

Expression and stabilization of galactose oxidase in *Escherichia coli* by directed evolution

Lianhong Sun¹, Ioanna P.Petrounia¹, Makoto Yagasaki², Geethani Bandara and Frances H.Arnold³

Division of Chemistry and Chemical Engineering 210-41, California Institute of Technology, Pasadena, CA 91125, USA

¹L.S. and I.P.P. contributed equally to this work.

²Present address: Kyowa Hakko Kogyo Co. Ltd, 1-1 Kyowa-chou, Hofu-shi, Yamaguchi 747-8522, Japan

³To whom correspondence should be addressed. E-mail: frances@cheme.caltech.edu

We have used directed evolution methods to express a fungal enzyme, galactose oxidase (GOase), in functional form in *Escherichia coli*. The evolved enzymes retain the activity and substrate specificity of the native fungal oxidase, but are more thermostable, are expressed at a much higher level (up to 10.8 mg/l of purified GOase), and have reduced negative charge compared to wild type, all properties which are expected to facilitate applications and further evolution of the enzyme. Spectroscopic characterization of the recombinant enzymes reveals a tyrosyl radical of comparable stability to the native GOase from *Fusarium*.

Keywords: directed evolution/galactose oxidase/random mutagenesis/StEP recombination

Introduction

Galactose oxidase (Cooper *et al.*, 1959; Avigad *et al.*, 1962) (D-galactose: oxygen 6-oxidoreductase, GOase; EC 1.1.3.9) is a copper enzyme secreted by a number of fungal species. The GOase from *Fusarium* NRRL 2903 (Ito *et al.*, 1991; McPherson *et al.*, 1992, 1993; Baron *et al.*, 1994; Whittaker *et al.*, 1998; Whittaker and Whittaker, 1988; Rogers *et al.*, 2000), a glycoprotein (carbohydrate content ~1.7%) of 639 amino acid residues and molecular mass ~68 kDa, catalyzes the oxidation of primary alcohols to corresponding aldehydes coupled with reduction of O₂ to H₂O₂ (Figure 1). GOase accepts substrates ranging from glycerol and allyl alcohol to D-galactose, galactopyranosides, oligo- and polysaccharides (Schlegel *et al.*, 1968; Avigad, 1985; Bretting and Jacobs, 1987; Mendonca and Zancan, 1987). X-ray crystallography revealed a multi-domain structure dominated by β -sheets with a square pyramidal copper active center consisting of ligands from Tyr272, Tyr495, His496 and His581 (Ito *et al.*, 1991, 1994). A novel thioether bond between Cys228 and Tyr272 supports the radical mechanism first proposed based on spectroscopic studies (Whittaker and Whittaker, 1988; Whittaker *et al.*, 1989).

GOase has numerous applications in biosensors, chemical synthesis and diagnostics. Sensors incorporating GOase have been used to measure D-galactose, lactose and other GOase substrate concentrations (Vega *et al.*, 1998; Tkac *et al.*, 1999) in process monitoring (Szabo *et al.*, 1996), blood samples (Vrbova *et al.*, 1992) and quality control in the dairy industries (Adanyi *et al.*, 1999; Mannino *et al.*, 1999). Enzymatic synthesis of carbohydrates by GOase circumvents the require-

ment for protecting the hydroxyl groups (Root *et al.*, 1985; Mazur and Hiler, 1997; Liu and Dordick, 1999). GOase-catalyzed oxidation of cell surface polysaccharides is an essential step in the radiolabeling of membrane bound glycoproteins (Calderhead and Lienhard, 1988; Gahmberg and Tolvanen, 1994). The enzyme can also be used to detect a disaccharide tumor marker in colon cancer and precancer (Yang and Shamsuddin, 1996; Said *et al.*, 1999).

