Characterization of Recombinant Wheat Germin Expressed by *Pichia pastoris*

Heng-Yen Pan

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The thesis "Characterization of Recombinant Wheat Germin Expressed by *Pichia pastoris*" by Heng-Yen Pan has been examined and approved by the following Examination Committee:

_____

James W. Whittaker, Advisor
Associate Professor

_____

Gebretateos Woldegiorgis
Associate Professor

_____

Peter Zuber
Professor

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ABBREVIATIONS

ABTS: 2,2'-azinobis(3-ethybenzthiazoline-6-sulfonic acid)

AOX: Alcohol Oxidase

BMGY: Buffered Glycerol-complex medium

BMMY: Buffered Methanol-complex medium

ds-DNA: Double Stranded DNA

ddH$_2$O: Double Deionized water

DTT: Dithiothreitol

ECM: Extracellular Matrix

E. coli: *Escherichia coli*

ER: Endoplasmic Reticulum

GlcNAc: N-Acetylglucosamine

GLPs: Germin-Like Proteins

GPI : Glycophosphatidylinositol

HEPES: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid

HRP: Horse Radish Peroxidase

LBZ medium: Luria-Bertani medium with Zeocin

MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight
   Mass Spectrometry

NaAc: Sodium Acetate

NYZ$^+$ medium: NZ amine/Yeast extract

OD: Optical Density

OXO: Oxalate Oxidase

PCR: Polymerase Chain Reaction

pI: Isoelectric Point

P-I-D: Proportional Integral Derivative

SDS-PAGE: Sodium Dodecyl Sulfate-PolyacrylAmine Gel Electrophoresis

SOD: Superoxide Dismutase

ss-DNA: Single Stranded DNA

YPD medium: Yeast extract/Peptone/Dextrose medium

YPDS/Z: Yeast extract/Peptone/Dextrose medium contain sorbitol and Zeocin

Y-PER: Yeast Protein Extraction Reagent

ABSTRACT

Characterization of Recombinant Wheat Germin Expressed by *Pichia pastoris*

Heng-Yen Pan, B.S.

M.S., OGI School of Science & Engineering

at Oregon Health & Science University

August 2005

Thesis Advisor: Dr. James W. Whittaker

Germin, a protein found in germinating wheat, has been identified as an oxalate oxidase (oxalate: oxygen oxidoreductase, EC 1.2.3.4) catalyzing the conversion of oxalate to carbon dioxide and hydrogen peroxide. Wheat germin is a homohexameric glycoprotein (~150 kDa) composed of 26 kDa subunits, each containing two potential N-glycan glycosylation sites.

Wheat germin was mutated within its two potential NXS glycosylation sites (N47A, S49A, and N52A), and the recombinant enzyme was produced in a *Pichia pastoris* yeast expression system. One of these mutational variants (S49A) was prepared by high-density fermentation yielding 160 mg of purified oxalate oxidase. Wheat germin was also mutated within its putative oxalate binding site (Lys 44), and the recombinant enzyme was produced by the same expression system in shake flask culture.

The recombinant protein expression level and the carbohydrate content were analyzed by SDS-PAGE. Retention of carbohydrates in the N52A mutant

indicated Asn 52 is not an N-glycan glycosylation site, even though it is in an N-glycan NXS target sequence motif. The K44A mutation did not alter the oxalate specific activity of the enzyme, indicating that Lys 44 is not likely to function as an oxalate binding site. Replacement of either N47 or S49 by alanine resulted in secretion of non-glycosylated protein.

The recombinant proteins (S49A and K44A) contained approximately 0.24 g-atom Mn/monomer and exhibited a specific activity of close to 5 U/mg, similar to the properties reported for the recombinant wild-type wheat germin. The mutation S49A caused the loss of glycosylation; however, losing glycans (carbohydrates) did not alter the efficiency of manganese binding by oxalate oxidase or catalytic activity.

CHAPTER 1

INTRODUCTION

1.1   Cupin Superfamily

Germins and germin-like proteins are a large family of polypeptides present in all plants.   The term "germin" was adopted based on the discovery of wheat germin in a study of germinating wheat grains in 1980 [1].   Germin was initially thought to be a relatively rare protein that was only present in germinating wheat.   However, with the advent of genome sequencing, germin and related germin-like proteins have been found to be ubiquitous in the plant kingdom.

Germins are members of an extended family of the protein called the cupin superfamily, which includes proteins from both prokaryotes and eukaryotes.   The term "cupin" (from the Latin *cupa*, for a small barrel or cask) was based on the well-defined β-barrel structural domain present in all members of the family.   The protein is characterized by two histidine-containing sequence motifs [$G(X)_5HXH(X)_{11}G$ and $G(X)_5P(X)_4H(X)_3N$, where X can be any amino acid] [Figure 1.1].   The overall organization of the protein can be composed of either a single domain (cupin), or a duplicated, two-domain structure (bicupin).   Plant germins and germin-like proteins belong to the single domain structure; whereas some related bacterial cupins (e.g., *B. subtilis* oxalate decarboxylase) belong to the

```
WheatGermin    MGYSKTLVAGLFAMLLLAPAVLATDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFL 60
BarleyOXO      MGYSKNLGAGLFTMLLLAPAIMASDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFL 60
RyeOXO         MAYFKTLAAGLFALLFLAPFIMATDPDPLQDFCVADLDGKEVSVNGHPCKPMSEAGDDFL 60
Arab.GLP       ----------MLRTIFLLSLLFALSNASVQDFCVANLKRAETPAGYPCIRPIHVKATDFV 50
TobaccoGLP     ---------MFFQAFFIFSLLFLSSDAAVLDFCVGDLSVPDGPGGYACKKPSAVTANDFV 51
MaizeGLP       -----MAKMVLLCVLVSFLLMPLASLALTQDFCVADLTCSDTPAGYPCK--SSVTANDFY 53
               *      ****.:        . .            . **


WheatGermin    FSSKLAKAGNTSTPNGSAVTELDVAEWPGTNTLGVSMNRVDFAPGGTNPPHIHPRATEIG 120
BarleyOXO      FSSKLTKAGNTSTPNGSAVTELDVAEWPGTNTLGVSMNRVDFAPGGTNPPHIHPRATEIG 120
RyeOXO         FSSKLAKAGNTSTPNGSAVTELDVAEWPGTNTLGMSMNRVDFAPGGTNPPHIHPRATEIG 120
Arab.GLP       FSG-LGTPGNTTNIINAAVTPAFAAQFPGLNGLGLSTARLDLAPKGVIPMHTHPGASEVL 109
TobaccoGLP     FSG-LATPVKLNPLIKAAVTPAFAPQFPGLNGLGISMARLDLAIGGVIPMHTHPGASEVL 110
MaizeGLP       FHG-LAGQGKINPLIKAAVTPAFVGQFPGVNGLGISAARLDIEVGGVVPLHTHPAGSELL 112
               * . *    :  .       :***   . ::** * **:*   *:*:   *. * *  ** .:*:


WheatGermin    IVMKGELLVGILGSLDSGNKLYSRVVRAGETFLIPRGLMHFQFNVGKTEASMVVSFNSQN 180
BarleyOXO      MVMKGELLVGILGSLDSGNKLYSRVVRAGETFVIPRGLMHFQFNVGKTEAYMVVSFNSQN 180
RyeOXO         IVMKGELLVGIIGSLDSGNKLYSKVLRAGETFLIPRGLMHFQFNVGKTEASMVVSFNSQN 180
Arab.GLP       FVLTGSITAGFVSS---ANAVYVQTLKPGQVMVFPQGLLHFQINAGKSSASAVVTFNSAN 166
TobaccoGLP     YVVTGEICAGFISSS--DNKVFFKNLKQGDIMVFPQGLLHFQINSGKTTGLAIVSFSSPT 168
MaizeGLP       FVTQGTVAAGFISSG--SNTVYTKTLYAGDIMVFPQGLLHYQYNAGTGAAVGLVAFSSPN 170
               *    * : .*::.*    *  :: : :  *: :::*:**:*:* * *.  .   :*:*.* .


WheatGermin    PGIVFVPLTLFGSNPPIPTPVLTKALRVEARVVELLKSKFAAGF 224
BarleyOXO      PGIVFVPLTLFGSDPPIPTPVLTKALRVEAGVVELLKSKFAGGS 224
RyeOXO         PGIVFVPLTVFGSNPPIPTAVLTKALRVDAGVVELLKSKFAGGF 224
Arab.GLP       PGLQILDFALFANS--LPTELVVGTTFLDATTVKKLKGVLGGTG 208
TobaccoGLP     PGLQITDFALFAND--LATELVQATTFLDAATIKKLKGVLGGTN 210
MaizeGLP       PGLQITDFALFANN--LPSAVVEKVTFLDDAQVKKLKSVLGGSG 212
               **: :  :::*...  :.: ::  .   ::   :: **. :..
```

Figure 1.1 Sequence alignment for germins and germin-like proteins.   Germin oxalate oxidase (wheat[*], barley[†], and ryegrass[‡]), and germin-like proteins (Arabidopsis[§], tobacco[**], and maize[††]) are compared.   Lys 44 is shown in **red**; two possible N-glycan glycosylation sites are underlined; two histidine-containing sequence motifs characteristic of the cupin domain are shown in **blue**. The sequence alignment was calculated using ClustalW [2].

---

[*] Wheat germin. GI: 170693

[†] Barley oxalate oxidase. GI: 2266668

[‡] Ryegrass oxalate oxidase. GI:11967813

[§] Arabidopsis thaliana germin-like protein. GI: 1934728

[**] Tobacco nectarin I germin-like protein. GI: 31711506

[††] Zea mays germin-like protein. GI: 37623878

the two-domain structure [3].   The two histidine-containing sequence motifs have been recently identified as manganese metal ion binding sites for germins and germin-like proteins based on a germin/oxalate oxidase 3D model [4] and the X-ray crystal structure of barley oxalate oxidase [5].

Germins are a homogeneous group [Figure 1.1] composed of proteins with more than 90% identity.   It is a protein marker of the onset of growth in germinating wheat [4].   Germins are present in all monocotyledon plants, such as barley, maize, oats, rice, rye, and wheat, but they are not present in most dicotyledon plants.   In contrast to germins, germin-like proteins are heterogeneous [Figure 1.1] based on their amino acid identities ranging from 25% to 100% [6], and they are present in both monocotyledon and dicotyledon plants. Germin-like proteins are expressed in all parts of the plants and at all developmental stages, from "root to shoot," and from germination to fruit development [4, 6].

1.1.1   Cupin structure

In the cupin superfamily, barley and wheat germin have been extensively studied.   Their amino acid sequences are 95% identical [7] and the cDNA for wheat germin has been isolated [Figure 1.2].   Germin is a water-soluble protein [8] with extreme resistance to heat and to chemical degradation by proteases or hydrogen peroxide [5].   The resistance to extremes of environment may be a consequence of the structure.

The X-ray crystal structure of germin from barley [Figure 1.3] was recently determined at 1.6 Å resolution [5], revealing that germin is a hexamer instead of

```
ACC GAC CCA GAC CCT CTC CAG GAC TTC TGT GTC GCC GAC CTC
 T   D   P   D   P   L   Q   D   F   C   V   A   D   L

GAC GGC AAG GCG GTC TCG GTG AAC GGG CAC ACG TGC AAG CCC
 D   G   K   A   V   S   V   N   G   H   T   C   K   P

ATG TCG GAG GCC GGC GAC GAC TTC CTC TTC TCG TCC AAG TTG
 M   S   E   A   G   D   D   F   L   F   S   S   K   L

GCC AAG GCC GGC AAC ACG TCC ACC CCG AAC GGC TCC GCC GTG
 A   K   A   G   N   X   S   T   P   N   X   S   A   V

ACG GAG CTC GAC GTG GCC GAG TGG CCC GGT ACC AAC ACG CTG
 T   E   L   D   V   A   E   W   P   G   T   N   T   L

GGT GTG TCC ATG AAC CGC GTG GAC TTT GCT CCC GGA GGC ACC
 G   V   S   M   N   R   V   D   F   A   P   G   G   T

AAC CCA CCA CAC ATC CAC CCG CGT GCC ACC GAG ATC GGC ATC
 N   P   P   H   I   H   P   R   A   T   E   I   G   I

GTG ATG AAA GGT GAG CTT CTC GTG GGA ATC CTT GGC AGC CTC
 V   M   K   G   E   L   L   V   G   I   L   G   S   L

GAC TCC GGG AAC AAG CTC TAC TCG AGG GTG GTG CGC GCC GGA
 D   S   G   N   K   L   Y   S   R   V   V   R   A   G

GAG ACG TTC CTC ATC CCA CGG GGC CTC ATG CAC TTC CAG TTC
 E   T   F   L   I   P   R   G   L   M   H   F   Q   F

AAC GTC GGT AAG ACC GAG GCC TCC ATG GTC GTC TCC TTC AAC
 N   V   G   K   T   E   A   S   M   V   V   S   F   N

AGC CAG AAC CCC GGC ATT GTC TTC GTG CCC CTC ACG CTC TTC
 S   Q   N   P   G   I   V   F   V   P   L   T   L   F

GGC TCC AAC CCG CCC ATC CCA ACG CCG GTG CTC ACC AAG GCA
 G   S   N   P   P   I   P   T   P   V   L   T   K   A

CTC CGG GTG GAG GCC AGG GTC GTG GAA CTT CTC AAG TCC AAG
 L   R   V   E   A   R   V   V   E   L   L   K   S   K

TTT GCC GCT GGG TTT
 F   A   A   G   F
```

Figure 1.2 Wheat germin nucleotides and mature protein coding sequence. Two potential N-glycan glycosylation sites are underlined.   The putative oxalate binding site (Lys 44) is marked in **red**.   Two histidine-containing sequence motifs are marked in **blue**.
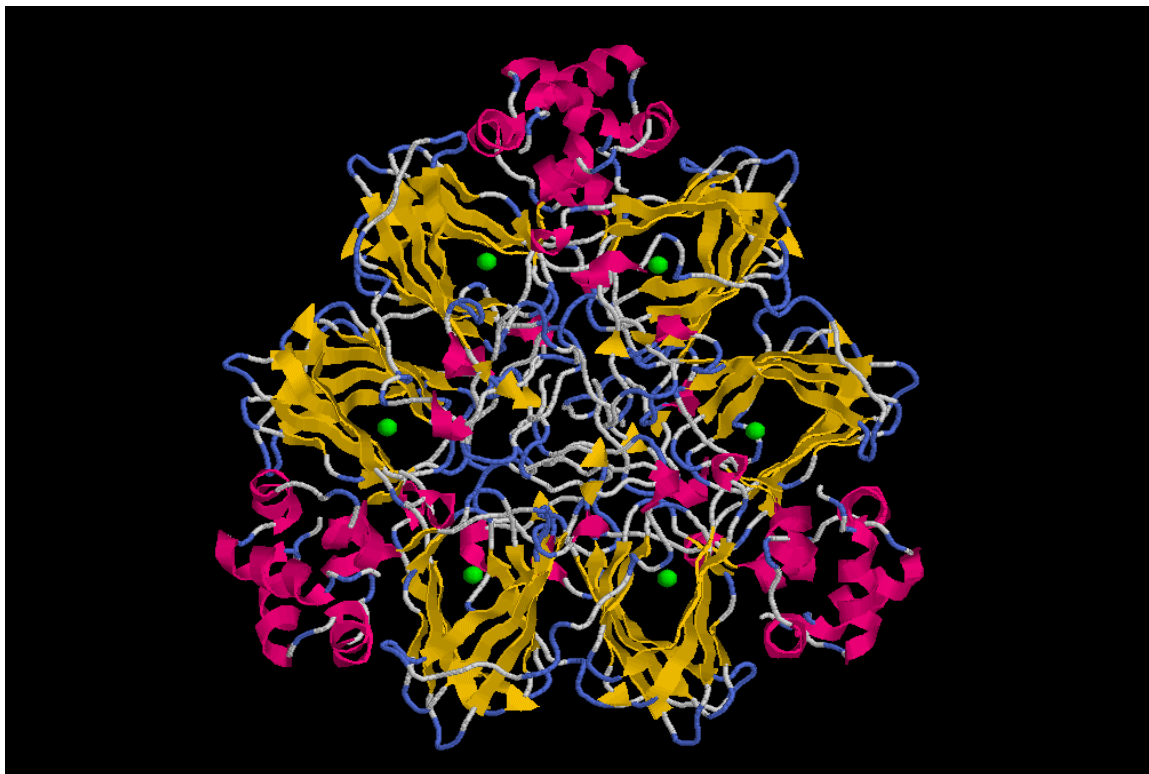
Figure 1.3 The X-ray crystal structure of barley oxalate oxidase. Manganese ions are in **green**. Based on PDB ID 1FI2 and rendered using Rasmol [9].

a pentamer as previously thought. The structure was found to be composed of β-jellyroll monomers locked together into a homohexamer (a trimer of dimers) [5]. The N-terminal of each monomer has an irregular extension, and the C-terminal is composed of three α-helices. The trimer of dimers is locked together by the clasping of the C-terminal α-helical domain [5], and these interlocking surfaces account for the high stability of germin. Each monomer consists of 201 amino acids with a single tightly bound manganese ion. The manganese ion is coordinated by three histidines (His 88, 90, 137) and one glutamate (Glu 95), with two water molecules occupying adjacent positions to form a six-coordinate active metal complex [5] [Figure 1.4]. This metal ion binding site is believed to be the oxalate oxidase catalytic active site [4].

