Published in final edited form as:

Biochem Biophys Res Commun. 2007 May 18; 356(4): 925–929.

Characterization of wheat germin (oxalate oxidase) expressed by *Pichia pastoris*

Heng-Yen Pan^a, Mei M. Whittaker^a, Romaric Bouveret^b, Anne Berna^b, François Bernier^b, and James W. Whittaker^{a,*}

^a Department of Environmental and Biomolecular Systems, Oregon Health and Sciences University, Beaverton, Oregon, 97006-8921

^b IBMP-Institut de Botanique, Strasbourg, France

Abstract

High-level secretory expression of wheat (*Triticum aestivum*) germin/oxalate oxidase was achieved in *Pichia pastoris* fermentation cultures as an α -mating factor signal peptide fusion, based on the native wheat cDNA coding sequence. The oxalate oxidase activity of the recombinant enzyme is substantially increased (7-fold) by treatment with sodium periodate, followed by ascorbate reduction. Using these methods, approximately 1 g (4×10^4 U) of purified, activated enzyme was obtained following eight days of induction of a high density *Pichia* fermentation culture, demonstrating suitability for large-scale production of oxalate oxidase for biotechnological applications. Characterization of the recombinant protein shows that it is glycosylated, with N-linked glycan attached at Asn47. For potential biomedical applications, a nonglycosylated (S49A) variant was also prepared which retains essentially full enzyme activity, but exhibits altered protein-protein interactions.

Keywords

germin; oxalate oxidase; *Pichia pastoris*; glycoprotein; cupin; glycan

Introduction

Wheat germin is a water-soluble, heat stable, protease-resistant glycoprotein that serves as a marker of embryonic development in germinating wheat grains [1–3]. In a broader context, both germans (which are restricted to grasses) and the related germin-like proteins (which are ubiquitous plant proteins) are members of the functionally diverse cupin superfamily, based on a β -barrel domain containing two conserved histidine-containing sequence motifs. Wheat germin, like all germans, possesses oxalate oxidase activity [4]. In contrast, none of the germin-like proteins are oxalate oxidases although several possess superoxide dismutase activity [3].

Oxalate oxidases catalyze the oxidative cleavage of oxalate to carbon dioxide with reduction of dioxygen to hydrogen peroxide [5,6]:

* Address correspondence to: James W. Whittaker, Department of Environmental and Biomolecular Systems, Oregon Health and Science University, 20000 N.W. Walker Road, Beaverton, Oregon 97006-8921, Tel. 503 748-1065; Fax. 503 748-1464; E-Mail: jim@ebs.ogi.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The enzyme requires manganese for catalysis [7], and the X-ray crystal structure of barley (*Hordeum vulgare*) oxalate oxidase (HvOXO) shows that the metal ion is bound by three His and a Glu from the conserved cupin sequence motifs [8,9]. Two N-glycan motifs (NXS/T) are also present in the protein sequence (at Asn47 and Asn52: NTSTPNGS), and HvOXO purified from barley seedlings is a glycoprotein [7], as is the recombinant HvOXO produced by *Pichia pastoris* [10]. On standing, recombinant HvOXO loses its carbohydrate, but retains full catalytic activity. The X-ray crystal structure of highly deglycosylated recombinant HvOXO reveals a single GlcNAc bound to Asn47 [11]. Barley HvOXO (GenBank accession no. CAA74595) and wheat (*Triticum aestivum*) germin (TaOXO) (GenBank accession no. P15290) share the same N-glycan attachment motifs, and a single N-glycan moiety was found to be attached to each monomer of wheat germin in earlier studies [12].