These applications of GOase would benefit from access to enzyme variants that are more stable and active towards non-natural substrates. A prerequisite to enzyme modification by powerful directed evolution methods (Arnold, 1998; Petrounia and Arnold, 2000) is functional expression in a host organism that permits creation and rapid screening of mutant libraries. *Escherichia coli* is an excellent host for directed evolution, but does not support functional expression of many important eukaryotic enzymes. To date, all biochemical studies of GOase have been performed on the enzyme obtained from its natural source or from fungal (McPherson *et al.*, 1993; Xu *et al.*, 2000) and yeast (Whittaker and Whittaker, 2000) expression systems not suitable for directed evolution. Expression of GOase in *E.coli* has been attempted (McPherson *et al.*, 1993), but functional enzyme was obtained only as a *lacZ* fusion (Lis and Kuramitsu, 1997). Biochemical characterization of the *E.coli*-expressed GOase was not reported. Herein we report on functional expression of GOase in *E.coli* achieved by directed evolution.

Materials and methods

Materials

All chemicals were reagent grade or better. 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), D-galactose and horseradish peroxidase (HRP) were from Sigma (St Louis, MO). Native *Fusarium* GOase was obtained from Worthington Biochemical Corporation (Lakewood, NJ). *Escherichia coli* strain BL21(DE3) and vector plasmid pUC18 were purchased from Novagen (Madison, WI). Restriction enzymes and ligase were obtained from Boehringer Mannheim (Indianapolis, IN), Life Technologies (Grand Island, NY) or New England Biolabs (Beverly, MA).

Bacterial strain and plasmids

Bacterial strain BL21(DE3) was used for cloning and library construction. Plasmid pR3 containing the gene for mature GOase fused to the 5'-end of the *lacZ* fragment was kindly provided by Dr Howard K. Kuramitsu (Department of Oral Biology, State University of New York at Buffalo). The GOase gene was amplified from pR3 by PCR to introduce a *Hind*III

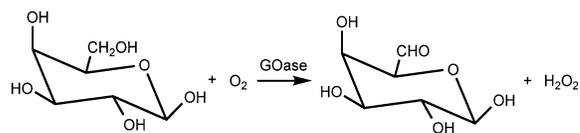


Fig. 1. Reaction catalyzed by GOase.

restriction site followed by an ATG initiation codon immediately upstream from the mature GOase sequence and *Xba*I site immediately downstream from the stop codon. The PCR product was subcloned into a modified vector pUC18 (containing a double *lac* promoter and lacking the *Pst*I site) to yield pGAO-036.

Construction of GOase mutant libraries

GOase was expressed in *E. coli* using plasmid pGAO-036. Two approaches were followed for directed evolution: (A) random mutagenesis of the complete GOase gene (bases 1–1917) by error-prone PCR (generations A1 and A2) and StEP recombination of improved variants from library A2 (generation A3) and (B) sequential random mutagenesis of a region of the GOase gene (bases 518–1917) by error-prone PCR (generations B1–B4).

Random mutagenesis and StEP recombination of the complete GOase gene. Error-prone PCR and StEP recombination (Zhao *et al.*, 1998) were carried out using primers 5'-AATTCGAAGCTTATGGCCTCAGCACCTATCGGAAGC-3' (*Hind*III site underlined) and 5'-CCTCCTTCTAGATTACTGAGTAACGCGAATCGT-3' (*Xba*I site underlined). The mutagenic PCR reaction contained 10 mM Tris-HCl, 50 mM KCl buffer (pH 8.5 at 25°C), ~0.3 µg plasmid DNA as template, 30 pmol of each primer, 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, 1 mM dTTP, 7 mM MgCl₂, 0.1 mM MnCl₂ and 1.5 U *Taq* polymerase (Perkin-Elmer, Gaithersburg, MD or Qiagen, Valencia, CA) in a total volume of 100 µl. PCR reactions were carried out on an MJ Research (Watertown, MA) thermal cycler (PTC-200) for 30 cycles with the following parameters: 94°C for 30 s, 50°C for 30 s and 72°C for 60 s. StEP recombination of four improved variants identified in generation A2 was performed in a 100 µl reaction containing 10 mM Tris-HCl, 50 mM KCl buffer (pH 8.5), ~0.3 µg (total) plasmid DNA as template (prepared by mixing equal amounts of the four plasmids), 10 pmol of each primer, 0.5 mM of each dNTP, 2.5 mM MgCl₂ and 5 U *Taq* polymerase. PCR conditions were as follows: 95°C for 3 min and 100 cycles of 94°C for 30 s and 58°C for 10 s. Mutagenic PCR or recombination products were purified using a DNA purification kit (Qiagen or Zymo Research, Orange, CA) and cloned (using the *Hind*III and *Xba*I restriction sites) back into the expression vector. Ligation mixtures were transformed into BL21(DE3) cells by electroporation.