1.2   Enzyme Activity

Germin was identified as an oxalate oxidase (oxalate: oxygen oxidoreductase, EC 1.2.3.4) in 1993 using a SDS-PAGE blot assay [7] to compare with a commercial preparation of barley oxalate oxidase. Oxalate oxidase (OXO) converts oxalic acid to carbon dioxide and hydrogen peroxide, and was first reported in 1912 from a study of wheat flour [10].

$$(COOH)_2 + O_2 \rightarrow 2CO_2 + H_2O_2$$
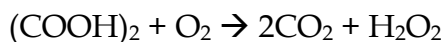
This enzyme is a dehydrogenase that is able to use oxygen as a hydrogen acceptor. The function of germin-like proteins is unknown and most do not have any oxalate oxidase activity [11, 12]. However, two germin-like proteins have been identified as manganese-containing superoxide dismutases (SODs).
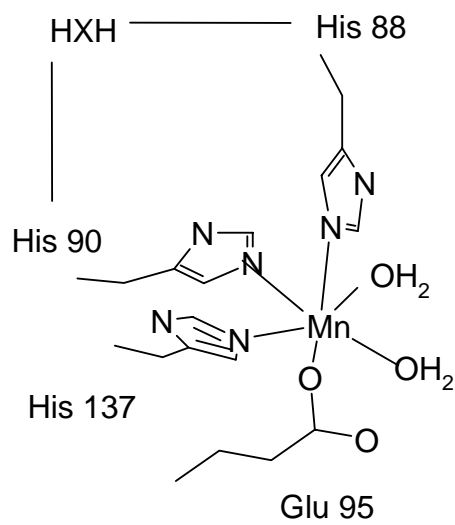
Figure 1.4 The active site of germin based on the X-ray crystal structure [4].

One was isolated from the moss *Barbula unguiculate* [13] and the other is the major tobacco nectar protein, nectarin I [12]. Both SODs were shown to be extracellular oligomeric glycoproteins [3].

1.3   Germin Is a Glycoprotein

In 1987, germin was discovered to be a glycoprotein [14]. A glycoprotein consists of sugar residues attached to the protein backbone through post-translational modification called glycosylation. Glycosylation is one of the most frequent post-translational modifications, and virtually all eukaryotic proteins that pass through the secretory apparatus are glycosylated [14]. In this process, oligosaccharides are covalently attached to the proteins forming glycoproteins, and some proteins do not fold properly without glycosylation. There are three types of glycoprotein carbohydrate chains: (1) *N*-linked oligosaccharides (N-glycans), (2) O-linked oligosaccharides (O-glycans), and (3) glycophosphatidylinositol (GPI)-membrane anchors [Figure 1.5].

All of the N-linked glycosylation starts in the endoplasmic reticulum (ER) lumen with sugar residues usually linking to the amide nitrogen of asparagine in the protein with the target sequence of NXS (asparagine-X-serine), where X can be any amino acid except proline or aspartic acid. Then the synthesis process carries on to the Golgi apparatus and finally the proteins are directed to their destinations. All examples of germin and germin-like proteins are glycosylated; however, the potential glycosylation site(s) vary in position with the protein.

(1)

(2)

(3)
Protein

Glycine

P

P

Asn

Ser/Thr

Plasma membrane

GlcNAC   Mannose   Fucose   Inositol

Galactose   Sialic acid   Ethanolamine   P: phosphate
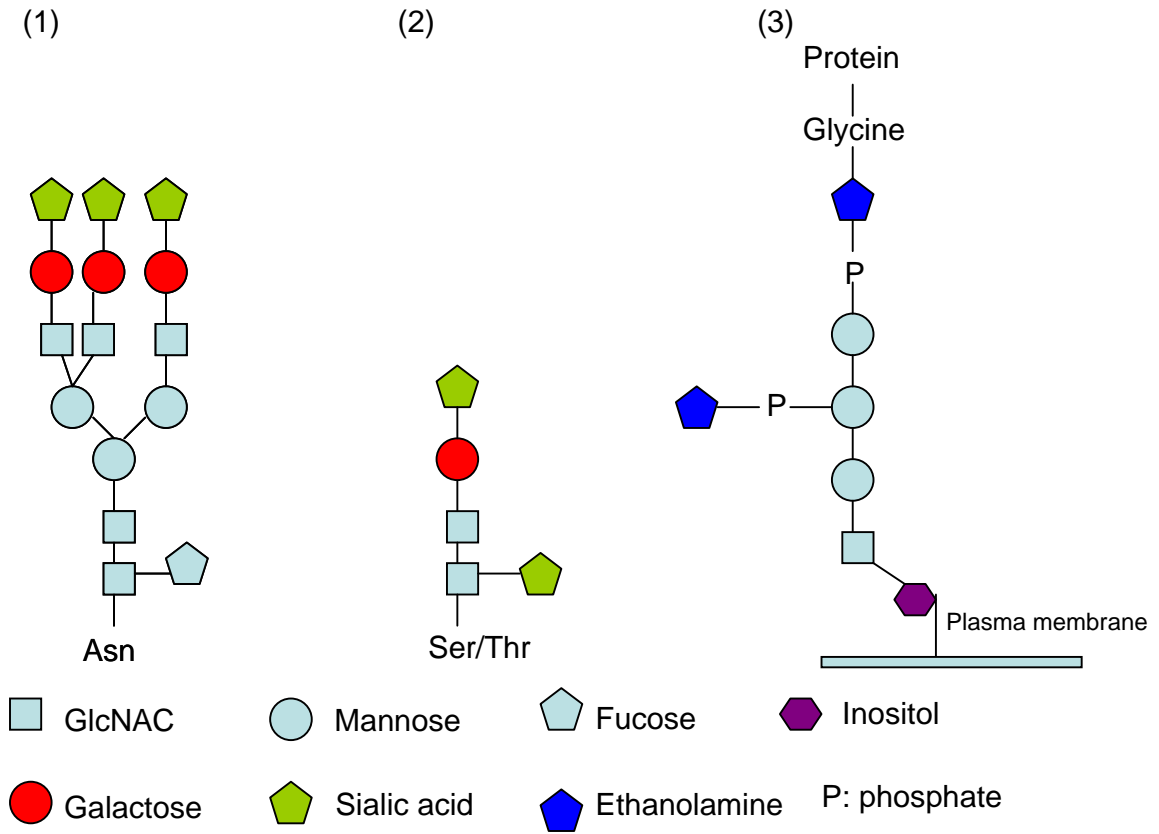
Figure 1.5   Schematic illustration of the proposed structures for the glycoprotein carbohydrate chains.   (1) *N*-linked oligosaccharides (N-glycans), (2) O-linked oligosaccharides (O-glycans), (3) glycophosphatidylinositol (GPI)-membrane anchors.

Germin contains two potential N-glycan glycosylation sites (Asn 47 and Asn 52) [15] [Figure 1.2]. The evidence for N-glycan glycosylation is that the increased mobility during SDS-PAGE of the release of carbohydrates when N-glycans were removed by specific enzyme treatment [16] indicates that germin does not contain O-glycans [17]. Preliminary data from the X-ray crystal structure of barley oxalate oxidase indicates that N47 is the site of N-glycan attachment, but the carbohydrate is very poorly defined and disordered [5].

Sequence analysis predicts that the native wheat germin and its closely related barley oxalate oxidase should have molecular weights of 21.2 kDa for the non-glycosylated proteins, while apparent molecular weights of about 26 kDa have been estimated for the glycosylated forms based on SDS-PAGE [10].

1.4   Functions of Germin

Plants defend themselves against pathogen attack by utilizing a variety of mechanisms that include the production of specific antimicrobial compounds and germins are involved in these plant defense responses. When wheat leaves were infected with a powdery mildew (*Erysiphe graminis*), germin mRNA levels were strongly induced and oxalate oxidase activity also increased [18]. The accumulation of oxalate oxidase also generated hydrogen peroxide in response to *E. graminis* infection. Hydrogen peroxide has been reported to mediate developmental signaling, cross-linking, and defense reactions in plants [8]. Plant recognition of pathogens results in a hypersensitive response involving the rapid tissue death at the site of infection that restricts pathogen growth and

spread.   Hydrogen peroxide that accumulates by this mechanism could act as a secondary messenger to activate the defense response, and it also mediates lignification creating a barrier to pathogen invasion [18].   Hydrogen peroxide also acts as a powerful oxidizing agent, defending against some organisms (e.g., *Sclerotinia sclerotiorum*) which secrete high concentration of oxalic acid as a toxin.

Oxalate oxidase activity and germin transcription were shown to be induced in barley and wheat following fungal infection.   Since oxalate oxidase is not naturally found in most dicotyledon plants, its gene can be transferred to create transgenic plants (e.g., soybeans, potatoes, and sunflowers) that are more resistant to fungal invasions [1].

1.4.1   Significance of oxalic acid and oxalate oxidase to humans

Oxalic acid and oxalate are well known to be toxic to humans in high concentrations and can cause medical problems even at low concentrations. Spinach is a dietary source containing particularly high concentrations of oxalic acid.   Fortunately, there are many improved breeds of spinach available with a lower oxalate content.   Oxalate also can be an environmental contaminant from the smelting industries.   Burning, burial in landfill sites, and bioremediation can be used as disposal methods [19].

Oxalate oxidase also has been used in biomedical applications, including determining the level of oxalate in biological fluids.   High concentration levels, which can be assayed by kits containing oxalate oxidase, lead to deposition of calcium oxalate throughout the body (hyperoxaluria) or the incidence of kidney or bladder stone formation [19].

An increasing number of plant glycoproteins have been linked to allergies over the years, including germin and germin-like proteins that are also being recognized as potential food allergens [20].   The presence of complex types of N-glycans [21] is thought to be important, especially in inducing the allergic response [22].   Germin and germin-like proteins have been shown to be responsible for eliciting allergies based on their N-glycan carbohydrate moieties [22].

1.5   Recombinant Oxalate Oxidase Expression

With the advent of recombinant protein techniques, prokaryotic expression in *Escherichia coli* has generally been the first choice for several reasons.   *E. coli* is one of the most studied organisms in the world, and abundant information is available about its genetics, molecular biology, biochemistry, physiology, and general biology.   It is a single-celled organism and does not require elaborate facilities for growth and maintenance.   *E. coli* only requires a simple culture medium and its rapid growth have made it a favorite research organism [23].   However, germins that have been synthesized in *E. coli* are either unstable or lack biological activity, due to improper folding or loss of post-translational modifications (e.g., glycosylation).   To avoid these problems, transgenic plants (e.g., tobacco plants) have been used as a protein expression system.   In order for a plant to be used to produce specific molecules, a gene is inserted into its chromosome.   A regulatory sequence inserted with that gene dictates to the plant where to produce the desired protein in its leaves, roots, or

seeds.   However, transgenic plants generally do not produce very much germin [24].   So, a yeast expression system was used, and *Pichia pastoris* is a good candidate for the system.

P. pastoris has become a highly successful system for the expression of heterologous genes.   Several factors have contributed to its rapid acceptance: (1) *P. pastoris* has a strong, methanol-inducible alcohol oxidase (AOX1) promoter controlling the expression of alcohol oxidase [25]; it has a strong preference for respiratory growth that greatly facilitates its culturing at high cell densities; and (3) it does not secrete large amounts of endogenous proteins, which simplifies the purification of secreted recombinant proteins.

P. pastoris is a single-celled microorganism that is easy to manipulate and culture; it is also generally regarded as being faster, easier, and less expensive to use, and usually gives higher expression levels [26].   *P. pastoris* is a eukaryote that is capable of many of the post-translational modifications performed by higher eukaryotic cells, such as proteolytic processing, folding, disulfide bond formation, and glycosylation.

P. pastoris is methyltropic yeast; it can utilize methanol as a carbon source in the absence of a repressing carbon source (e.g., glucose).   The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde and hydrogen peroxide by the enzyme alcohol oxidase (AOX).   This step happens in the peroxisome, which degrades hydrogen peroxide to oxygen and water to avoid the toxicity of hydrogen peroxide [27].   Alcohol oxidase has a poor affinity for oxygen, and methylotrophic *P. pastoris* compensates for this deficiency by synthesizing large amounts of the enzyme [26].

pPICZ [Figure 1.6] is a commercially available expression vector and has been used extensively for *P. pastoris* expression.   It is designed for simple cloning, selection, and high-level secretory expression of recombinant protein. The basic features of this vector include: the AOX1 promoter, one or more unique restriction sites for insertion of the foreign gene, the transcription termination sequences from the *P. pastoris AOX1* gene, an origin of replication, and the Zeocin resistance gene [27].   This Zeocin marker allows isolation of multicopy integrants.   Increasing the number of copies of the interested gene can result in higher expression levels.

The *P. pastoris* expression system has been used to produce more than 200 different biologically active proteins from different organisms [28].   In the laboratory of Dr. James W. Whittaker, recombinant barley oxalate oxidase [29] and wheat germin proteins have been successfully expressed by the *P. pastoris* expression system.