Oxalate oxidase is widespread in nature and has been found in bacteria [13], fungi [14] and various plant tissues [1–3]. In plants, OXO appears to play important roles in antimicrobial defense and signaling. A number of fungal pathogens of plants produce millimolar concentrations of oxalic acid during infection, etching the cell surface and interfering with guard cell function [15,16]. Plant cells express OXO as an important countermeasure to fungal invasion, eliminating the oxalic acid and producing hydrogen peroxide, which can serve both as a fungicidal agent and as a signal for plant defenses and development [1]. Several species of plants have been rendered fungus-resistant by heterologous expression of wheat OXO [17–21]. In addition to these biological roles, OXO has significant bioanalytical applications and is routinely used in the clinical determination of oxalate in biological fluids [22], and has potential applications in treatment of calcium oxalate kidney stones [6]. Other applications of OXO include the prevention of formation of calcium oxalate deposits in paper manufacture [23].

Wheat germin has been successfully expressed in transgenic tobacco [24], and HvOXO has been expressed in *Pichia pastoris* [10], using a synthetic gene. Heterologous expression in *E. coli* has also been reported, and activity was detected in an *E. coli* strain optimized for disulfide formation for both wheat germin and barley OXO cDNA clones [23]. In the present study we demonstrate high level production of TaOXO by *Pichia pastoris* using the native wheat germin GF-2.8 cDNA sequence. Earlier work has demonstrated that the allergenicity of germin and germin-like proteins was due to IgE binding, most likely *via* a carbohydrate determinant [25]. Thus, a nonglycosylated variant (S49A TaOXO) was also produced for potential biomedical applications.

Materials and Methods

Biological materials

Pichia pastoris X33 and the vector pPICZαB were from Invitrogen (San Diego, CA). Competent *E. coli* XL-2 Blue and BL21(DE3) cells were from Stratagene (La Jolla, CA).

Expression vector construction

The *Pichia* expression vector was based on the sequence of wheat germin GF-2.8 cDNA. The sequence encoding the mature wheat germin polypeptide (lacking the native N-terminal signal peptide) was amplified from a pEMBL18 vector containing the cDNA template [24] using a pair of primers designed to introduce a 5'-*Xho*I restriction site required for splicing into the α-mating factor coding sequence in pPICZαB (5'-CGA CTC GAG AAA AGA ACC GAC CCA GAC CCT CTC CAG GAC TTC-3') and a 3'-*Xba*I restriction site (5'-CGA TCT AGA TTA AAA CCC AGC GGC AAA CTT GGA CTT G-3'). Both the pPICZαB vector and the TaOXO PCR product were digested with *Xho*I and *Xba*I restriction enzymes. Because the wheat germin sequence contains an internal *Xho*I site, ligation was performed in two steps. First, the double-

digested vector arms and PCR product were ligated and a vector containing the 3' end of the PCR product (250 nt) was isolated. This product was then cut by *Xho*I, treated with alkaline phosphatase, and ligated with *Xho*I-digested TaOXO PCR. A vector containing the complete germin coding sequence with correct orientation was identified by restriction digestion and PCR analysis, and the sequence of the insert was verified by nucleotide sequence analysis (Molecular Biology Core Facility, Oregon Regional Primate Research Center, Beaverton, OR). The expression cassette was linearized with *Pme*I and electrotransformed into electrocompetent *P. pastoris* X33 according to standard methods [26]. High copy number chromosomal integrants were selected using YPDS agar containing 1 mg/mL Zeocin.

Site directed mutagenesis

The wheat germin pPICZaBTaOXO expression vector served as a template for production of mutational variants using the QuikChange Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA) with three 5'-phosphorylated primers: N47A, 5' -G GCC AAG GCC GGC GCC ACG TCC ACC CCG-3'; S49A, 5'-C GGC AAC ACG GCC ACC CCG ACC-3'; N52A, 5'-CG TCC ACC CCG GCC GGC TCC GCA GTG AC-3'). Silent mutagenesis of the N52A primer (GCA to GCC) was used to prevent strong secondary structure. The sequences of the mutant plasmids were verified by nucleotide sequence analysis.