Random mutagenesis of GOase gene region 518–1917. Error-prone PCR was carried out using primers 5'-TTGTTCTGCGGCTGCAGCAATTGAACCG-3' (*Pst*I site underlined) and 5'-TGCCGGTCGACTCTCTAGATTACTGAGTAACG-3' (*Xba*I site underlined). Mutagenic PCR was performed in a 100 µl reaction mixture containing 10 mM Tris-HCl, 50 mM KCl buffer (pH 8.3 at 25°C), 10 ng plasmid DNA as template, 50 pmol of each primer, 0.2 mM of each dNTP, 7 mM (generations B1 and B2) or 4 mM MgCl₂ (generations B3 and B4) and 5 U *Taq* polymerase (Boehringer Mannheim). PCR conditions were as follows: 94°C for 2 min and 25 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 60 s. Purified restricted inserts from PCR reactions were ligated with an expression vector generated by *Pst*I-*Xba*I digestion of pGAO-036. Ligation products were transformed into BL21(DE3) cells by a modified chemical transformation method (SuperComp protocol, Bio 101, Inc., Carlsbad, CA).

Screening GOase libraries

Transformed cells were plated on Luria-Bertani (LB) agar plates supplemented with 100 µg/ml ampicillin and grown overnight at 30°C.

Screening of libraries A1–A3. Single colonies were picked into deep-well plates (well depth 2.4 cm, volume 1 ml; Beckton Dickinson Labware, Lincoln Park, NJ) and cells were grown for 10 h at 30°C and 270 r.p.m. in 200 µl LB medium containing 100 µg/ml ampicillin (LB-Amp). The master plates were duplicated by transferring a 10 µl aliquot to a new deep-well plate containing 300 µl LB-Amp and 1 mM IPTG and grown for 12 h at 30°C and 250 r.p.m. The cultures were then centrifuged for 10 min at 5000 r.p.m. and the cell pellet was resuspended in 300 µl 100 mM sodium phosphate (NaPi) buffer, pH 7.0, containing 0.4 mM CuSO₄. Following addition of 0.5 mg/ml lysozyme (35 min at 37°C) and 2.5% (w/v) SDS (overnight at 4°C), the GOase activity was assayed using a GOase-HRP coupled assay (Baron *et al.*, 1994). Aliquots of the cell extracts were reacted with D-galactose (50 mM for generation A1 or 25 mM for generations A2 and A3) at pH 7.0. The initial rate of H₂O₂ formation was followed by monitoring the HRP-catalyzed oxidation of ABTS at 405 nm on a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA). Thermostability was assayed as follows: aliquots of the cell extracts were heated at 55–70°C for 10 min and then chilled on ice for 10 min. The samples were equilibrated to room temperature, at which point they were assayed for activity. The ratio of residual to initial activity was used to characterize thermostability. 1500–2000 clones from each library were screened and the clones with improved activity or enhanced thermostability accompanied with activity comparable to the parents were picked for further verification. *Screening of libraries B1–B4.* Single colonies were picked into deep-well plates (well depth 4.4 cm, volume 2.2 ml; Qiagen) and cells were grown for 8 h at 30°C and 270 r.p.m. in 500 µl LB-Amp. The master plates were duplicated by transferring a 10 µl aliquot to a new deep-well plate containing 500 µl LB-Amp and 1 mM IPTG and grown overnight at 30°C and 270 r.p.m. An aliquot of the culture was transferred to a microtiter plate. Following addition of 0.5 mg/ml lysozyme (30 min at 37°C) and 0.4% (w/v) SDS–0.4 mM CuSO₄ in 100 mM NaPi buffer, pH 7.0 (4 h at 4°C), the GOase activity was assayed using the GOase-HRP coupled assay as described above. The galactose concentration was 25 mM (generations B1 and B2) or 10 mM (generations B3 and B4). Approximately 1000 clones from each library were screened.