1.6   Aim of This Study

The characteristics of recombinant wheat germin were investigated in this study.   The sequence alignment for detailed characterization shown in Figure 1.1 identifies two potential N-glycan glycosylation sites (Asn 47 and Asn 52) associated with NXS target sequence motifs.   In addition, Lys 44 has been considered a putative oxalate binding site, because it is conserved throughout the germin family (e.g., wheat germin, barley oxalate oxidase, and rye oxalate oxidase) [Figure 1.1].   Since Lys in a positively charged amino acid residue, it
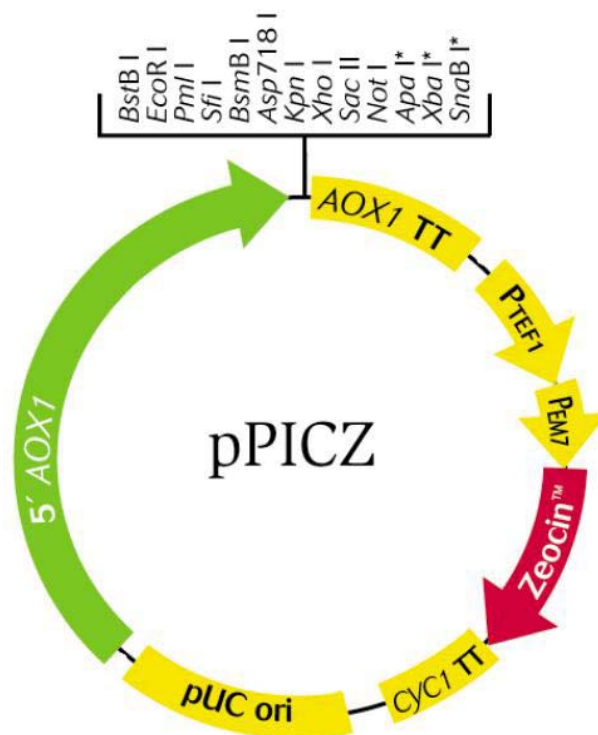
Figure 1.6 Physical map of pPICZ vector for the *P. pastoris* expression system with unique restriction sites. 5′ *AOX1*, *P. pastoris AOX1* promoter sequence; *AOX1* TT, alcohol oxidase transcription termination region; P_{TEF1}, promoter region of transcription elongation factor 1; P_{EM7}, promoter conferring Zeocin resistance; Zeocin, a selectable marker; Cyc1 TT, cytochrome c1 transcription termination region; Ori, origin of replication.

forms a complex with the negatively charged oxalate by an ionic linkage. The wheat germin gene was mutated in these two N-glycan glycosylation sites and the putative binding site, and was used to produce a functional recombinant enzyme in the *P. pastoris* yeast expression system. This study aimed to investigate the role of Asn 47 and Asn 52 in N-glycan glycosylation and Lys 44 in an oxalate binding site through site-directed mutagenesis, expression, and biochemical analysis of recombinant protein.

CHAPTER 2

MATERIALS AND METHODS

2.1   Biological Materials

P. pastoris X33 [26] was obtained from Invitrogen (San Diego, CA).

Competent *E. coli* XL2 cells were obtained from Stratagene (La Jolla, CA).

2.2   Expression Cassette Design

The expression cassette design was followed by basic protocols, which are

described in detail in the following sections.

2.2.1   Vector construction

Plasmid pEMBL18 with a 2.8 kb insert containing the wheat germin

coding sequence was kindly provided by Dr. François Bernier (Institut de

Botanique, Strasbourg, France).

A vector for the experiment of heterologous recombinant wheat germin

was constructed by Dr. Whittaker from a commercially available

pPICZBα expression vector (Invitrogen) [Figure 2.1].   After transformation into

E. coli [Appendix], the DNA was purified by Mini prep [Appendix], analyzed by
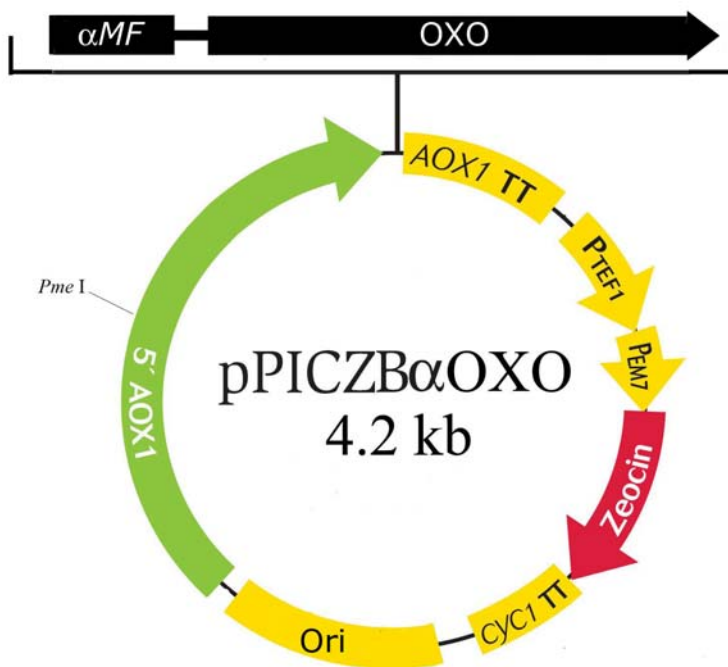
Figure 2.1 Physical map of the pPICZBαOXO expression vector for production of recombinant wheat OXO by the methylotrophic yeast *P. pastoris*. 5′ *AOX1*, *P. pastoris AOX1* promoter sequence; *AOX1* TT, alcohol oxidase transcription termination region; P_{TEF1}, promoter region of transcription elongation factor 1; P_{EM7}, promoter conferring Zeocin resistance; Zeocin, a selectable marker; Cyc1 TT, cytochrome c1 transcription termination region; Ori, origin of replication; αMF, *Saccharomyces cerevisiae* α mating factor prepro sequence; OXO, wheat germin mature protein coding sequence.

endonuclease digestion, and purified on a large scale by Midi prep [Appendix].

The yield of pPICZBαOXO was about 342 μg.

2.2.2 Site-directed mutagenesis

Based on the wheat germin coding sequence [Figure 1.2], six mutagenic

primers were designed and obtained from Invitrogen targeting predicted

N-linked glycosylation sites with NXS sequence motifs (N47, S49, N52), and a

putative catalytic residue (K44).   In the part of the wild-type wheat germin

DNA sequence below, the two possible N-linked glycosylation sites are

underlined:

```
       TCC  AAG  TTG  GCC  AAG  GCC  GGC  AAC  ACG  TCC  ACC  CCG
   40  Ser  Lys  Leu  Ala  Lys  Ala  Gly  Asn  Thr  Ser  Thr  Pro
                                          N    X    S


       AAC  GGC  TCC  GCC  GTG  ACG  GAG  CTC  GAC  GTG  GCC
   52  Asn  Gly  Ser  Ala  Val  Thr  Glu  Leu  Asp  Val  Ala  62
        N    X    S
```

The primers were designed to be between 25 and 45 bases in length with a

melting temperature (T_m) of ≥ 75°C and were 5′ phosphorylated for maximum

ligation efficiency.

Five mutagenic primers were designed to introduce six mutations in the

protein sequences (see below as labeled):

N47A: 5'-P- G GCC AAG GCC GGC **GC**C‡‡ ACG TCC   ACC   CCG -3'
             Ala Lys Ala Gly **Ala** Thr Ser   Thr   Pro

N52A: 5'-P- CG TCC ACC CCG **GC**C GGC TCC **GCA**§§,*** GTG AC -3'
             Ser Thr Pro **Ala** Gly Ser **Ala**   Val

S49A: 5'-P- C GGC AAC ACG **G**CC ACC CCG AAC -3'
            Gly Asn Thr **Ala** Thr Pro Asn

K44A: 5'-P- GC TCC AAG TTG GCA **GCA** GCC GGC AAC ACG C -3'
             Ser Lys Leu Ala **Ala** Ala Gly Asn Thr

S49A/ 5'-P- C GGC AAC ACG **G**CC ACC CCG **GCA** GGC TCC GC -3'
N52A: Gly Asn Thr **Ala** Thr Pro **Ala** Gly Ser

N47A/ 5'-P- G GCC AAG GCC GGC **GC**C ACG TCC ACC CCG **GC**C GGC TCC **GCA** GTG AC-3'
N52A: Ala Lys Ala Gly **Ala** Thr Ser Thr Pro **Ala** Gly Ser **Ala** Val

     The N47A/N52A double mutation was based on the mutations N47A and N52A (above). The plasmid containing the N47A mutation was the template for N52A wheat germin, because the N52A primer contained a silent mutation. The silent mutation at the 3' end of N52A was designed to relieve the very strong secondary structure predicted from the mature sequence.

     Site-directed mutagenesis was performed using the QuikChange Multi procedure from Stratagene. The reaction mixture was placed in a thermal cycler (MJ Research Minicycler Thermal cycler, Reno, NV) for 30 reaction cycles to complete the mutant strand synthesis reaction. Each reaction cycle included

---

‡‡ **Red** color indicates where the mutations are in the codons.
§§ **Blue** color indicates where the silent mutation is in the codon.
*** A silent mutation for GCC. The codon usage in *P. pastoris* for GCC and GCA is similar with frequencies 0.014860 and 0.016856 per thousand so that GCA is suitable for the silent mutation.

DNA denaturation (1 min, 95°C), mutagenic primer annealing (1 min, 55°C), and extension nick ligation (8 min, 65°C), requiring about 5.25 h to complete the 30-cycle reaction.   The QuikChange reaction mixture was treated with 15 U of *Dpn*I to digest the methylated and hemimethylated template DNA to reduce wild-type background in the next step.   The reaction mixture was transformed to *E. coli* cells.   In this step, the mutant closed circular single-stranded DNA was taken up by the *E. coli* cells, which converted it into closed circular double-stranded DNA.   The transformation reaction mixture was plated on low salt LBZ agar plates [Appendix] for 2 days for colony selection.   High salt inhibits Zeocin and reduces the selection.   Six independent colonies were selected and streaked onto a fresh low salt LBZ plate.   Plasmid DNA was isolated from 200 mL of culture by Midi prep.   The sequence of purified DNA was determined by the Oregon Regional Primate Research Center (Beaverton, OR).

2.2.3   Plasmid linearization

The pPICZBαOXO plasmid (20 µg) in a 100 µL reaction was linearized by digestion with 100 U of *Pme*I in a 37°C water bath overnight, which cuts at a unique site within the P_{AOX1} promoter.   The DNA was precipitated with 0.5 volume (50 µL) of 7.5 M ammonium acetate, and 2.0 volume (300 µL) of 100% ethanol.   The DNA was cooled down to –80°C for 30 min and centrifuged briefly.   The DNA pellet was washed with 70% ethanol to remove salts.   The washed, linearized plasmid DNA was dissolved in 10 µL of doubly deionized water (ddH$_2$O) to a final concentration of approximately 2 µg/µL.

2.3   **Transformation of *Pichia pastoris***

A *P. pastoris* X33 culture was prepared by inoculating from a fresh agar culture into 50 mL of sterile YPD medium [Appendix] in a 250 mL baffled flask in a 30°C water bath shaker (New Brunswick Scientific, Edison, NJ) overnight. The starter culture was used to inoculate in 500 mL of fresh sterile YPD medium to $OD_{600nm} \sim 0.2$ and grown at 30°C to $OD_{600nm} \sim 1.0$.   The cells were centrifuged in four sterile 250 mL centrifuge bottles in a Sorvall RC58 refrigerated super speed centrifuge (Du Pont, Newtown, CT) at 4,000 rpm for 5 min.   The supernant was discarded, and 50 mL of sterile YPD medium was used to suspend the cells.   Dithiothreitol (DTT, 0.4 g) was dissolved in 2 mL of ddH_2O and sterilized with a 0.22 µm syringe filter (Millipore Corporation, Bedford, MA). The DTT and 2.0 mL of 1 M HEPES, pH 8.0, solutions were added to the cell suspension and incubated in a 30°C water bath shaker at 200 rpm for 15 min. The cell suspension was then diluted to 500 mL with sterile cold distilled water and centrifuged at 4,000 rpm and 4°C for 5 min.   The supernant was discarded and the cell pellet was suspended in 500 mL of sterile cold distilled water and centrifuged again at 4,000 rpm and 4°C for 5 min.   The supernant from this step was discarded and the cell pellet was suspended in 250 mL of sterile cold distilled water and centrifuged again at 4,000 rpm and 4°C for 5 min.   The cells were then suspended in 20 mL of cold 1 M sorbitol solution and recentrifuged. The supernant was discarded and the cells were carefully resuspended in 0.5 mL of 1 M cold sorbitol solution.   Electrocompetent *P. pastoris* X33 cells (40 µL) were transformed with the linearized plasmid (4 µg) by electroporation (Brinkmann

Instruments, Eppendorf electroporator 2510, Germany) in an ice cold 2 mm gap

sterile electroporation cuvette and an applied voltage of 2500 V (12.5kV/cm).

The electroporation mixture was immediately mixed with 1 mL of ice cold 1 M

sorbitol and transferred to a 14 mL polypropylene Falcon tube (Becton Dickinson

Labware, Franklin Lakes, NJ).   After 1 h recovery at 30°C with shaking, the cells

was treated with 1 mL of 1 M sorbitol YPD and incubated at 30°C with shaking

for another hour.   The cells were plated on YPDS/Z agar plates [Appendix] at

low and high cell density.   The high Zeocin concentration allows selection for

multicopy integration of the pPICZBαOXO expression cassette.   At high Zeocin

concentrations, colonies exhibiting the strongest antibiotic resistance are

expected to contain the greatest number of expression cassettes integrated into

the genome.   Thus, the largest colonies were selected for expression screening.

The transformation efficiency is estimated at greater than 1×10^4

transformants/µg DNA.

2.4   Protein Expression and Purification

2.4.1   Expression screening

Large single colonies corresponding to hyper-resistant clones were picked

from YPDS/Z plates [Appendix] after 3 days incubation at 30°C, inoculated into

20 mL of BMGY medium [Appendix], and grown in an incubator shaker at 30°C

and 200 rpm until they reached saturation.   The cultures were centrifuged at

10,000 rpm for 5 min and resuspended in 2 mL of BMMY medium [Appendix].

Methanol (0.5%) was fed to the cells daily for 6 days.   Samples were collected

every 24 h to monitor expression level by 12% Tris-HCl SDS-PAGE (Bio-Rad,
Hercules, CA).

2.4.2   Intracellular localization of expression products

Parts of the cell pellets from the last step were also screened for
intracellular localization of the recombinant protein.   Cell culture samples were
suspended with 1 mL of Buffer EB and centrifuged at 5,000 rpm for 3 min.   The
supernants were discarded.   Buffer Y1 (12 mL) (QIAGEN, Valencia, CA) was
added and the samples were vortexed to homogenous mixtures.   β-Mercapto
ethanol (12 μL) and Zymolyase (6 mg) were added to the mixtures.   The
mixtures were incubated in a 37°C water bath for 1.5 h until a large amount of
cells were aggregated as spheroplasts.   The mixtures were centrifuged at 4,000
rpm for 10 min.   The supernants were carefully decanted and discarded, and the
cells were suspended with 5 mL of Y-PER (yeast protein extraction reagent,
Pierce, Rockford, IL) and incubated at room temperature for 10 min.   Cell debris
was removed by centrifugation at 10,000 rpm for 10 min.   The supernants were
saved for SDS-PAGE analysis.

2.4.3   High-density fermentation

P. pastoris high-density fermentation was performed to produce a large
quantity of recombinant protein.   Fermentation was conducted in a BioFlo 3000
benchtop fermentor (New Brunswick Scientific) with about 5 L working volume.
The *P. pastoris* X33 strain transformed with a pPICZBαOXO S49A expression
cassette was inoculated into three 500 mL baffled flasks, each containing 100 mL

of BMGY medium in an incubator shaker at 30°C and 200 rpm overnight.   The overnight shake cultures were inoculated in the fermentor containing 5 L of FM22 salt medium [Appendix] with 5 mL of 1000× PTM4 trace metal solution [Appendix] and 10 mL of 500× Biotin solution.   The temperature was controlled at 29°C.   The dissolved oxygen was set at 40% and the pH was set at 4.35.   An anti-foaming reagent [5% KFO673 (Kabo Chemicals, Jackson Hole, WY) in methanol] was added as required to control foaming.   Ammonium hydroxide solution (7 N) served both to adjust the pH and as a nitrogen source for the yeast. Silicone tubing was used to deliver the ammonium hydroxide (NH_4OH) to prevent air gaps, since it is very volatile and air gaps in feed lines might cause problems during the fermentation [30].   Dissolved oxygen control was maintained at 40% by P-I-D (proportional integral derivative), which delivered a mixture of pure oxygen and air to the culture.   Expression with *P. pastoris* is performed in fermentor cultures using a standard three-step procedure: (1) glycerol batch phase, (2) glycerol fed-batch phase, and (3) methanol induction phase.   After 21 h of the first phase (glycerol batch phase), the optical density (OD_{600nm}) reached 86 and the oxygen consumption began to decrease when glycerol was depleted.   This was followed by the glycerol fed-batch phase, which started with 400 mL of 50% glycerol at 84 mL/h delivery rate.   The methanol induction phase was started after 50 h of cell growth when oxygen consumption decreased.   Manganese sulfate was added to a final concentration of 1 mM in the fermentor culture at the beginning of the induction phase, and the temperature was lowered to 25°C to reduce the amount of green pigment formed. Teflon tubing was used to deliver methanol because of the volatility of methanol,

which could cause air gaps to develop.   Air gaps would cause irregular feeding

of methanol, which can kill the *Pichia* culture by generating toxic metabolites [30].