Protein expression and purification

Pichia pastoris transformants to be screened for recombinant wheat germin expression were cultivated in 20 mL of BMGY medium [26] at 30°C for 24 h. The cells were collected by centrifugation and resuspended in 2 mL of BMMY medium [26]. Methanol (0.5% of the culture volume) was added daily for 6–8 days. Aliquots of each culture were tested for TaOXO expression by SDS gel electrophoresis. For small-scale protein production, the cells were grown in 2 L of BMGY medium in two 2 L baffled shake flasks to O.D.600 nm ~40. The cultures were centrifuged and the cells resuspended in 180 mL FM22 medium [26] (including trace metals and biotin) plus 20 mL of sterile 0.5 M potassium phosphate buffer, pH 7, and 0.5% methanol was added daily for 8 days. Large-scale protein production was accomplished by high density fermentation [27] as previously described [10], except that 5 mM MnCl₂ was added either at the beginning of the fermentation or after the start of the induction phase. TaOXO was purified as described for HvOXO [10].

Biochemical methods

TaOXO activity was measured by a coupled assay based on 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) oxidation by horseradish peroxidase [7]. TaOXO activity was also measured using a thermostated (25°C) Clark oxygen electrode as previously described [28] calibrated with the protocatechuic acid/protocatechuate dioxygenase reaction [29]. Assay mixtures typically contained 50 mM sodium succinate buffer (pH 4) with 20 mM oxalate. Protein concentration was estimated by the method of Lowry *et al* [30]. The molecular mass of denatured protein was estimated by SDS-PAGE using Bio-Rad protein molecular weight standards. Proteins resolved by SDS-PAGE were stained with GelCode Blue staining reagent (Pierce Biotechnology, Inc., Rockford, IL). Gels were digitized using a scanner and analyzed with timage Measurement and Analysis program [31]. SDS-PAGE gels were stained for glycoproteins using periodate oxidation and conjugation with pararosaniline [32]. A glycoprotein (*Phanerochatae chrysosporium* glyoxal oxidase) and a nonglycosylated protein (*Lactobacillus plantarum* manganese catalase) served as experimental controls. Metal ion analyses were performed using a Varian Instruments SpectraAA 20B graphite furnace atomic absorption spectrometer. Protein samples for mass spectrometry were dialyzed extensively against 5 mM ammonium acetate, and sandwich spots of the protein in a sinapinic acid matrix were analyzed on a QSTAR XL mass spectrometer (Applied Biosystems, Foster City, CA).

equipped with an oMALDI 2 ion source (by Dr. Cory Bystrom, Proteomics Shared Resource, OHSU Proteomic Core Laboratory). The pH-dependence of protein solubility of WT and S49A TaOXO was compared by dialyzing starting solutions (6 mg/mL protein in 25 mM sodium acetate buffer, pH 5, 0.5 mL samples) against 25 mM sodium acetate (pH 5 (control), 5.5), or 25 mM potassium phosphate (pH 6, 6.5, 7). After 24 h, the samples were transferred to a 1.5 mL microcentrifuge tube and the dialysis tubing was rinsed with sufficient dialysis buffer to give a final weight of 1 g for each sample. Insoluble protein was separated by centrifugation, and the protein concentration in the supernatant was determined by the Lowry method [30].

Activation of TaOXO with sodium periodate

TaOXO (5 mg/mL in 10 mM sodium succinate buffer, pH 4) was titrated with 5 mM sodium periodate (NaIO_4) to the end point (approximately 1.2 equivalents based on the Mn content), with oxidation being monitored by the increase in near UV absorption (250 – 500 nm). The reaction mixture was immediately desalted by gel filtration. Pooled protein fractions were made 1 mM in ascorbic acid by addition of 100 mM ascorbic acid stock solution, and excess ascorbic acid was removed by a second gel filtration column. Alternatively, ascorbate was added (to 2 mM) directly to the protein mixture after periodate oxidation, followed by gel filtration. The activity of the oxidized enzyme was determined as described above.