Protein purification and characterization

Escherichia coli cultures were grown for 16 h at 30°C in LB medium with 100 µg/ml ampicillin. Cells were harvested by centrifugation, resuspended in 100 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and disrupted by sonication. Cell debris was removed by centrifugation and the resulting supernatant was made 0.4 mM in CuSO₄ and stirred for ~2 h at 4°C. (NH₄)₂SO₄ was added to 25% saturation (w/v) and after centrifugation the supernatant was further saturated to 65% of (NH₄)₂SO₄. The pellet was dissolved in 100 mM CH₃COONH₄ buffer, pH 7.2, and chromatographed on Sepharose 6B (Amersham Pharmacia, Piscataway, NJ) according to published procedures (Hatton and Regoeczi, 1982). Fractions with the highest GOase activity were collected and precipitated by addition of (NH₄)₂SO₄ to 95% saturation. The pellet was dissolved in 100 mM NaPi, pH 7.0, and

Table I. Mutants identified during directed evolution of GOase

Generation	GOase name	Base substitutions	Mutations identified ^a	Relative total activity for D-galactose
0	Wild-type			1.0
A1	A1.D2	A1609G	N537D	1.6
A1	A1.D12	T408C/T1481C	<i>P136/V494A</i>	5.9
A2	A2.D3	T408C/T1481C/T28C	<i>P136/V494A/S10P</i>	11.1
A2	A2.C3	T408C/T1481C/A9C/ G584C	<i>P136/V494A/A3/ G195E</i>	9.1
A2	A2.D6	T408C/T1481C/T654C/ A936G/A1603G	<i>P136/V494A/T218/ L312/N535D</i>	11.7
A2	A2.E12	T408C/T1481C/A208G	<i>P136/V494A/M70V</i>	16.4
A3	A3.E7	T408C/T1481C/T28C/ A208G/G584A/A1603G	<i>P136/V494A/S10P/ M70V/G195E/N535D</i>	63.4
B1	B1.D4	A1237G	N413D	2.4
B2	B2.G4	A1237G/T1650A	N413D/ <i>S550</i>	3.8
B3	B3.H7	A1237G/T1650A/T1481C	N413D/ <i>S550/V494A</i>	10.5
B4	B4.F12	A1237G/T1650A/T1481C/ T1830A	N413D/ <i>S550/V494A/ S610</i>	15.2

^aSynonymous mutations are indicated by italics.

dialyzed extensively against the same buffer (the first dialysis buffer contained 0.2 mM CuSO₄). The dialyzed protein was filtered through a 0.2 μm filter and frozen immediately at -80°C. Fungal GOase was purified by passage through a Sepharose 6B column. The eluted protein was incubated with 0.1 mM CuSO₄ for 4 h at 4°C and desalted using a Biogel column (Bio-Rad, Hercules, CA).

The purified protein ran as a single band during SDS-PAGE (Novex, San Diego, CA). Protein concentrations were determined from the absorbance at 280 nm ($\epsilon = 1.05 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) (Ettinger, 1974). Kinetic measurements were performed in 100 mM NaPi buffer, pH 7.0, over a range of D-galactose concentrations from 15 to 250 mM using the HRP-ABTS coupled assay (Baron *et al.*, 1994). The rate of absorbance change was monitored by a Shimadzu (Columbia, MD) UV-Vis spectrophotometer at 420 nm ($\epsilon = 42.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) (Yamazaki *et al.*, 1997). A Lineweaver-Burk plot was applied, and the kinetic parameters were calculated using the program KinetAsyst (IntelliKinetics, State College, PA).