The feed medium was prepared from 100% methanol, 4 mL of 1000× PTM4, and

4 mL of 500× Biotin solution.   Methanol feeding was continued for 5 days at a

constant rate (12.5 mL/h) using a Watson Marlow 101 U/R pump

(Watson-Marlow Bredel, Inc., Wilmington, MA).   After 140 h of cell growth, the

optical density (OD_{600nm}) reached 282.   Samples were collected and measured at

OD_{600nm} every hour for 5 h and subsequently 24 h to monitor protein expression

levels by SDS-PAGE.   The cell culture was centrifuged in four 1 L centrifuge

bottles at 5,000 rpm for 20 min with a KAD 9.1000 composite rotor (KOMPspin,

Mountain View, CA).   After a week, the cell mass was about 0.9 kg from a 5 L

culture representing approximately 18% of the fermentation volume.

2.4.4   Purification of recombinant oxalate oxidase

The culture supernatant was pre-filtered with a capsule disposable

cartridge filter (Millipore Opticap 4″, pore size 0.5 µm, Millipore Corporation) to

remove particulates, and subsequently it was concentrated by a tangential flow

Millipore Pellicon-2 cassette filter unit (Millipore Corporation, Billerica, MA)

using three PLCGC composite regenerated cellulose membranes with a total

membrane area of 0.3 m².   The pump speed was adjusted to keep the inlet

pressure below 14 psi to avoid damaging the membranes.   The concentrated

protein solution (about 190 mL) was loaded onto a DE-52 cellulose (Whatman Inc,

Fairfield, NJ) anion exchange chromatography column (5×50 cm).   The column

was equilibrated and eluted with 2 L of 20 mM potassium phosphate buffer, pH

7.0, containing 0.4 mM EDTA buffer. The eluted protein was collected by an FC-100 Micro-Fractionator (Gilson Medical Electronics, Inc., Middleton, WI) in 20 mL fractions. Optical density at absorbance 280 (OD_{280nm}) and enzyme activity [Materials and Methods 2.5.1] were measured for all the combined fractions. The assayed protein fractions that exhibited oxalate oxidase activity were pooled and concentrated by ultra filtration with an YM-10 membrane (Amicon, Danvers, MA). The concentrated protein was dialyzed against 1 L of 25 mM sodium acetate (NaAc) buffer, pH 5.0, overnight with stirring. The protein was purified further with a CM-52 cellulose cation exchange chromatography column (2.5×15 cm). The column was equilibrated with 100 mL of 25 mM NaAc and eluted with a 0–0.4 N gradient buffer consisting of 400 mL of 25 mM NaAc, pH 5.0, and 400 mL of 25 mM NaAc containing 0.4 N of NaCl. The eluted protein was collected in 15 mL fractions. OD_{280nm} and enzyme activity were determined for each fraction. The assayed protein fractions exhibiting oxalate oxidase activity were pooled and concentrated. The concentrated protein was dialyzed against 1 L of 25 mM NaAc buffer, pH 5.0, overnight with stirring.

The isoelectric point (pI) of oxalate oxidase is 6.4. Therefore, in buffer having a pH greater than 6.4, oxalate oxidase is negatively charged, allowing it to be purified by anion exchange chromatography. On the other hand, at lower pH, the protein is positively charged, allowing it to be purified by cation exchange chromatography.
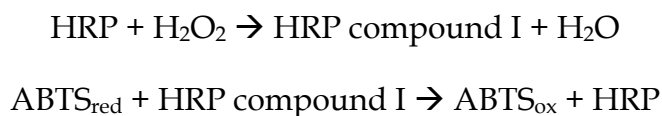
2.5   Protein Characterization and Metal Ion Analysis

2.5.1   Enzyme assays

Oxalate oxidase activity was measured by a coupled assay reaction based on hydrogen peroxide-dependent peroxidase oxidation of ABTS: 2,2'-azinobis(3-ethybenzthiazoline-6-sulfonic acid).   Activity was measured in absorbance at 650 nm [Figure 2.2] resulting from the oxidation product of ABTS ($ABTS_{ox}$) at room temperature.   Assay mixtures contained 50 mM sodium succinate buffer, pH 4.0, with horseradish peroxidase (HRP) (24 units; as defined by supplier), ABTS (5 mM), oxalic acid (50 mM) and oxalate oxidase added last, in a total volume of 1 mL.

Coupled reaction:

$$HRP + H_2O_2 \rightarrow HRP \text{ compound I} + H_2O$$
$$ABTS_{red} + HRP \text{ compound I} \rightarrow ABTS_{ox} + HRP$$

The presence of an endogenous oxalate oxidase in *P. pastoris* would interfere with this enzyme assay giving a background activity, complicating purification of recombinant oxalate oxidase.   Fortunately, no endogenous oxalate oxidase is detected in *Pichia pastoris* cultures.

2.5.1.1   Protein analysis.   Total protein (mg) was estimated by the Lowry Protein Test [25].   Purified oxalate oxidase protein concentration was determined by optical absorption measurements, using the extinction coefficient $\varepsilon = 8400$ M^{-1} cm^{-1} at 278 nm for the monomer [31].
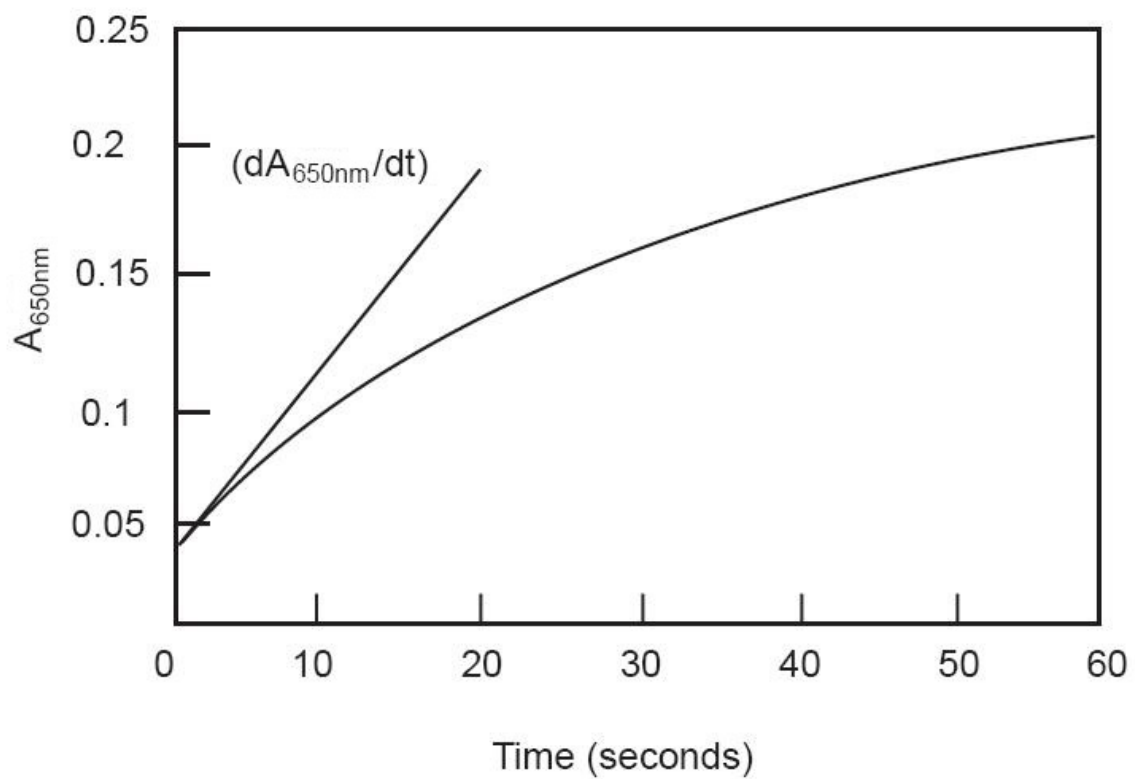
Figure 2.2   Illustration of initial rate analysis of enzyme activity.

2.5.1.2 Molecular weight determination. The monomeric molecular weight was determined by SDS-PAGE with a commercial protein molecular weight standard (Bio-Rad).   The proteins were denatured in the sample buffer at 100°C for 10 min prior to loading the gel.   Proteins were stained with Gel-Code Blue stain reagent (Pierce Chemical).

2.5.1.3 Glycosylation. The SDS-PAGE was stained for glycoprotein using 50 mL of 12.5% trichloracetic acid for 30 min and rinsed with distilled water for 15 min.   The gel was immersed in 50 mL of 1% periodic acid for 50 min and washed 8× for 10 min, each in 200 mL of distilled water.   The last wash was checked with silver reagent solution for IO_3^-.   The gel was immersed in fuchsin-sulfite glycoprotein stain solution in the dark for 50 min and washed 3× with 0.5% metabisulfite for 10 min each.   Finally, the gel was washed in a sequential change of distilled water with agitation until excess stain was removed [32].

2.5.1.4 Metal ion analysis. All the protein samples were diluted to approximately 5 µg/mL for manganese ion analysis.   A Varian Instruments SpectrAA-20 atomic absorption spectrometer equipped with a GTA-96 graphite furnace was used for measuring 1 blank (10 µL of distilled water), 3 standard manganese solutions (10 µL each), whose concentrations were 1 ppb, 2 ppb, and 3 ppb as a standard curve, and the protein samples.   The standard curve was used to estimate how much manganese ion was present in each protein sample.

2.5.1.5 MALDI-TOF mass spectrometry. The recombinant oxalate oxidase S49A, K44A, and wild type were analyzed by MALDI-TOF MS by Dr.

Cory Bystrom, Proteomics Shared Resource, OHSU Proteomics Core Laboratory
(Portland, OR).

CHAPTER 3

RESULTS

Recombinant oxalate oxidases were: (1) tested for the recombinant
protein expression level by SDS-PAGE, which also determined the apparent
molecular weigh; (2) purified by ion exchange chromatography; (3) stained for
protein and carbohydrate forms by SDS-PAGE with different staining solutions;
(4) analyzed for manganese content and enzyme activity; and (5) analyzed by
MALDI-TOF MS.

3.1   Oxalate Oxidase Protein Expression

Recombinant oxalate oxidase S49A was produced by high-density
fermentation, and other recombinant oxalate oxidases (N47A, N52A,
N47A/N52A, and K44A) were produced in small-scale shake cultures.   Six
independent clones were analyzed for each mutational variant of recombinant
oxalate oxidase.

3.1.1   Oxalate oxidase S49A

As shown in Figure 3.1., the protein expression level of recombinant
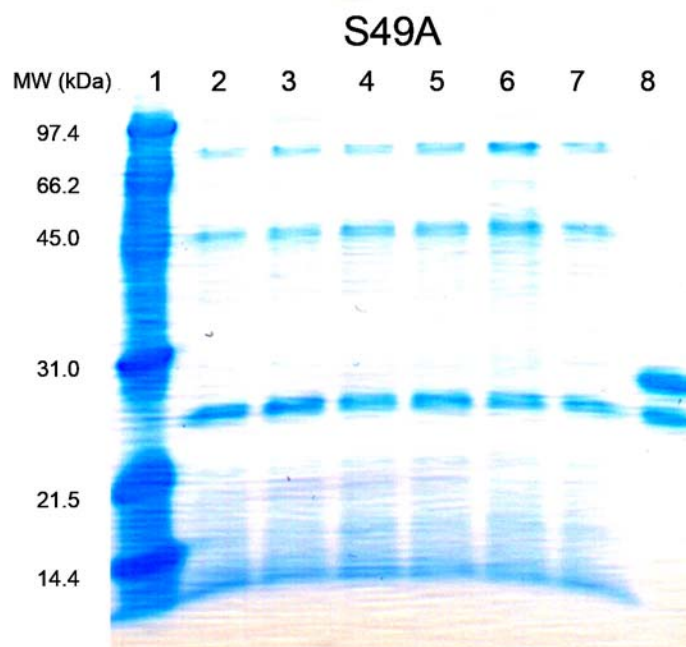oxalate oxidase S49A was uniformly high over all six independent clones (lanes

Figure 3.1   SDS-PAGE analysis of protein expression of recombinant oxalate oxidase S49A.   Lane 1, molecular weight standards; lanes 2–7 are six independent clones of recombinant oxalate oxidase S49A; lane 8, wild-type recombinant wheat oxalate oxidase.   Supernants were from 3-day cultures.

2–7), and the apparent molecular weight is approximately 23 kDa based on comparison with molecular weight standards (lane 1).   Wild-type recombinant wheat oxalate oxidase (lane 8) is heterogeneous.   The apparent molecular weight for the upper band is approximately 26 kDa and 23 kDa for the lower band based on comparison with molecular weight standards.   According to a previous study on barley oxalate oxidase [16], the heterogeneity is likely due to the loss of carbohydrates, with the upper band being the glycosylated form and the lower band being the non-glycosylated form.   The recombinant oxalate oxidase S49A samples all migrated consistent with them being non-glycosylated.

3.1.2   Oxalate oxidase (N47A, N52A, S49A/N42A, N47A/N52A, and K44A)

In Figure 3.2, the protein expression level of recombinant oxalate oxidase N47A was uniformly very low over all six independent clones.   In contrast, the recombinant oxalate oxidase N52A protein expression level was uniformly high over all six independent clones.   The apparent molecular weight of recombinant oxalate oxidase N47A is approximately 23 kDa, and for recombinant oxalate oxidase N52A is approximately 26 kDa based on comparison with molecular weight standards.   The recombinant oxalate oxidase N47A migrated consistent with it being non-glycosylated, and recombinant oxalate oxidase N52A migrated consistent with it being glycosylated.