Results

Heterologous expression of wheat germin in *Pichia pastoris*

P. pastoris transformed with the pPICZαBTaOXO expression cassette secreted active TaOXO into the medium continuously beyond one week (Fig. 1). High-density fermentation (5 L starting volume) yielded ~1 g of purified TaOXO with a specific activity of 5.8 ± 0.1 U/mg and containing 0.286 ± 0.04 Mn/monomer. On SDS-PAGE the protein appears as one major band of 26 kDa and a minor band of 23 kDa (Fig. 2, Top, lane 2; Bottom, lane 1'). Only the higher MW band is visible when the SDS-PAGE gel is stained for carbohydrate (Fig. 2, Bottom, lane 1).

Glycan composition of recombinant TaOXO

Two major species are resolved by MALDI-TOF mass analysis of WT TaOXO (21278 amu and 23307 amu; calculated: 21279 amu) (Fig. 3A). Each of these species gives rise to a progression of mass peaks separated by regular intervals. A single interval in the lower mass region (204 amu) corresponds to the mass of a single N-acetyl glucosamine moiety. The interval between the lower and higher mass regions ($23144.2 \text{ amu} - 21278 \text{ amu} = 1865.8 \text{ amu}$) is consistent with the presence of two N-acetyl glucosamine residues and nine simple hexose sugars (e.g., mannose, 162 amu). The higher mass region exhibits four regular intervals of approximately 162 amu. In addition, both progressions include an additional pattern offset by approximately +60 amu, consistent with the presence of a bound transition metal ion.

Expression of nonglycosylated TaOXO

Substitution of Ala for the Asn in the two NXS motifs resulted in proteins with distinct properties. While N52A TaOXO shows the same electrophoretic mobility as the higher MW component of WT TaOXO, N47A TaOXO behaves like the smaller, nonglycosylated component on SDS-PAGE (Fig. 2, Top, lanes 2, 4 and 5), indicating that the glycan is attached to Asn47. However, the expression level of N47A TaOXO is very low (Fig. 2, Top, lane 4). In contrast, disruption of the first NXS motif by substitution at serine (S49A) also results in expression of nonglycosylated protein (Fig. 2, Top, lane 3; Bottom, lanes 2 & 2') but with a much higher yield, comparable to that observed for glycosylated N52A TaOXO (Fig. 2, Top, lanes 3 and 5). After 4 days of methanol induction, a 5 L culture yielded ~160 mg of purified

S49A TaOXO, containing 0.197 ± 0.001 Mn/monomer, with the same specific activity as the WT enzyme (5.75 ± 0.06 U/mg). The MALDI-TOF MS analysis of this protein shows a single species (21263 amu; calculated: 21263 amu) (Fig. 3B).

Solubility of nonglycosylated S49A TaOXO versus WT TaOXO

During the purification of S49A TaOXO the concentrated protein was found to precipitate at neutral pH, in contrast to the WT protein. The solubility of WT and S49A TaOXO was investigated over a range of pH (5.0 – 7.0), and the solubility of the S49A TaOXO variant was found to be significantly reduced at higher pH, compared to the WT TaOXO under the same conditions (Table 1). The precipitate formed by the S49A TaOXO solutions dissolves in 25 mM sodium acetate buffer (pH 5) without loss of activity.

Activation of TaOXO by periodate

Wheat OXO activity is dramatically increased by treatment with periodate and ascorbate. For the WT protein, the activity after oxidation is 42.2 ± 5.5 U/mg (corresponding to 147 ± 19 U/mg/Mn), an increase of more than 7-fold. For S49A TaOXO, the activity increases to 21.9 ± 1.5 U/mg (corresponding to 111 ± 8 U/mg/Mn), an increase of approximately 4-fold.

Discussion

Large quantities of active recombinant wheat OXO were isolated from high density fermentation cultures of *Pichia pastoris* containing an expression cassette based on the native cDNA sequence, with the amount of protein increasing linearly beyond one week (Fig. 1). The efficient translation of the plant cDNA sequence demonstrates that *Pichia* be generally useful for functional studies on germins (and germin-like proteins) from a variety of sources. Activation of purified TaOXO by oxidation demonstrates that, as previously found for HvOXO [28], the Mn(III) species is the catalytically active species, rather than the Mn(II) which is the majority in the as-isolated enzyme [7,10]. The specific activity of purified TaOXO increased 7-fold for WT TaOXO following activation. More than 4×10^4 U of TaOXO was produced in a single 5 L fermentation run following 8 days methanol induction, an expression level that should be suitable for most industrial applications.