UV-Vis spectra of wild-type and mutant GOases were recorded from 320 to 900 nm. Oxidation of GOase was performed in 100 mM NaPi, pH 7.0, by incubation with 100 mM K₃[Fe(CN)₆] for 10 min followed by removal of the oxidant on a Biogel column at 4°C (Whittaker and Whittaker, 1988).

Thermostability of the purified native, wild-type and mutant GOases was assessed by measuring residual activity/initial activity over the temperature range 24–75°C. Enzymes (30 μl each, 0.054 mg/ml) were incubated at each temperature for 10 min in an MJ Research thermal cycler and chilled on ice before they were assayed in a microtiter plate. Stability at ambient temperature was measured by incubating 0.04 mg/ml GOase in the presence of 0.5 mM CuSO₄ and 3 U of catalase in a total volume of 100 μl and measuring activity as a function of time.

Results and discussion

GOase expression and directed evolution in *E.coli*

The GOase gene from pR3 was amplified to introduce the ATG initiation codon as well as the *Hind*III and *Xba*I restriction

sites, and the amplified fragment was subcloned into a pUC18 vector. Introduction of a second *lac* promoter led to increased expression (data not shown) and yielded plasmid pGAO-036. The cloned *gaoA* gene includes a pro-sequence encoding 41 amino acid residues, the N-terminal part of which has been proposed to be associated with secretion (McPherson *et al.*, 1993) and which is cleaved in a copper-mediated self-processing reaction (Rogers *et al.*, 2000). Functional expression of low levels of mature GOase in *E.coli* was accomplished in the absence of the pro-sequence.

To increase the total activity of GOase in *E.coli*, random mutagenesis was applied to the entire mature GOase gene and also to just the region of the gene encoding domains II and III which are responsible for catalytic activity (McPherson *et al.*, 1993). Adjusting the concentration of Mn²⁺ or Mg²⁺ during the PCR led to an error rate of ~2–3 base substitutions per gene. Mutant libraries were screened for activity on D-galactose, using HRP to detect the hydrogen peroxide produced during the reaction (Baron *et al.*, 1994). Higher-activity mutants identified in each round were subjected to further mutagenesis or recombination.

Screening 1600 mutants in the first round of mutagenesis of the complete GOase gene (library A1) generated two variants with 6- and 1.5-fold higher total activity toward D-galactose than the wild type (Table I). The most active variant A1.D12 showed enhanced thermostability as well and was used as template for the second generation. Screening ~1600 clones in the second generation identified four variants with improved activity and enhanced thermostability (A2.C3). Recombination of these four generated A3.E7 with ~60-fold higher total activity than wild-type GOase.

Screening the first generation library created by random mutagenesis of domains II and III (library B1) produced variant B1.D4 with approximately twice the total activity of the wild type. Three more rounds of mutagenesis and screening yielded variant B4.F12 with ~15-fold higher total activity than the wild type. Recombining the best mutants (A3.E7 and B4.F12) and screening ~3200 clones yielded no further improvement in total activity.

Protein purification, kinetics and spectroscopy

A rapid two-step procedure consisting of fractionation by (NH₄)₂SO₄ and chromatography on Sepharose 6B was

Table II. Yields and kinetic parameters of purified native fungal, wild-type and mutant GOases

GOase	Yield (mg l ⁻¹)	K _M (mM)	k _{cat} (s ⁻¹)	k _{cat} /K _M (M ⁻¹ s ⁻¹)
Native		95 ± 8	1391 ± 115	14 600 ± 1700
Wild-type	0.6	57 ± 3	1070 ± 43	18 800 ± 1200
B4.F12	4.7	57 ± 3	1195 ± 77	21 000 ± 1700
A3.E7	10.8	43 ± 2	1376 ± 35	32 000 ± 1700

developed for purification of GOase. Recombinant wild-type GOase and mutants B4.F12 and A3.E7 were purified and characterized. All three migrated on SDS-PAGE with an apparent molecular mass of 66 kDa rather than the 68.5 kDa predicted by the DNA sequence. The faster migration rate indicates that the thioether bond between Cys228 and Tyr272 is formed in all enzyme samples (Baron *et al.*, 1994; Rogers *et al.*, 2000), in contrast to results with an earlier GOase *E.coli* expression system in which the majority of the enzyme produced was inactive because it lacked the thioether bond (McPherson *et al.*, 1993).