In Figure 3.3, the apparent molecular weight of the recombinant protein S49A/N52A double mutant is approximately 23 kDa based on comparison with molecular weight standards, and the protein expression was relatively poor. On the other hand, the apparent molecular weight of the recombinant protein
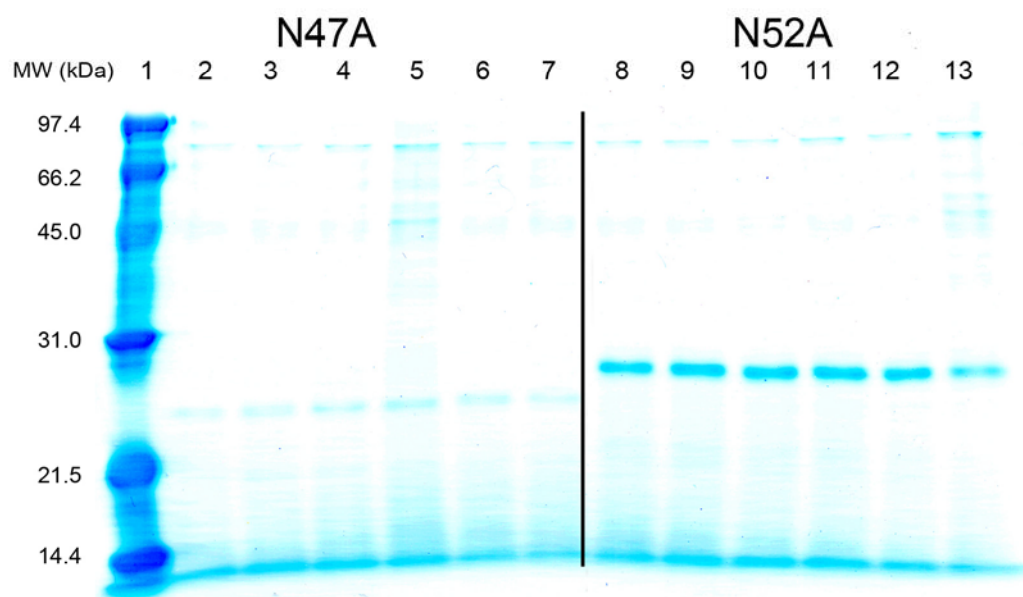
Figure 3.2 SDS-PAGE analysis of protein expression of recombinant oxalate oxidase N47A and N52A.   Lane 1, molecular weight standards; lanes 2–7 are six independent clones of recombinant oxalate oxidase N47A; lanes 8–13 are six independent clones of recombinant oxalate oxidase N52A.   Supernants were from 3-day cultures.
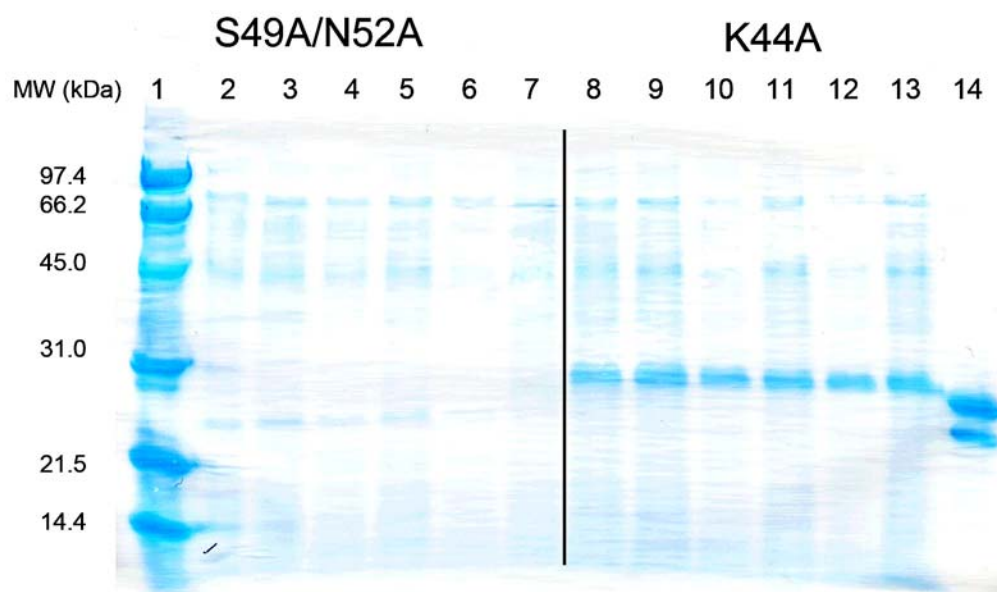
Figure 3.3 SDS-PAGE analysis of protein expression of recombinant oxalate oxidase S49A/N52A and K44A. Lane 1, molecular weight standards; lanes 2–7 are six independent clones of recombinant oxalate oxidase S49A/N52A; lanes 8–13 are six independent of recombinant oxalate oxidase K44A; lane 14, wild-type recombinant wheat oxalate oxidase. Supernants were from 3-day cultures.

K44A is approximately 31 kDa based on comparison with molecular weight standards and the protein expression was good.

The recombinant oxalate oxidase N47A/N52A double mutant was expressed at extremely low levels according to SDS-PAGE analysis [Figure 3.4]. Cells isolated from the expression culture were disrupted and analyzed for intracellular accumulation of the recombinant protein to determine if it was being retained in the cells [Figure 3.5], and the result showed that there was no protein retained within the cells.   Although there is a nearly continuous staining of protein on the SDS-PAGE gel [Figure 3.5], there is no sign of a large intracellular pool of recombinant oxalate oxidase (lanes 2, 3, and 4).   Western blot analysis would in principle be used to detect small amounts of recombinant oxalate oxidase; however, the SDS-PAGE analysis shown was used only to determine whether larger amounts of recombinant protein were produced but not secreted.

3.2   Purification and Enzyme Assays of Oxalate Oxidase

Two mutational variants of recombinant oxalate oxidase were purified with ion exchange chromatography and analyzed with the HRP/ABTS coupled assay for enzyme activity.

3.2.1   Oxalate oxidase S49A

The elution profile of oxalate oxidase S49A from the DE-52 cellulose anion exchange column was monitored by OD_{280nm}, and the enzyme activity was
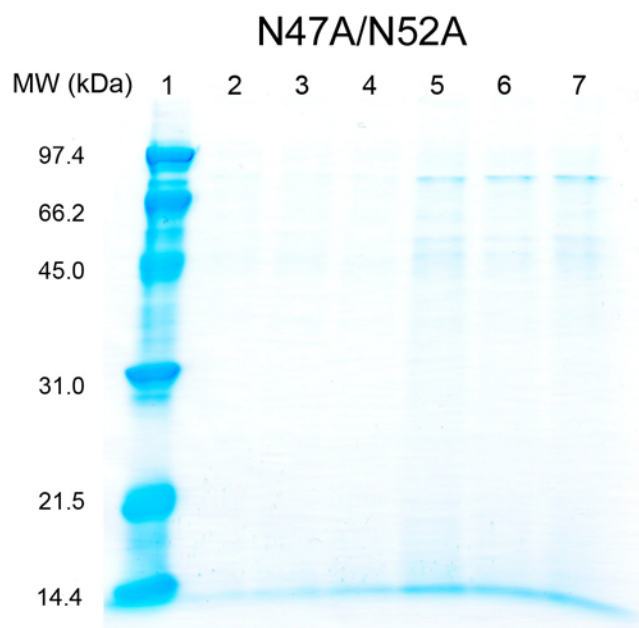
Figure 3.4 SDS-PAGE analysis of protein expression of recombinant oxalate oxidase N47A-N52A. Lane 1, molecular weight standards; lanes 2–7 are six independent clones of recombinant oxalate oxidase N47A/N52A. Supernants were from 3-day cultures.
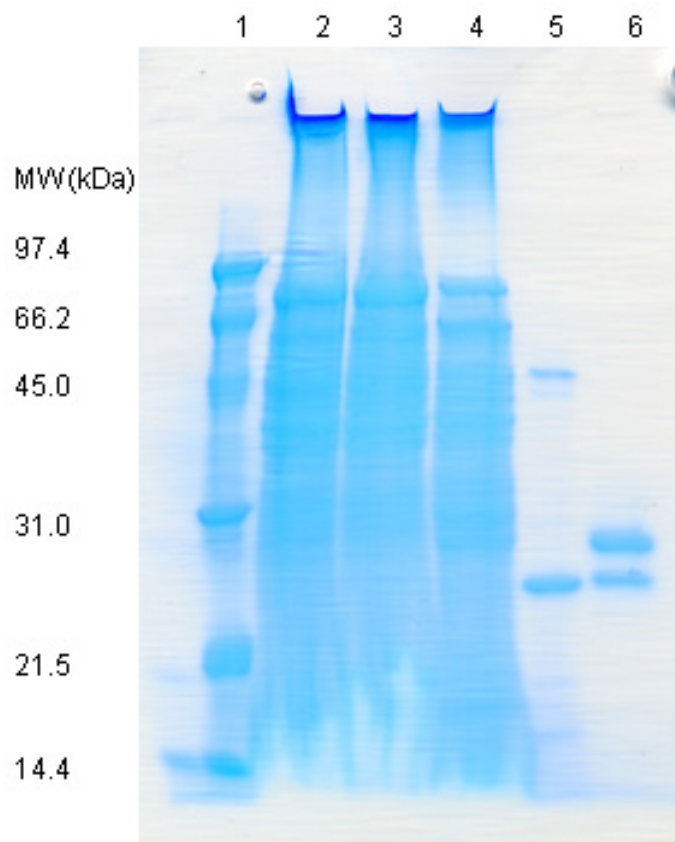
Figure 3.5 SDS-PAGE analysis of recombinant oxalate oxidase intracellular localization. Lane 1, molecular weight standards; lane 2, disrupted cells from recombinant oxalate oxidase N47A expression culture; lane 3, disrupted cells from recombinant oxalate oxidase N52A expression culture; lane 4, disrupted cells from recombinant oxalate oxidase N47A/N52A expression culture; lane 5, recombinant oxalate oxidase S49A/N52A; lane 6, wild-type recombinant wheat oxalate oxidase.

determined using the HRP/ABTS coupled assay [Figure 3.6].   Two peaks (peaks A and B) were resolved based on OD_{280nm}.   In Figure 3.6, peak A coincided with the activity peak identifying the fractions containing the recombinant oxalate oxidase S49A protein.   Peak B did not exhibit oxalate oxidase activity.   The protein in peak A was pooled and purified further by CM-52 cellulose cation exchange chromatography.

The elution profile of oxalate oxidase S49A from the CM-52 cellulose anion exchange column was monitored by OD_{280nm}, and the enzyme activity was determined using the HRP/ABTS coupled assay [Figure 3.7].   Two peaks (peak A and peak B) were resolved based on OD_{280nm}.   In Figure 3.7, peak B coincided with the activity peak identifying the fractions containing the recombinant oxalate oxidase S49A protein.   Peak A did not exhibit oxalate oxidase activity. The tail on peak B was checked by SDS-PAGE [Figure 3.7] to determine whether the protein was oxalate oxidase even though the activity was relatively low. The protein in peak B was pooled concentrated.   The protein was homogeneous based on SDS-PAGE analysis [Figure 3.7].

The purification results are summarized in Table 3.1.   The retentate was purified and concentrated from the crude extract of *P. pastoris* culture supernant. The overall yield was 56% from the retentate with a purification factor of 10 during purification.

When the recombinant oxalate oxidase S49A was dialyzed against 20 mM potassium phosphate buffer, pH 7.0, a white precipitate formed in the dialysis tube.   When the buffer was changed to 25 mM NaAc, pH 5.0, the white precipitate dissolved, indicating that absence of carbohydrates changes the
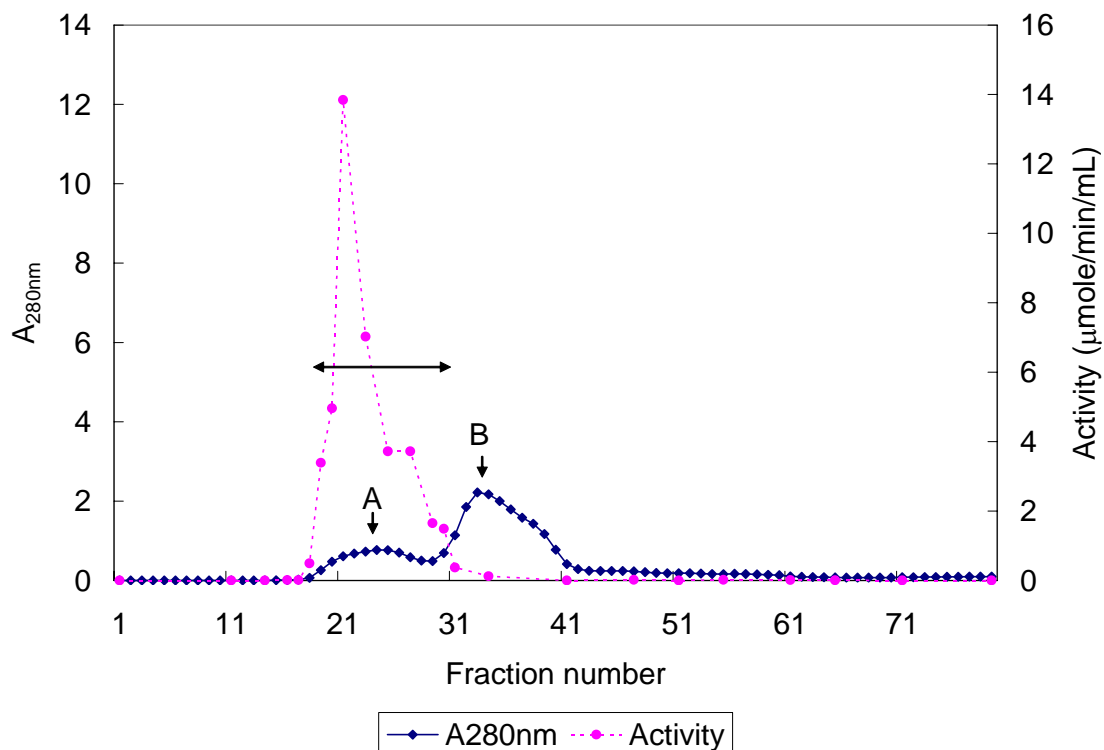
Figure 3.6 DE-52 cellulose anion exchange chromatography of recombinant oxalate oxidase S49A. The protein sample was applied to a 5 × 50 cm DE-52 column and equilibrated and eluted both with 20 mM potassium phosphate buffer with 0.4 mM EDTA at pH 7.0. Flow-through with highest oxalate oxidase activity was pooled (<—>, fraction 18–31).
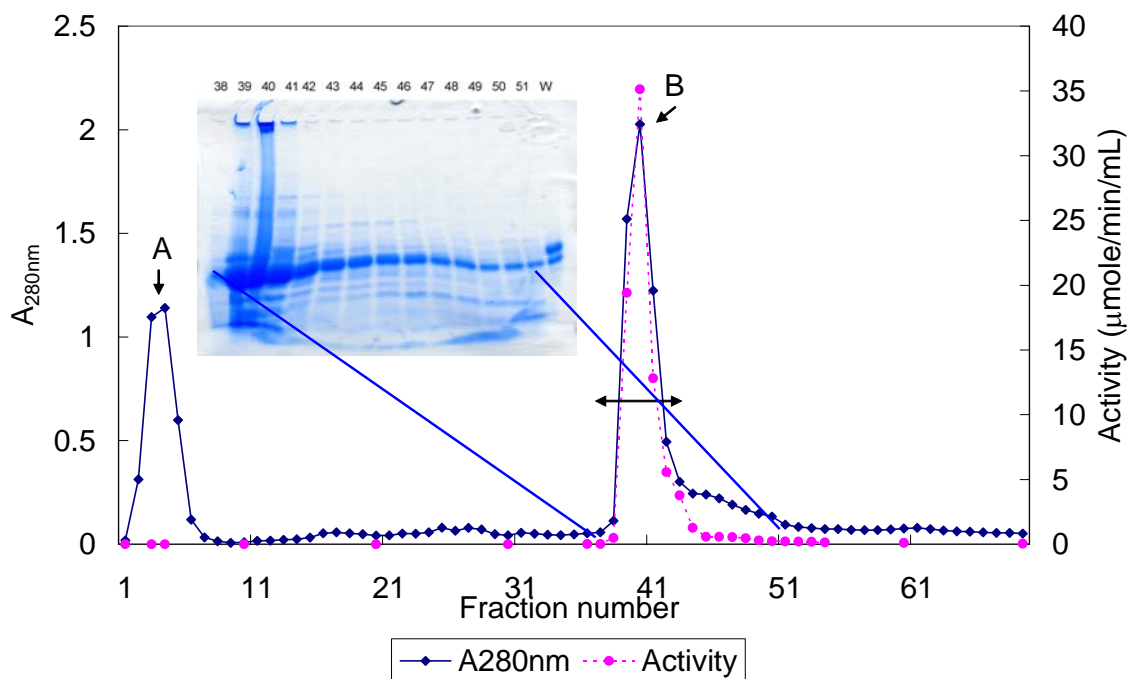
Figure 3.7 CM-52 cellulose cation exchange chromatography of recombinant oxalate oxidase S49A. The protein sample was applied to a 2.5 × 15 cm CM-52 column, equilibrated with 25 mM sodium acetate buffer, and eluted with a gradient from 0 to 0.4 N sodium chloride in 25 mM sodium acetate, pH 5.0, to develop the column. Flow-through with highest oxalate oxidase activity was pooled (<—>, peak B). *Insert:* SDS-PAGE analysis of elution profile for recombinant oxalate oxidase S49A. Fractions 38 to 51 were tested. Fraction 40 contained too much protein, which was not denatured completely. W, wild-type recombinant wheat oxalate oxidase.