Mass spectral studies on the purified WT TaOXO from *Pichia pastoris* show that the recombinant protein is more highly glycosylated ($\sim 2\times$) than the protein isolated from the natural source (wheat), which has been reported to contain an (GlcNAc)₃₋₄(Man)₃(Fuc)₁(Xyl)₁ N-linked glycan [12]. Hypermannosylation has been observed for heterologous proteins produced by *Pichia* [33], and is consistent with the TaOXO mass data. This glycan is lost on prolonged incubation, either as a result of spontaneous cleavage, or the activity of a co-purifying glycosidase. Nonglycosylated S49A TaOXO (Fig. 2, Top, lane 3) may be produced by disruption of the NXS motif, which efficiently blocks glycosylation. Since N52A TaOXO is glycosylated, even though the second potential NXS site is absent, glycosylation must be linked through Asn47. Surprisingly, disruption of the glycan attachment site by replacement of that Asn residue (N47A TaOXO) results in a dramatically lower level of protein expression (Fig. 2, Top, lane 4). Nonglycosylated S49A TaOXO is susceptible to aggregation at neutral pH (Table 1), suggesting that the bound carbohydrate may block protein-protein interactions under these conditions. Aggregation of the nonglycosylated protein may contribute to the difficulty of expression in *E. coli* [23]. A variant TaOXO (S49A/N52A) in which both potential NXS motifs are disrupted has been expressed in both *Pichia pastoris* [34] and transgenic tobacco [35]. In the former case, nonglycosylated protein was produced in low yield, and in the latter, the expression level was lower than WT TaOXO, and the product was active although nonglycosylated. Evidence that the N-glycan moiety of OXO contributes to its allergenicity [25] suggests that nonglycosylated

OXO will be required in any therapeutic applications of the enzyme, and the ability to produce fully active, nonglycosylated OXO may be expected to benefit these efforts.

Acknowledgements

Support for this project (from the National Institutes of Health, GM46980 to J. W. W. and from C.N.R.S. to F. B.) is gratefully acknowledged.

