Phenylethylamine Obtained by the Glycine Oxidase Assay Screening in Crude Lysate Opposed to Results Obtained by Screening with the Acetophenone Assay

		Acetophenone-Assay	GO-Assay	
(*R*)-Selective	AspFum	+	++	(*R*)-PEA
	AspOry	+	++	
	AspTer	+	+	
	ATA117	+	++	
	GamPro	+	+	
	JanSp	+++	+++	
	LabAle	+	++	
	MycVan	+	+++	
	NeoFis	+	++	
	RhiEtl	+++	++++	
(*S*)-Selective	3I5T	0	0	
	3FCR	0	0	
	Cvi	0	0	
	Vfl	0	+	
	3HMU	+	+	
(*R*)-Selective	AspFum	0	0	(*S*)-PEA
	AspOry	0	0	
	AspTer	0	0	
	ATA117	0	0	
	GamPro	0	0	
	JanSp	0	0	
	LabAle	0	0	
	MycVan	0	0	
	NeoFis	0	0	
	RhiEtl	0	0	
(*S*)-Selective	3I5T	++	++	
	3FCR	+	++	
	Cvi	+++	+++	
	Vfl	++++	++++	
	3HMU	++++	++++	
		Activity	Activity	

transaminase activities (<0.5 mU per well), the discrimination of actual transaminase activity from background noise is difficult in the glycine oxidase assay and may result in false positives and/or negatives. Prolonged measurements and increased amounts of lysate can help to solve these problems.

For prescreening and substrate profiling, 0.12 mg/mL glycine oxidase (200 mU/well) proved to be sufficient for discrimination of a total transaminase activity of 0.5 mU per well (150 μL). Hence, one liter of shake flask fermentation broth provides enough glycine oxidase (47.5 mg of glycine oxidase yield) for prescreening of more than 20 microtiter plates (150 μL for each well).

Establishment of a Solid-Phase Assay. Initially, it was planned to apply purified glycine oxidase together with horseradish peroxidase onto the assay plates to screen colonies having expressed exclusively the transaminase of interest. However, this approach was discarded as only diffuse color formation in the whole assay agar was observed in preliminary experiments (data not shown) as reported by Willies et al. for the alanine racemase solid-phase assay.[15] Additionally, limited expression yields of glycine oxidase would have been a bottleneck for high-throughput screening of large transaminase libraries. Hence, a coexpression approach was followed in which glycine oxidase is expressed together with the transaminase of interest. In this way, high local concentrations of the reactants and the involved enzymes are achieved, which makes accurate staining of the colonies possible. Additionally, no purification of the glycine oxidase is required. Cells carrying the glycine oxidase gene on a pET28a plasmid were made electrocompetent and transformed with different transaminases

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coded on incompatible pET22 plasmids for proving the assay's principle. As transformation efficiencies amounted to only 20 transformants per 50 ng of incompatible plasmid DNA, later the glycine oxidase gene was subcloned into a compatible pCDF-1b vector to improve transformation efficiencies. Usually, more than 10 000 transformants per 50 ng of plasmid DNA are obtained by application of the compatible glycine oxidase pCDF-1b construct providing the possibility to transform error prone PCR based libraries for directed evolution.

For all published solid-phase assays so far, horseradish peroxidase substrates derived from staining protocols of immunohistochemistry or immunocytochemistry (like 4-chloronaphthol or 3,3-diaminobenzidine) have been applied as chromogenic substrates,[14−18] as they are initially soluble for good distribution and then precipitate *in situ* upon oxidation by horseradish peroxidase to prevent diffusion and to allow accurate staining.[32] However, the pH-working range of these horseradish peroxidase substrates is below pH 8.0,[33] while many transaminases are reported to have a narrow pH optimum above pH 9.0.[20] However, in all experiments involving 4-chloronaphthol and α-phenylethylamine, a yellow side product was formed preferably at pH values higher than pH 7.5. Hence, an alternative horseradish peroxidase substrate offering a higher sensitivity and screening at pH values above pH 9.0 was required. In this study, we successfully applied 4-aminoantipyrine and phenol or vanillic acid, respectively, as horseradish peroxidase staining substances for detection of hydrogen peroxide in a solid-phase assay at pH 9.0 and pH 9.5. In this way, we were able to detect low transaminase activities of approximately 50 mU/mg of an ordinarily overexpressed transaminase without problems of diffuse color formation or dye diffusion, despite the fact that the quinone imine dye is soluble. Thus, even transaminase activities corresponding to the activity of 3FCR toward (S)-α-phenylethylamine (0.4 U/mL crude lysate) can be detected in less than 1 h of incubation of the membrane on assay plates. In case of decently active transaminases like the 3HMU transaminase, staining begins already within 5 min of incubation at pH 9.0 or pH 9.5 on assay agar instead of hours of incubation at pH 7.5 by application of 4-chloronaphthol, as screening conditions could be harmonized to the pH optimum of 3HMU. Membranes can be taken off the assay agar after up to approximately 5 h of incubation depending on the amount of dye that has been formed and can be dried before considerable diffusion of the dye is relevant (Supporting Information 2.5.1).

In order to allow discrimination of low initial transaminase activities for acceptance of a desired amine compound, it is required that absolutely no staining of the colonies occurs that is not due to conversion of that amine. Hence, it should be investigated whether background color formation of an active transaminase occurs when no amine is supplemented in the assay agar. To do so, BL21(DE3) *E. coli* cells having coexpressed glycine oxidase and highly active 3HMU transaminase were placed on assay agar without amine donor. However, all colonies placed on assay agar without amine showed considerable color formation most likely due to hydrogen peroxide generated by oxidation of intracellular glycine or transamination of other intracellular amines. In order to solve these problems of high and unspecific background noise, an on-membrane dialysis step at 4 °C was introduced. After cell permeabilization, the nitrocellulose membranes were placed on a 0.45% agarose agar plate overnight to allow low molecular weight compounds to diffuse into the dialysis agar. Membranes after dialysis did not show any unspecific color formation on assay plates without amine, while controls, that were placed on LB agar plates at 4 °C and treated with chloroform for cell permeabilization afterward, showed unspecific color formation (Figure 2).