Kinetic parameters for fungal GOase and *E.coli*-expressed wild-type and mutant GOases are reported in Table II, as are the yields of purified enzymes. The fungal and wild-type recombinant GOases exhibit similar kinetic behavior. Variant B4.F12 shows an 8-fold increase in production of GOase at shake-flask level while retaining the catalytic efficiency of the wild type. The 30-fold increase in total activity for variant A3.E7 relative to wild-type reflects an 18-fold increase in GOase expression and a 1.7-fold increase in catalytic efficiency. This variant yields 10.8 mg/l purified enzyme.

The broad substrate specificity of wild-type GOase (Schlegel *et al.*, 1968; Avigad, 1985; Bretting and Jacobs, 1987; Mendonca and Zancan, 1987) is retained in the evolved enzymes. Glycerol, xylitol, β-D-lactose and IPTG are oxidized by the wild type at 0.4, 1.5, 5 and 40% the rate of galactose oxidation, respectively. Similar relative activities were observed for the variant GOases (data not shown).

Solutions of pure GOase as isolated are a mixture of two oxidation states, oxidized (Cu²⁺-Tyr^o) and semireduced (Cu²⁺-Tyr) (Whittaker and Whittaker, 1988). These states may be interconverted by treatment with oxidizing or reducing agents and are distinguished by their absorption spectra: the semireduced form shows weak absorption over the visible region whereas the oxidized form is characterized by an intense absorption band at 445 nm and a broad band with a maximum near 810 nm (Whittaker and Whittaker, 1988; Baron *et al.*, 1994). Treatment of wild-type and evolved GOases with K₃[Fe(CN)₆] at 4°C led to the generation of the tyrosyl radical and the spectrum of the oxidized species was found to persist for several hours at ambient temperature (Figure 2) indicating that the oxidized form of the enzyme produced in *E.coli* has redox potential and stability comparable to the GOase from *Fusarium* (Baron *et al.*, 1994; Reynolds *et al.*, 1997).

Stability of GOase and mutants

The thermostabilities of the purified fungal and *E.coli*-expressed wild-type and mutant GOases are shown in Figure 3. The T₅₀ (the temperature at which the enzyme loses 50% of its activity following incubation for 10 min) is increased to 67°C for both B4.F12 and A3.E7 relative to a T₅₀ of 63°C for wild type. However, the *E.coli*-expressed wild type is less stable than the fungal enzyme, whose T₅₀ is 67°C. Similar

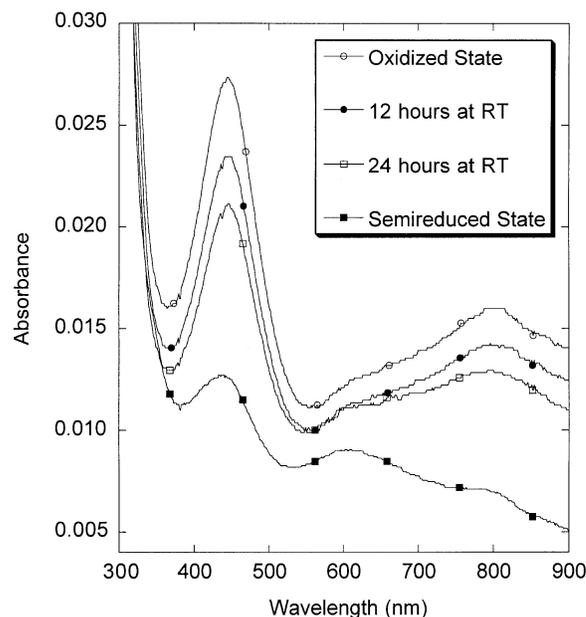


Fig. 2. Optical absorption spectra of 16 μM A3.E7 GOase in the semireduced and oxidized states. Spectra of oxidized GOase were recorded immediately and at 12 and 24 h following redox-activation by K₃[Fe(CN)₆].