References

1. Lane BG. Oxalate, germins, and higher plant pathogens. *IUBMB Life* 2002;53:67–75. [PubMed: 12049198]
2. Lane BG. Oxalate oxidase and differentiating surface structures in wheat: germins. *Biochem J* 2000;349:309–321. [PubMed: 10861243]
3. Bernier F, Berna A. Germins and germin-like proteins: Plant do-all proteins, But what do they do, exactly? *Plant Physiol Biochem* 2001;39:545–554.
4. Lane BG, Dunwell JM, Ray JA, Schmitt MR, Cumming AR. Germin, a protein marker of early plant development, is an oxalate oxidase. *J Biol Chem* 1993;268:12239–12242. [PubMed: 8509360]
5. Sugiura M, Yamamura H, Hirano K, Sasaki M, Morikawa M, Tsuboi M. Purification and properties of oxalate oxidase from barley seedlings. *Chem Pharm Bull* 1979;27:2003–2007.
6. Svedružić D, Jónsson S, Toyota CG, Reinhardt LA, Ricagno S, Lindqvist Y, Richards NG. The enzymes of oxalate metabolism: unexpected structures and mechanisms. *Arch Biochem Biophys* 2005;433:176–192. [PubMed: 15581576]
7. Requena L, Bournemann S. Barley (*Hordeum vulgare*) oxalate oxidase is a manganese-containing enzyme. *Biochem J* 1999;343:185–190. [PubMed: 10493928]
8. Woo EJ, Dunwell JM, Goodenough PW, Pickersgill RW. Barley oxalate oxidase is a hexameric protein related to seed storage proteins: evidence from X-ray crystallography. *FEBS Lett* 1998;437:87–90. [PubMed: 9804177]
9. Woo EJ, Dunwell JM, Goodenough PW, Marvier AC, Pickersgill RW. Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. *Nat Struct Biol* 2000;7:1036–1040. [PubMed: 11062559]
10. Whittaker MM, Whittaker JW. Characterization of recombinant barley oxalate oxidase expressed by *Pichia pastoris*. *J Biol Inorg Chem* 2002;7:136–145. [PubMed: 11862550]
11. Opaleye O, Rose RS, Whittaker MM, Woo EJ, Whittaker JW, Pickersgill RW. Structural and spectroscopic studies shed light on the mechanism of oxalate oxidase. *J Biol Chem* 2006;281:6428–6433. [PubMed: 16291738]
12. Jaikaran ASI, Kennedy TD, Dratewka-Kos E, Lane BG. Covalently bonded and adventitious glycans in germin. *J Biol Chem* 1990;265:12503–12512. [PubMed: 2115520]
13. Koyama H. Purification and characterization of oxalate oxidase from *Pseudomonas* sp. OX-53. *Agric Biol Chem* 1988;52:743–748.
14. Escutia MR, Bowater L, Edwards A, Bottrill AR, Burrell MR, Polanco R, Vicuña R, Bournemann S. Cloning and sequencing of two *Ceriporiopsis subvermispura* bicupin oxalate oxidase allelic isoforms: implications for the reaction specificity of oxalate oxidases and decarboxylases. *Appl Environ Microbiol* 2005;71:3608–3616. [PubMed: 16000768]
15. Guimarães RL, Stotz HU. Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. *Plant Physiol* 2004;136:3703–3711. [PubMed: 15502012]
16. Livingstone DM, Hampton JL, Phipps PM, Grabau EA. Enhancing the resistance to *Sclerotinia minor* in peanut by expressing a barley oxalate oxidase gene. *Plant Physiol* 2005;137:1354–1362. [PubMed: 15778458]
17. Welch AJ, Stipanovic AJ, Maynard CA, Powell WA. The effects of oxalic acid on transgenic *Castanea dentata* callus tissue expressing oxalate oxidase. *Plant Sci* 2007;172:488–496.
18. Chippis TJ, Gilmore B, Myers JR, Stotz HU. Relationship between oxalate, oxalate oxidase activity, oxalate sensitivity, and white mold susceptibility in *Phaseolus coccineus*. *Phytopathology* 2005;95:292–299.