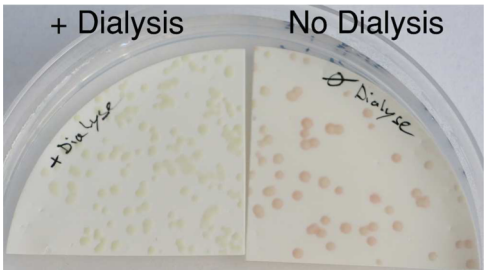

Figure 2. On-membrane dialysis diminishes background color formation. BL21(DE3) *E. coli* cells having coexpressed 3HMU and glycine oxidase after 2 h of incubation at 37 °C on an assay plate without amine donor. The left membrane was treated with chloroform after expression for cell permeabilization and placed on a dialysis plate overnight at 4 °C before screening. The right membrane was placed on an LB agar plate at 4 °C overnight after expression and then treated with chloroform before screening. Overnight dialyzed samples did not show unspecific staining compared to samples that had not been dialyzed.

To investigate the sensitivity of the solid-phase assay, the (S)-selective transaminases 3HMU and 3FCR were applied to the solid-phase assay procedure (Scheme 2) and screened against 10 mM racemic α-phenylethylamine at 37 °C for 30 min (Figure 3). Colonies expressing 3HMU became intensely red

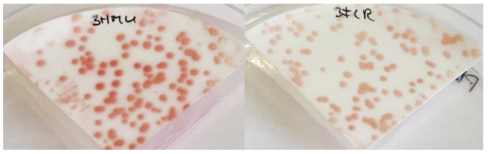

Figure 3. (S)-selective transaminases 3HMU (left) and 3FCR (right) showed different color intensities after 30 min of incubation at 37 °C on assay agar supplemented with 10 mM rac-α-phenylethylamine, owing to the difference in their activity.

colored within minutes of incubation on the assay agar, while the 3FCR sample, which showed a 40-fold lower volumetric activity in crude lysate screened in the acetophenone assay (0.4 U/mL instead of 16 U/mL) was less intensely colored, while samples incubated on assay agar without amine did not develop color at all (Supporting Information 2.5.2). As the detection limit depends also on the expression level of the transaminase of interest, no specific activities shall be compared.

To prove the applicability of the solid-phase assay as a prescreening method for error-prone PCR libraries that usually contain many inactive variants due to stop-codon and/or deleterious mutations incorporation, mixtures of cells carrying plasmids coding for glycine oxidase and different transaminases have been plated out. Mixtures of cells carrying transaminases of variable activity, namely 3HMU (16 U/mL), 3FCR (0.4 U/mL) and 3GJU (inactive), were prepared. These plates were screened against 2 mM (S)-phenylethylamine and 4 mM glyoxylate. Active transaminases 3HMU and 3FCR (Supporting Information 2.5.3) could be discriminated from inactive

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transaminase 3GJU carrying colonies. Mixtures of 3HMU and 3FCR transaminases containing colonies showed different staining intensities of colonies on the membrane according to their volumetric activities (Supporting Information 2.5.3).

Currently, the solid-phase assay is applied in our group for screening epPCR libraries. To evaluate the viability of the variants, the libraries are screened against α-phenylethylamine acceptance, a substrate well accepted by the template. The screening results in different pattern of coloring, as expected (Supporting Information 2.5.4). Colored colonies were picked as positive hits, and noncolored colonies were negative (inactive variants). These variants were expressed and were tested for activity in the acetophenone assay against α-phenylethylamine. The results were in line with the solid-phase assay; the positive hits have shown activity, while the negative ones did not have any detectable activity.

Screening of an epPCR library of 3HMU that possesses an average mutation frequency of 10 mutations per gene against the more bulky (*S*)-α-phenylpropylamine in the solid-phase assay resulted in a small number of colored colonies (Figure 4). These colonies were picked, and their activity toward (*S*)-phenylpropylamine was confirmed by a photometric assay, similar to the acetophenone assay (data not shown).

Figure 4. Solid-phase assay screening of a 3HMU epPCR library that possesses an average mutation frequency of 10 mutations per gene against (*S*)-α-phenylpropylamine led to a small number of colored colonies that are currently under investigation.

CONCLUSION

In this study, we demonstrated the proof of principle for a novel assay that allows screening and substrate profiling of transaminases for acceptance of a range of (*R*)- or (*S*)-amines. Expression and purification of glycine oxidase is easily manageable in shake flasks using *E. coli* as expression strain leading to high yields of recombinant protein sufficient to screen more than 20 microtiter plates of transaminase variants with GO obtained from one liter of LB fermentation broth. In addition, the assay is amenable for high-throughput prescreening of transaminase libraries on nitrocellulose membranes enabling directed evolution of transaminases for enhanced activity toward any amine substrate. The solid-phase assay provides a sensitivity that allows discrimination of specific activities of 50 mU/mg of an ordinary overexpressed transaminase from inactive variants.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.S.W. and M.H. developed the assay. M.S.W., M.H., and U.T.B. designed the experiments. M.W.S. and I.V.P. conducted the experiments. All authors interpreted the data, wrote the manuscript, and have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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