Table 3.1

Purification of Recombinant Oxalate Oxidase S49A

Purification step	Volume[a] (mL)	Total Protein[b] (mg)	Total Activity[c] (units)	Specific Activity[d] (units/mg protein)	Yield (%)	Purification factor
Retentate	190	2831	1413	0.5	100	1.0
DE-52	325	309	1276	4.1	90	8.3
CM-52	75	161	790	4.9	56	9.8

[a] Total volume used in each purification step

[b] Estimated protein amounts (mg) from Lowry protein test

[c] Activity (from HRP/ABTS coupled assay) × Volume

[d] Total activity / Total protein

solubility and self-aggregation properties of the protein. However, since the S49A mutant exhibits nearly the same specific activity [Table 3.1] as wild-type recombinant oxalate oxidase, glycosylation does not appear to be required for activity.

3.2.2 Oxalate oxidase K44A

The recombinant oxalate oxidase K44A was produced in BMMY medium in a shake flask culture. This rich medium contains a large amount of proteins that appear in the elution profile of the DE-52 cellulose anion exchange column. After enzyme assays, two activity peaks (peaks A and B) were resolved, indicating fractions containing recombinant oxalate oxidase K44A [Figure 3.8]. *P. pastoris* produces a green pigment during the expression phase that binds very tightly to the top of the DE-52 column and may interfere with elution of protein resulting in the artifactual appearance of two activity peaks. Alternatively,
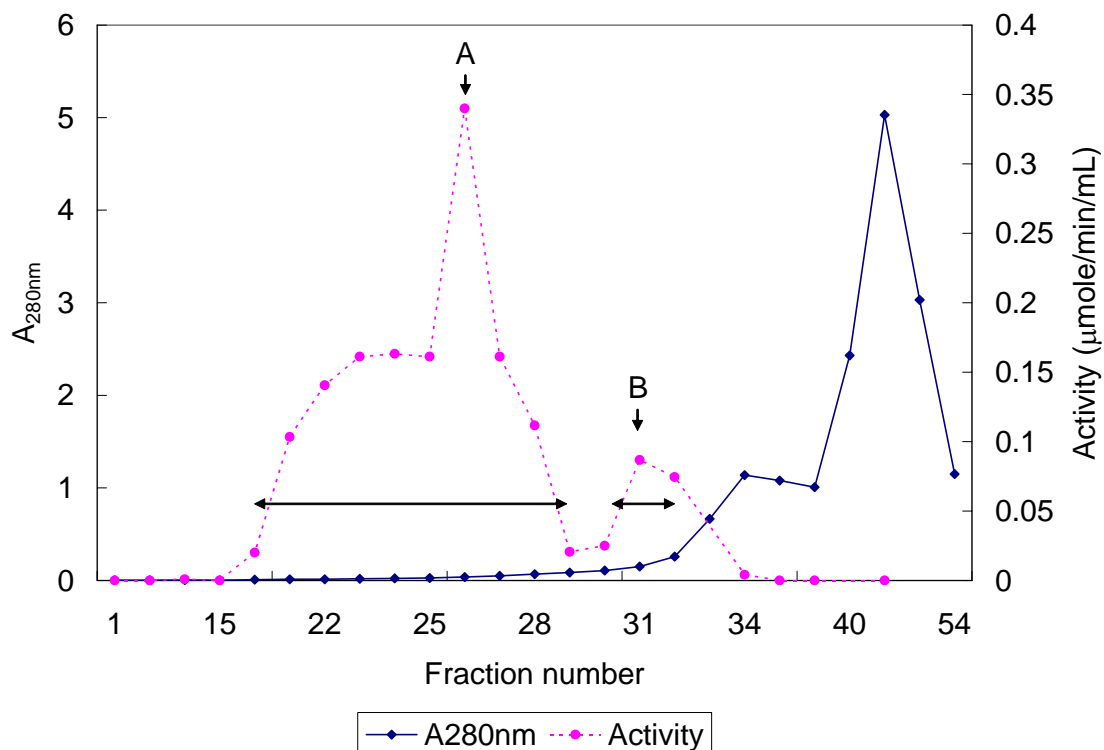
Figure 3.8 DE-52 cellulose anion exchange chromatography of recombinant oxalate oxidase K44A.   The protein sample (195 mL) was applied to a 5 × 50 cm DE-52 column, and equilibrated and eluted with 20 mM potassium phosphate buffer with 0.4 mM EDTA at pH 7.0.   Flow-through with highest oxalate oxidase activity was pooled (<—>; peak A, fractions 20–29; peak B, fractions 30–32).

there may be some carbohydrate differences between the proteins eluting in the two peaks.

In Figure 3.9, peak A (from the DE-52 column) resolved into two components (peaks A and B) on the CM-52 column based on OD_{280nm}.   Peak B coincided with the activity peak identifying that those fractions contained the recombinant oxalate oxidase K44A protein.

Peak A did not exhibit oxalate oxidase activity.   In Figure 3.10, peak B (from the DE-52 column) was also resolved into two components (peaks A and B) on the CM-52 column based on OD_{280nm}.   Again, peak B coincided with the activity peak identifying the fraction containing the recombinant oxalate oxidase K44A protein.   Peak A did not exhibit oxalate oxidase activity.

The purified recombinant oxalate oxidase K44A was analyzed at each step of the purification results are summarized in Table 3.2.   The overall yield for peak A was 21%, whereas peak B was 30%.   The purification factors were 2.3 for peak A and 49 for peak B.

3.3   Glycoprotein Test

Recombinant oxalate oxidase S49A and K44A proteins were analyzed for protein and carbohydrate by SDS-PAGE after two purification steps.   An SDS-PAGE gel was stained with Gel-Code Blue for protein analysis and with fuchsin-sulfite stain for carbohydrate analysis.   In Figure 3.11, the protein stained gel indicated a successful purification step based on the fact that no other

Figure 3.9   CM-52 cellulose cation exchange chromatography of recombinant oxalate oxidase K44A (DE-52, peak A).   The protein sample was applied to a 2.5 × 15 cm CM-52 column, equilibrated with 25 mM sodium acetate buffer, and eluted with a gradient from 0 to 0.4 N sodium chloride in 25 mM sodium acetate, pH 5.0, to develop the column.   Flow-through with highest oxalate oxidase activity was pooled (<—>, peak B).
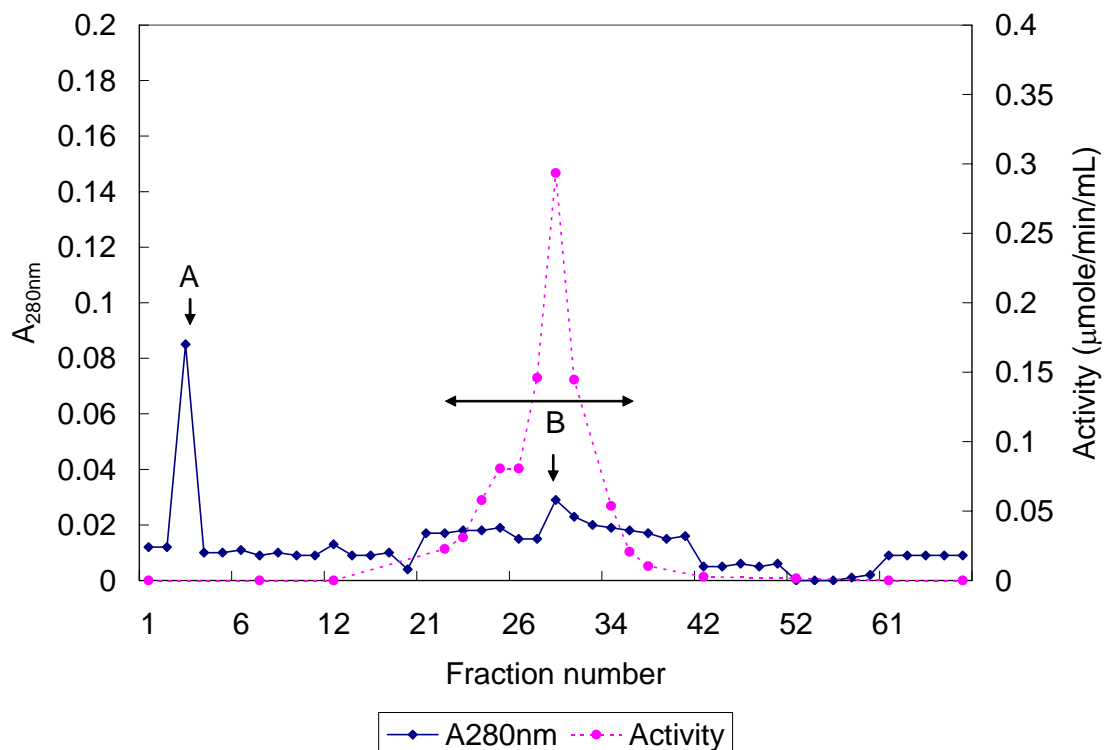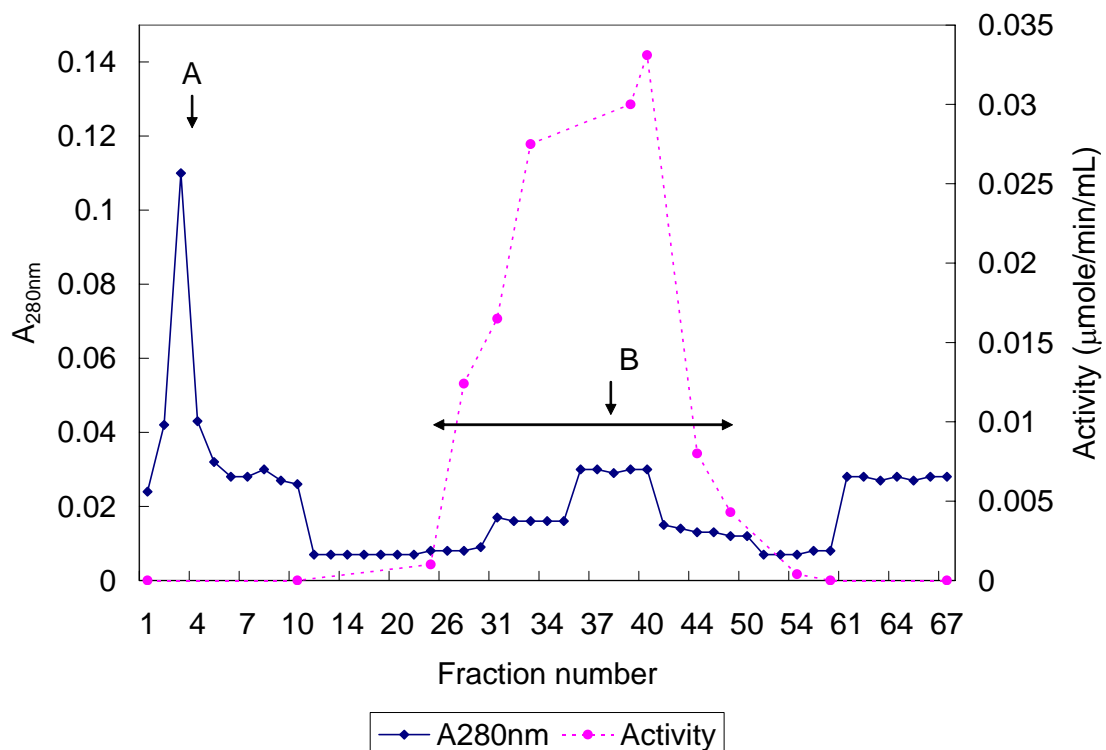
Figure 3.10 CM-52 cellulose cation exchange chromatography of recombinant oxalate oxidase K44A (DE-52, peak B). The protein sample was applied to a 2.5 × 15 cm CM-52 column, equilibrated with 25 mM sodium acetate buffer, and eluted with a gradient from 0 to 0.4 N sodium chloride in 25 mM sodium acetate, pH 5.0, to develop the column. Flow-through with highest oxalate oxidase activity was pooled (<—>, peak B).

Figure 3.11 SDS-PAGE stain of recombinant oxalate oxidase forms.
SDS-PAGE gels were stained for protein (left) and carbohydrate (right).   Lane 1,
recombinant oxalate oxidase K44A; lane 2, recombinant oxalate oxidase S49A;
lane 3, wild type recombinant oxalate oxidase.   Each lane contains 3 μg of
protein.

Table 3.2

Purification of Recombinant Oxalate Oxidase K44A
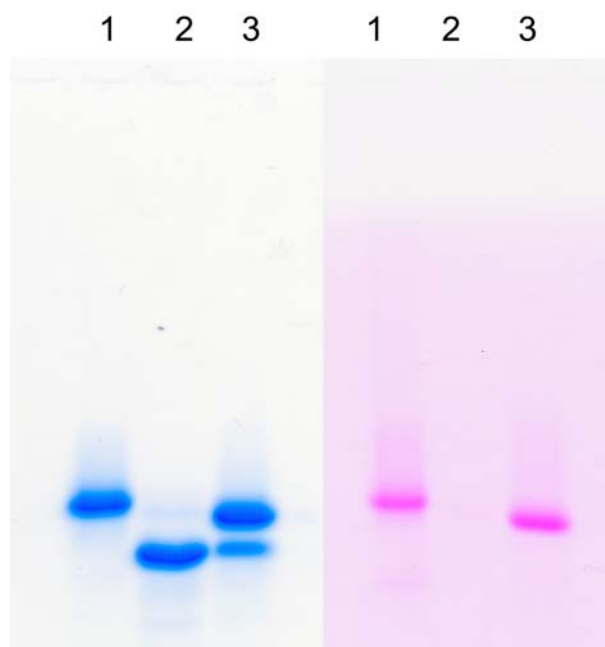
Purification step	Volume[a] (mL)	Total Protein[b] (mg)	Total Activity[c] (units)	Specific Activity[d] (units/mg protein)	Yield (%)	Purification factor
DE- 52 Peak A	240	19	37	1.9	100	1.0
DE- 52 Peak B	70	46	5.5	0.1	100	1.0
CM-52 Peak A	1.2	1.7	7.7	4.5	21	2.3
CM-52 Peak B	8.5	0.3	1.6	5.8	30	49

[a] Total volume used in each purification step

[b] Estimated protein amounts (mg) from Lowry protein test

[c] Activity (from HRP/ABTS coupled assay) × Volume

[d] Total activity / Total protein

proteins were stained on the gel. The recombinant oxalate oxidase K44A (lane 1) was a glycoprotein based on the carbohydrate stained gel. On the other hand, the recombinant oxalate oxidase S49A was not a glycoprotein. Interestingly, there was a very faint band of the recombinant oxalate oxidase S49A at higher molecular weight on both protein and carbohydrate stained gels, indicating that there were some carbohydrates attached to a small fraction of the recombinant oxalate oxidase S49A. SDS-PAGE analysis of the purified recombinant oxalate oxidase S49A (3 µg/lane) [Figure 3.11] shows that it is nearly homogeneous, although at much higher loading (50 µg/lane) [Figure 3.7] other protein contaminants can be detected.