19. Altpeter F, Varshney A, Abderhalden O, Douchkov D, Sautter C, Kumlehn J, Dudler R, Schweizer P. Stable expression of a defense-related gene in wheat epidermis under transcriptional control of a novel promoter confers pathogen resistance. *Plant Mol Biol* 2005;57:271–283. [PubMed: 15821882]
20. Liang H, Maynard CA, Allen RD, Powell WA. Increased *Septoria musiva* resistance in transgenic hybrid poplar leaves expressing a wheat oxalate oxidase gene. *Plant Mol Biol* 2001;45:619–629. [PubMed: 11430425]
21. Hu X, Bidney DL, Yalpani N, Duvick JP, Crasta O, Folkerts O, Lu G. Overexpression of a Gene Encoding Hydrogen Peroxide-Generating Oxalate Oxidase Evokes Defense Responses in Sunflower. *Plant Physiol* 2003;133:170–181. [PubMed: 12970484]
22. Li MG, Madappolly MM. Rapid enzymatic determination of urinary oxalate. *Clin Chem* 1989;35:2330–2333. [PubMed: 2591053]
23. Cassland P, Larsson S, Nilvebrant NO, Jönsson LJ. Heterologous expression of barley and wheat oxalate oxidase in an *E. coli* *trxB gor* double mutant. *J Biotechnol* 2004;109:53–62. [PubMed: 15063614]
24. Berna A, Bernier F. Regulated expression of a wheat germin gene in tobacco: oxalate oxidase activity and apoplastic localization of the heterologous protein. *Plant Mol Biol* 1997;33:417–429. [PubMed: 9049263]
25. Jensen-Jarolim E, Schmid B, Bernier F, Berna A, Kinaciyan T, Focke M, Ebner C, Scheiner O, Boltz-Nitulescu G. Allergologic exploration of germins and germin-like proteins, a new class of plant allergens. *Allergy* 2002;57:805–810. [PubMed: 12169176]
26. Higgins DR, Cregg JM. Introduction to *Pichia pastoris*. *Methods Mol Biol* 1998;103:1–15. [PubMed: 9680629]
27. Stratton J, Chiruvolu V, Meagher M. High cell-density fermentation. *Methods Mol Biol* 1998;103:107–120. [PubMed: 9680637]
28. Whittaker MM, Pan HY, Yukl ET, Whittaker JW. Burst kinetics and redox transformations of the active site manganese ion in oxalate oxidase: Implications for the catalytic mechanism. *J Biol Chem* 2007;282:0000–0000.
29. Whittaker MM, Ballou DP, Whittaker JW. Kinetic isotope effects as probes of the mechanism of galactose oxidase. *Biochemistry* 1998;37:8426–8436. [PubMed: 9622494]
30. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–275. [PubMed: 14907713]
31. Nelson, TJ. Tnimage Scientific Imaging Measurement and Analysis Lab Manual. The Johns Hopkins University; Baltimore: 2000.
32. Zacharius RM, Zell TE, Morrison JH, Woodstock JJ. Glycoprotein staining following electrophoresis on acrylamide gels. *Anal Biochem* 1969;30:148–152. [PubMed: 4183001]
33. Bretthauer RK, Castellino FJ. Glycosylation of *Pichia pastoris*-derived proteins. *Biotechnol Appl Biochem* 1999;30:193–200. [PubMed: 10574687]
34. Pan, HY. MS Thesis. Oregon Health and Science University; Portland: 2005. Characterization of Recombinant Wheat Germin Expressed by *Pichia pastoris*.
35. Bouveret, R.; Berna, A.; Bernier, F. unpublished results

Figure 1.

Expression of recombinant TaOXO by *Pichia pastoris*. Secretory expression of TaOXO was estimated by SDS-PAGE analysis of 10 μ L of culture supernatant following methanol induction as described in the *Materials and Methods*. (Top) SDS-PAGE. (Bottom) Protein estimation by densitometric analysis of the digitized gel image, as described in the *Materials and Methods*.

Figure 2.

SDS-PAGE analysis of TaOXO. (Top) Expression of TaOXO variants. (lane 1) Bio-Rad MW standards; (lane 2) 0.5 μ g of purified WT TaOXO; (lanes 3–5) 10 μ L of 7 day culture supernatant for cultures expressing: (lane 3) S49A TaOXO; (lane 4) N47A TaOXO; (lane 5) N52A TaOXO. (Bottom) Glycoprotein staining for TaOXO variants. SDS-PAGE gels were stained for protein (right) or glycoprotein (left) as described in the *Materials and Methods*. (lanes 1, 1') 3 μ g of WT TaOXO; (lanes 2, 2') 2 μ g of S49A TaOXO; (lanes 3, 3') 2 μ g glyoxal oxidase; (lanes 4, 4') 2 μ g manganese catalase.

Figure 3. MALDI-TOF Mass Spectrometric analysis of recombinant TaOXO. Samples were prepared for mass spectral investigation as described in the *Materials and Methods*. (A) WT TaOXO; (B) S49A TaOXO.

Table 1

pH dependent solubility of OXO variants

pH	Residual protein in solution ^a	
	WT OXO (μg/mL)	S49A OXO (μg/mL)
5.0	3150±140	3160±50
5.5	3280±40	3190±90
6.0	3150±70	1800±50
6.5	3380±40	1050±70
7.0	2900±70	690±30

^a Protein remaining in the supernatant following dialysis against buffer solutions and centrifugation as described in the *Materials and Methods*.