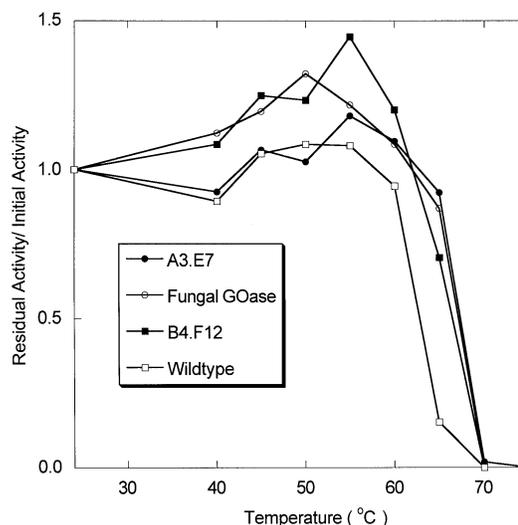


Fig. 3. Thermostability of wild-type, mutant and native GOases. Enzyme samples (1.6 μg) were assayed for thermal stability (residual activity/initial activity) after incubation for 10 min in 100 mM NaPi, pH 7.0, in a temperature range of 24 to 75°C.

results were obtained in the presence of 0.5 mM CuSO₄ (data not shown). Possible reasons for the reduced thermostability of the recombinant wild-type enzyme may be that the disulfide bonds do not form properly, or may be related to the lack of glycosylation.

The stabilities of the enzymes have also been determined under conditions used in the synthetic applications of GOase, i.e. at room temperature in the presence of CuSO₄ and catalase (Root *et al.*, 1985; Mazur and Hiler, 1997; Liu and Dordick, 1999). As shown in Figure 4, variants B4.F12 and A3.E7 and the fungal GOase are extremely stable, retaining ~80% activity following an 8-day incubation. In contrast, the wild type has lost ~50% activity under these conditions.

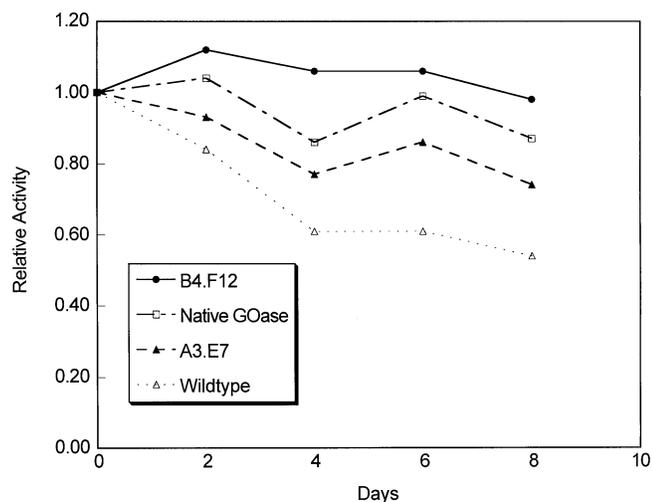


Fig. 4. Long-term stability of the native and *E.coli*-expressed GOases. Four micrograms of each enzyme were incubated at room temperature in the presence of 0.5 mM CuSO₄ and 3 U of catalase.