3.4   Manganese Test

The recombinant oxalate oxidase S49A and K44A proteins were tested to determine manganese content.   A standard curve [Figure 3.12] was constructed and the unknowns measured using an atomic absorption spectrometer.   The manganese content of recombinant oxalate oxidase S49A was about 0.22 g-atom Mn/subunit, whereas recombinant oxalate oxidase K44A (peak A) was about 0.26 g-atom Mn/subunit and peak B was about 0.23 g-atom Mn/subunit [Table 3.3].

Table 3.3

Manganese Elemental Analysis for Recombinant Oxalate Oxidase S49A and K44A

Mn standards	Readings	
1 ppb	0.042	
2 ppb	0.088	
3 ppb	0.134	
Samples	**Readings**	**Mn content (g-atom/subunit)**
S49A	0.128	0.22
K44A peak A	0.155	0.26
K44A peak B	0.136	0.23

3.5   MALDI-TOF MS Analysis

Matrix-Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS) is a technique in which a co-precipitate of a UV-light absorbing matrix and a biomolecule is irradiated by a nanosecond laser
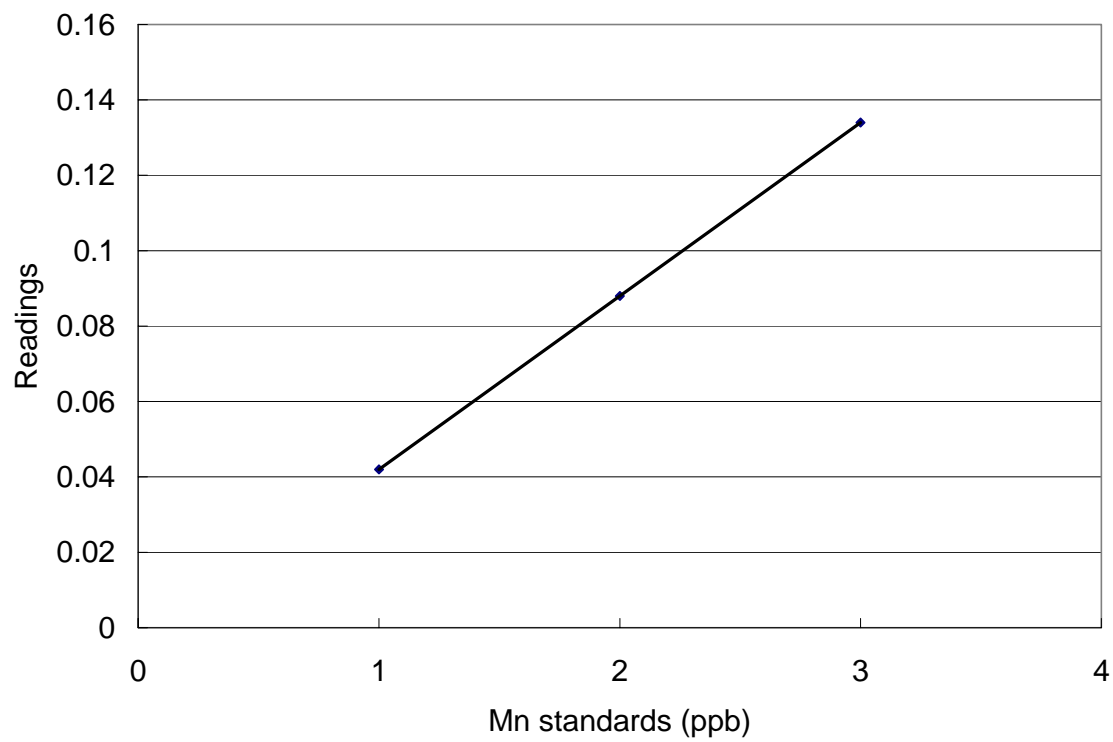
Figure 3.12   Standard curve of manganese test for recombinant oxalate oxidase S49A and K44A.

pulse.   Most of the laser energy is absorbed by the matrix, which prevents

unwanted fragmentation of the biomolecule.   The ionized biomolecules are

accelerated in an electric field and enter the flight tube, where molecules are

separated according to their mass-to-charge ratio and therefore reach the

detector at different times.   In this way, each molecule yields a distinct time of

flight (TOF) signal.   Protein identification by this technique has the advantage of

short measuring time and negligible sample consumption.   The recombinant

oxalate oxidases (wild type, S49A, and K44A) were analyzed by this method.

Figure 3.13 shows the MALDI-TOF mass spectrum of wild-type

recombinant oxalate oxidase.   Two major peaks (21,278 amu and 23,007 amu)

were resolved.   Figure 3.14 shows the MALDI-TOF mass spectrum of

recombinant oxalate oxidase S49A, and only one major peak (21,262 amu) was

resolved.   Figure 3.15 shows the MALDI-TOF mass spectrum of recombinant

oxalate oxidase K44A, and only one major peak (21,424 amu) was resolved.

Theoretical and experimental molecular masses determined by MALDI-TOF MS

are compared in Table 3.4.

Table 3.4

Molecular Masses of Recombinant Oxalate Oxidases
(WT, S49A, and K44A) Calculated Based on
Primary Structure and Determined Experimentally
by MALDI-TOF MS analysis.

Recombinant OXO	Molecular mass (amu)	
	Calc.	**MALDI-TOF**
WT	21,279	21,278
S49A	21,263	21,262
K44A	21,222	21,424
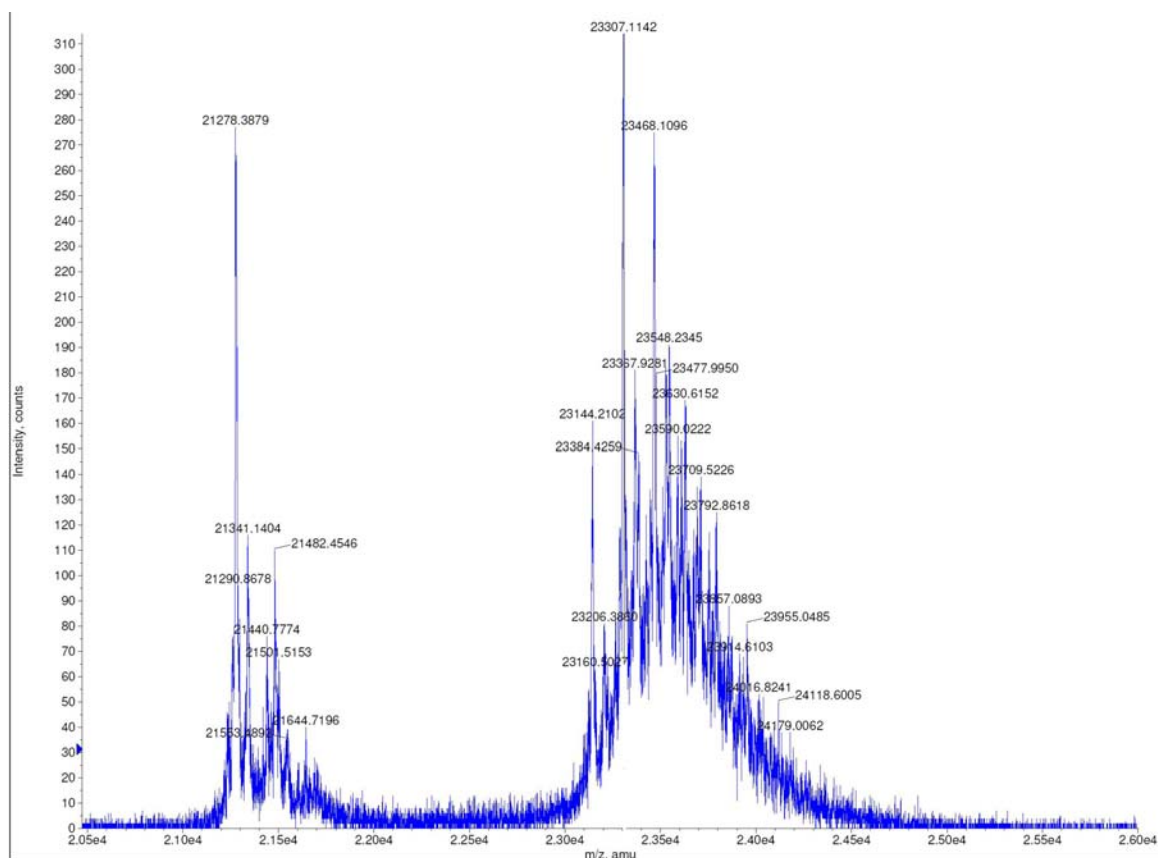
Figure 3.13 MALDI-TOF MS analysis of wild-type recombinant oxalate oxidase. X axis corresponds to mass (amu†††) and Y axis corresponds to intensity (counts).

††† Amu: atomic mass unit = 1.660538 x 10^{-27} kg

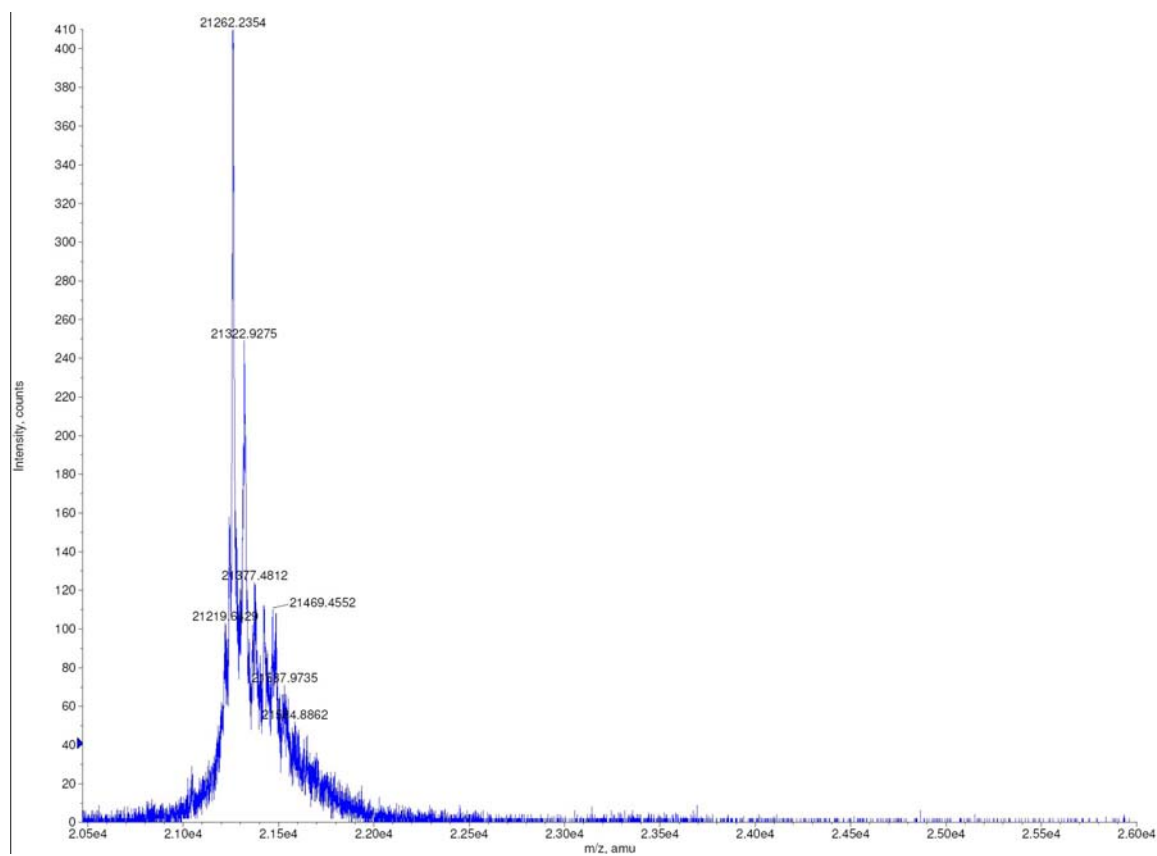**Figure 3.14** MALDI-TOF MS analysis of recombinant oxalate oxidase S49A. X axis is mass (amu) and Y axis is intensity (counts).
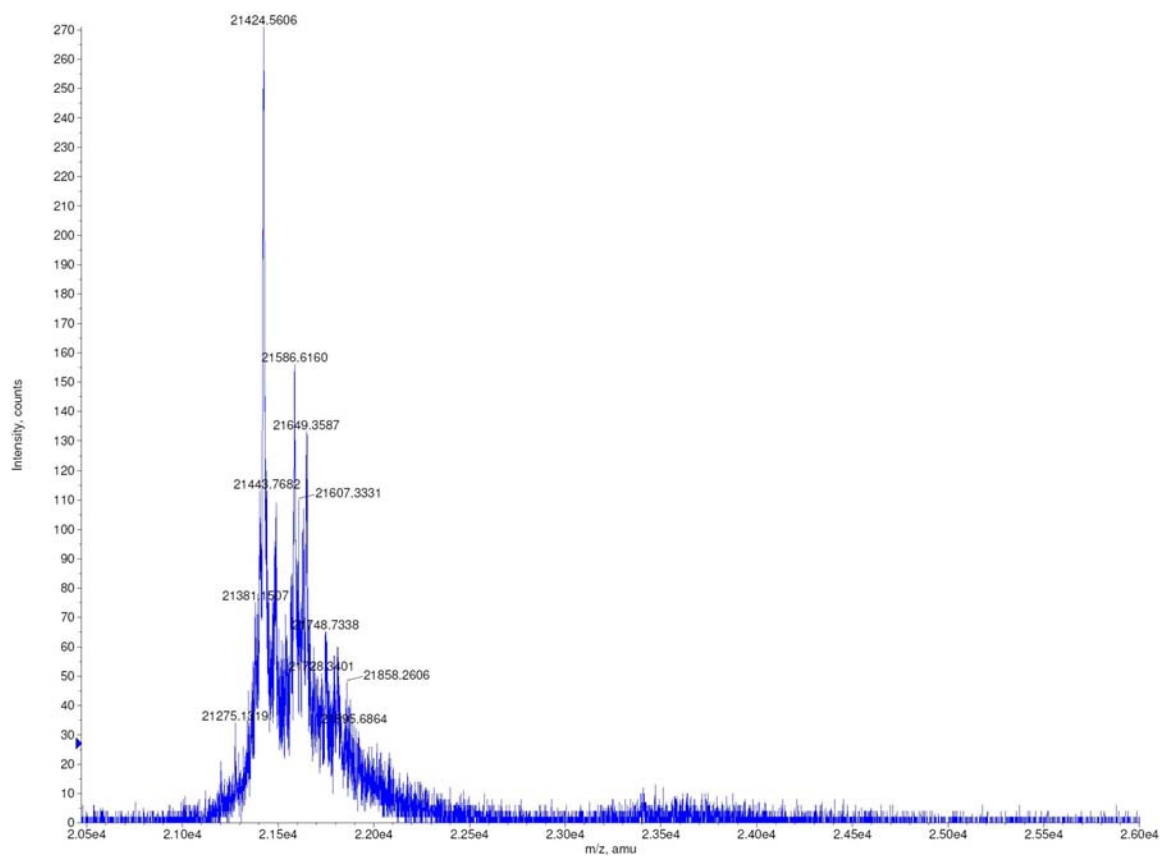
Figure 3.15   MALDI-TOF MS analysis of recombinant oxalate oxidase K44A.   X axis is mass (amu) and Y axis is intensity (counts).