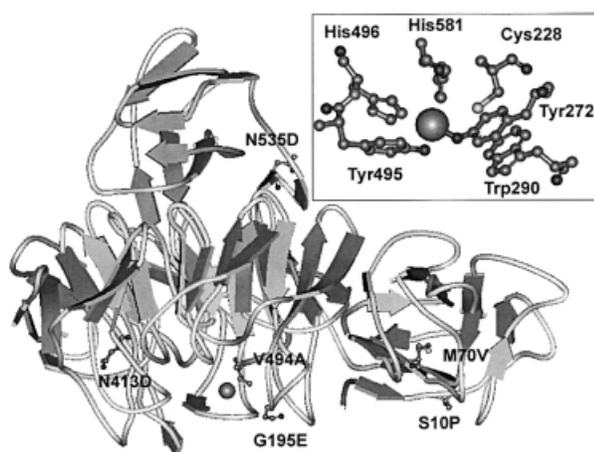


Fig. 5. Amino acid substitutions identified in GOase variants A3.E7 and B4.F12. Mutations are illustrated in ball-and-stick form. Inset shows the copper ion and residues comprising the GOase active site.

Sequences of evolved GOases

The mutations identified in the most highly expressed GOase variants are listed in Table I. Amino acid substitutions S10P, M70V and N413D as well as the synonymous mutations in codons S550 and S610 contribute to enhanced expression. The V494A substitution leads to increases in expression (variant B3.H7) and thermostability (variant A1.D12). G195E is a thermostabilizing mutation that appeared in clone A2.C3. The advantageous effect of N535D on expression became evident when recombined with other mutations in generation A3.

Identifying the mechanisms for the increased expression is difficult. However, we can provide some speculation. The mutation leading to amino acid substitution S10P is located at the N-terminal of the GOase gene; the nucleotide sequence in this region strongly influences gene transcription (Boer and Hui, 1990). Mutations could also contribute to changes in the secondary structure of GOase mRNA, which can affect protein expression (Cheong and Oriel, 2000). Synonymous mutations S550 and S610 generate codons that are much less frequently used (TCT, 17.4 per 1000 to TCA, 1.0 per 1000). Using rare codons in specific regions of the gene can be advantageous for protein expression (Komar *et al.*, 1999), probably by

inducing pauses in translation which result in a slower rate of protein synthesis and decreased levels of protein misfolding.

Screening the library made by recombining mutants A3.E7 and B4.F12 did not identify a more active variant than A3.E7. Nor did introduction of the N413D substitution into mutant A3.E7 by site-directed mutagenesis lead to a more active clone, demonstrating that the effects of the mutations are not cumulative.

Figure 5 shows the positions of the amino acid substitutions in variants B4.F12 and A3.E7. The V494A substitution is adjacent to the Cu(II) ligands Tyr495 and His496. The thermostabilizing mutation G195E occurs in a loop at the active site entrance and 10 Å away from copper. In wild type, Gly195 forms a hydrogen bond (2.90 Å) with Tyr189. A second charged hydrogen bond to Gly196 (2.40 Å) is introduced upon replacement of Gly with Glu, which may explain the beneficial influence of this mutation on GOase stability.

Three of the mutations identified in the evolved GOases (Figure 5) result in the replacement of neutral residues with negatively charged ones on the surface of the enzyme. Introduction of the negative charges reduces the unusually high basicity of the enzyme (pI = 12) (Mazur, 1991) and is expected to weaken interactions with other macromolecules. This will attenuate the sensitivity of GOase to inhibition and inactivation by macromolecular contaminants as well as promote reactivity with glycoconjugates attached on the cell membranes (Mazur, 1991). This effect, along with the thermostabilizing influence of V494A, need not be confined to *E.coli* as host for GOase expression; since these mutations are not detrimental to catalytic efficiency (Table II), introduction of these amino acid substitutions to GOase and expression of the enzyme in high-level expression hosts such as *Pichia pastoris* (Whittaker and Whittaker, 2000) and *Aspergillus oryzae* (Xu *et al.*, 2000) should generate active GOase of reduced basicity and increased stability. Enhanced thermostability and improved expression in *E.coli* facilitate protein purification and characterization. Enhanced expression also improves the reproducibility and sensitivity of screening. Thus the evolved GOase will be advantageous as a template for further evolution.

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