CHAPTER 4

DISCUSSION

In plants, the roles of N-glycans have been studied using different approaches, such as the use of N-glycosylation and N-glycan-processing inhibitors, site-directed mutagenesis of the N-glycosylation site, or the study of mutants affected in the attachment of N-glycans [33]. The diverse functions of glycoproteins have been shown to be a direct result of carbohydrate structures.

N-glycosylation is a major modification of proteins in plant cells, and it has a great impact both on their physicochemical properties and their biological functions. N-linked oligosaccharides may contain targeting information, or they may be directly involved in protein recognition or cell–cell adhesion processes. In plants, N-glycans can protect the protein from proteolytic degradation and thermal denaturation, as well as strongly influence the glycoprotein conformation, stability, solubility, and biological activity [33]. In this study, site-directed mutagenesis was used for investigating N-glycosylation sites and the putative oxalate binding site, which gave information about N-glycosylation and oxalate binding of the recombinant oxalate oxidase.

After Ser 49 was mutated to alanine, most of the recombinant oxalate oxidase migrated consistent with it being non-glycosylated [Figure 3.1]. This demonstrates that the N47 NXS site is the target sequence motif for N-glycan

glycosylation in recombinant wheat oxalate oxidase.   However, as shown in Figure 3.11, a very small amount of carbohydrate-containing protein was detected on the carbohydrate-stained gel.   This suggests that even though glycosylation was very inefficient after the mutation, a small fraction of the expressed protein was secreted with glycan attached.   This glycan may still be attached at Asn 47 in S49A oxalate oxidase (the site of glycan attachment) in spite of the fact that it is no longer in an NXS motif, since the amide group of Asn was not altered.

NX(S/T) is the target sequence motif for N-glycan glycosylation.   In Figure 1.2, N47 is within this motif (NTST, two threonines flanking serine).   The presence of two threonines following N47 may have compensated for the absence of serine in the canonical glycosylation motif.

Asn 47 is the potential N-glycan glycosylation site within the NXS target sequence motif.   After N47A mutagenesis, the recombinant oxalate oxidase migrated consistent with it being non-glycosylated, confirming that Asn 47 is an N-glycan glycosylation site [Figure 3.2].   N-glycosylation is major modification of proteins in plants cells, and the presence of N-glycans is necessary for efficient secretion of plant protein.   This may account for the poor expression of the recombinant oxalate oxidase N47A, since carbohydrates were lost after the mutation.   However, S49A also lacked glycans and was more efficiently expressed, so other structural features may be important as well.

Asn 52 is another potential N-glycan glycosylation site.   After it was mutated to alanine, the recombinant oxalate oxidase migrated consistent with it being glycosylated, indicating that Asn 52 is not an N-glycan glycosylation site

even though it is within an NXS target sequence motif.   The X-ray crystal structure of barley oxalate oxidase [5] shows that in that protein the corresponding residue is less exposed than N47, and this probably accounts for the lack of glycan attachment at N52.

Lys 44 has been considered to be a putative oxalate binding site.   After it was mutated to an alanine, the specific activity of oxalate oxidase [Table 3.2] did not decrease, indicating that Lys 44 is not likely to be an oxalate binding site.

According to Figure 3.4, recombinant oxalate oxidase N47A/N52A protein was expressed at extremely low levels according to the SDS-PAGE analysis.   One possibility is that the protein was expressed but not efficiently secreted and might accumulate in the cytosol or the secretory pathway.   Thus, a test for intracellular localization was done to determine whether the recombinant protein was retained in the cells.   However, there was no sign of accumulation of recombinant oxalate oxidase N47A/N52A inside the cells based on the SDS-PAGE [Figure 3.5].   The absence of the recombinant protein may have been the result of inefficient translation of mRNA, or the recombinant protein might have been proteolytically degraded.

MALDI-TOF MS analyses are shown in Figures 3.13, 3.14, and 3.15, and the molecular masses of recombinant oxalate oxidases (wild type, S49A, and K44A) are shown in Table 3.4.   Since the specific activity was similar for all the recombinant proteins in this study, it would be difficult to distinguish if the desired mutations were successful.   So, the highly accurate molecular mass determined by MALDI-TOF MS can be used to verify the S49A and K44A

mutations by comparison to the wild-type molecular weight.   The experiment confirmed that the mutations were present.

In Figure 3.13, the two major peaks from the wild type recombinant oxalate oxidase indicated one is a glycosylated form (23,307 amu) and the other one is a non-glycosylated form (21,278 amu).   This type of heterogeneity was also observed in SDS-PAGE even though the molecular weights were not exactly the same in the two analyses.   The different apparent molecular weights observed in SDS-PAGE may be explained by the anomalous migration of the glycosylated germin sample compared to the non-glycosylated molecular weight standards.

As shown in Figure 3.11, there was a very small amount of glycoprotein present in the recombinant oxalate oxidase S49A, but no glycoprotein was observed in the MALDI-TOF MS analysis.   This might be due to the fact that ionization of the small amount of glycosylated protein in the sample was suppressed by sodium (Na^+) or potassium (K^+) metal ions since carbohydrates tend to attach to cations very easily.   This also would explain why there is only one peak in the analysis of K44A [Figure 3.15].   The interference with Na^+ and K^+ explains the lack of signal from the glycosylated recombinant oxalate oxidase in the MALDI-TOF MS.   In Figure 3.14, the second peak (21,322 amu) was about 60 amu heavier than the major peak, consistent with the presence of transition metals such as Mn (55 amu) and Zn (65 amu) which are known to be bound in germins.   Only a small fraction of the protein was detected in the MALDI-TOF experiment that exhibited a mass increment consistent with a metal cofactor, suggesting that the majority of the metal was lost during ionization.

The recombinant oxalate oxidase K44A had a slightly greater molecular weight (202 amu) from the MALDI-TOF MS than the predicted molecular weight shown in Table 3.4.   This suggested that K44A was glycosylated based on the extra mass which is consistent with the presence of covalently attached acetylglucosamine residue (202 amu).   Evidence for glycosylation was also seen on the SDS-PAGE [Figure 3.3] where the K44A band had a much higher apparent molecular weight than other recombinant oxalate oxidases, suggesting hyper-glycosylation.   This may indicate an effect of the environment of the NXS site on the glycosylation pattern.

This study identified Asn 47 as the functional N-glycan glycosylation site in recombinant wheat oxalate oxidase expressed in *P. pastoris*.   Partial glycosylation of S49A oxalate oxidase showed that the flanking threonine residues may be able to compensate for lack of a NXS sequence motif.   This study also verified that N-glycan glycosylation sites within the NXS target sequence motif are not always the binding sites for N-glycans.   Asn 52 was identified as a potential N-glycan glycosylation site based on it being within an NXS target sequence motif; however, the study showed that it was not an N-glycan glycosylation site.

REFERENCES

[1] Lane, B. G. (2002) Oxalate, germins, and higher-plant pathogens. *IUBMB Life* **53**:67–75.

[2] ClustalW: A general purpose multiple sequence alignment program for DNA or proteins. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen.   Available for download from: www.ebi.ac.uk/clustalw

[3] Dunwell, J. M., Khuri, S., and Gane, P. J. (2000) Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin family. *Microbiol. Mol. Biol. Rev.* **64**:153–179.

[4] Gane, P. J., Dunwell, J. M., and Warwicker, J. (1998) Modeling based on the structure of vicilins predicts a histidine cluster in the active site of oxalate oxidase. *J. Mol. Evol.* **46**:488–493.

[5] Woo, E. J., Dunwell, J. M., Goodenough, P. W., Marvier, A. C., and Pickersgill, R. W. (2000) Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. *Nat. Struct. Biol.* **7**:1036–1040.

[6] Bernier, F., and Berna, A. (2001) Germins and germin-like proteins: Plant do-all protein. But what do they do exactly? *Plant physiological biochemistry* **39**:545–554.

[7] Lane, B. G., Dunwell, J. M., Ray, J. A., Schmitt, M. R., and Cuming, A. C. (1993) Germin, a protein marker of early plant development, is an oxalate oxidase. *J. Biol. Chem.* **268**:12239–12242.

[8]   Hurkman, W. J., and Tanaka C. K. (1996) Germin gene expression is induced in wheat leaves by powdery mildew infection. *Plant Physiol.* **111**:735–739.

[9]   RasMol: An easy and powerful software for looking at macromolecular structure and its relation to function.   For more information on this molecular visualization freeware, go to: www.umass.edu/microbio/rasmol/

[10]   Woo, E. J., Dunwell, J. M., Goodenough, P. W., and Pickersgill, R. W. (1998) Barley oxalate oxidase is a hexameric protein related to seed storage proteins: evidence from X-ray crystallography. *FEBS Lett.* **437**:87–90.

[11]   Dunwell, J. M., and Gane, P. J. (1998) Microbial relatives of seed storage proteins: conservation of motifs in a functionally diverse superfamily of enzymes. *Journal of molecular evolution* **46**:147–154.

[12]   Carter, C., Thornburg, R. W. (2000) Tobacco nectarin I. Purification and characterization as a germin-like, manganese superoxide dismutase implicated in the defense of floral reproductive tissues. *J. Biol. Chem.* **275**:36726–36733.

[13]   Yamahara, T., Shiono, T., Suzuki, T., Tanaka, K., Takio, S., Sato, K., Yamazaki, K., and Satoh, T. (1999) Isolation of a germin-like protein with manganese superoxide dismutase activity from cell of a moss, *Barbula unguiculata*. *J. Biol. Chem.* **274**:33274–33278.

[14]   Lane, B. G., Grzelczak, Z., Kennedy, T.D., Hew, C., and Joshi, S. (1987) Preparation and analysis of mass amounts of germin: demonstration that the protein with signals the onset of growth in germinating wheat is a glycoprotein. *Biochem. Cell Biol.* **65**:354–362.

[15]   Dratewka-Kos, E., Rahman, S., Grzelczak, Z. F., Kennedy, T. D., Murray, R. K., and Lane, B. G. (1989) Polypeptide structure of germin as deduced from cDNA sequencing. *J. Biol. Chem.* **264**:4896–4900.

[16]   Fitchette-Laine, A., Denmat, L., Lerouge, P., and Faye, L. (1998) Analysis of N- and O-glycosylation of plant proteins. In *Methods in Biotechnology: Recombinant Proteins from Proteins: Production and Isolation of Clinically Useful Compounds*, Vol. 3 (Cunningham, C., and Porter, A.J.R., Eds.), Humana Press, Totowa, NJ, pp. 271–289.

[17]   Jaikaran, A. S. I., Kennedy, T. D., Dratewka-Kos, E., and Lane, B. G. (1990) Covalently bonded and adventitious glycans in germin. *J. Biol. Chem.* **265**:12503–12512.

[18]   Lane, B. G. (1994) Oxalate, germin, and the extracellular matrix of higher plants. *FASEB J.* **8**:294–301.

[19]   Dunwell, J. M. (1998) Cupins: a new superfamily of functionally diverse proteins that include germins and plant storage proteins. *Biotechnol. Genet. Eng. Rev.* **15**:1–32.

[20]   Jensen-Jarolim, E., Schmid, B., Bernier, F., Berna, A., Kinaciyan, T., Focke, M., Ebner, C., Scheiner, O., and Boltz-Nitulescu, G. (2002) Allergologic exploration of germins and germin-like proteins, a new class of plant allergens. *Allergy* **57**:805–810.

[21]   Garcia-Casado, G., Sanchez-Monge, R., Chrispeels, M. J., Armetia, A., Salcedo, G., and Gomez, L. (1996) Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. *Glycobiology* **6**:471–477.

[22]   Batanero, E., Crespo, J. F., Monsalve, R. I., Martin-Esteban, M., Villalba, M., and Rodriguez, R. (1999) IgE-binding and histamine-release capabilities of the main carbohydrate component isolated from the major allergen of olive tree pollen *Ole e1*. *J. Allergy Clin. Immunol.* **103**:147–153.

[23]   Glick, B. R. and Pasternak, J. J. (2003) Molecular biotechnology biological systems. In *Molecular Biotechnology: Principles and Applications of Recombinant DNA*, 3rd Ed. (Glick, B. R. and Pasternak, J. J., Eds.), ASM Press, Washington, DC, pp. 14–17.

[24] Roy, N., and Agarwal, S. (2003) Therapeutic protein production--an overview. In *Business Briefing: Future Drug Discovery*, pp 79–82.

[25] Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. *Journal of biological chemistry* **193**:265–275.

[26] Higgins, D. R., and Cregg, J. M. (1998) Introduction to *Pichia pastoris*. *Methods Mol. Biol.* **103**:1–15.

[27] Cereghino, J. L., and Cregg, J. M. (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol. Rev.* **24**:45–66.

[28] Cregg, J. M., Cereghino, J. L., Shi, J., and Higgins, D. R. (2000) Recombinant protein expression in *Pichia pastoris*. *Mol. Biotechnol.* **16**:23–52.

[29] Whittaker, M. M., and Whittaker, J. W. (2002) Characterization of recombinant barley oxalate oxidase expressed by *Pichia pastoris*. *J. Biol. Inorg. Chem.* **7**:136–145.

[30] Stratton, J., Chiruvolu, V., and Meagher, M. (1998) High cell-density fermentation. *Methods Mol. Biol.* **103**:107–120.

[31] Gill, S. C., and von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**:319–326.

[32] Kaschnitz, R., Peterlik, M., and Weiss, H. (1969) Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.* **30**:148–152.

[33] Rayon, C., Lerouge, P., and Faye, L. (1998) The protein *N*-glycosylation in plants. *J. Exp. Bot.* **49**:1463–1472.

APPENDIX

A.1   Procedures

A.1.1 Transformation

An appropriate amount of plasmid DNA and 50 µL of XL2-blue cells were mixed well in a Falcon tube.   The tube was incubated on ice for 30 min and heat-pulsed in a 42°C water bath for 30 seconds.   The tube was then incubated on ice for 2 min.   NZY$^+$ broth (0.9 mL) was added and the tube was incubated at 37°C for 1 h with shaking at 225–250 rpm.

A.1.2 Mini prep purification

Bacterial cell cultures were plated on a fresh LBZ plate for two days grown.   The colonies on the streaked plate were resuspended in 250 µL of Buffer P1 (QIAGEN, Valencia, CA) and transferred to a microcentrifuge tube. Buffer P2 (250 µL) was added and the tube was gently inverted 4–6 times to mix. Buffer N3 (350 µL) was added and inverted immediately but gently 4–6 times. The tube was centrifuged for 10 min at maximum speed in a tabletop microcentrifuge.   The supernants were applied from the prior step to the QIAprep column by decanting or pipetting.   QIAprep spin column was washed by adding 0.75 mL of Buffer PE and centrifuged for 60 seconds.   The

flow-through was discarded, and the pellet was centrifuged for an additional 1 min to remove residual wash buffer.   Buffer BE (50 µL) was used to elute DNA to the center of each QIAprep column.   The column was allowed to stand for 1 min, and centrifuged for 1 min.   The DNA was transferred to a sterile microcentrifuge tube.

A.1.3 Midi prep purification

After transformation to *E. coli*, colonies were streaked onto a freshly made low salt LBZ plate and incubated for 2 days.   The colonies on the streaked plate were inoculated into a starter culture of 200 mL of LB medium containing 50 µL of 25 µg/ml Zeocin and grown at 37°C for 1 day with shaking at 225–250 rpm.   The bacterial cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C and supernants were discarded.   The bacterial pellet was resuspended in 5 mL of Buffer P1.   Buffer P2 (5 mL) was added and mixed gently with the pellet but thoroughly by inverting 4–6 times, and incubated at room temperature for 5 min.   Chilled Buffer P3 (5 mL) was added and mixed immediately but gently by inverting 4–6 times, and incubated on ice for 30 min. The mixture was centrifuged at 15,000 rpm for 30 min at 4°C.   Supernants containing plasmid DNA were removed promptly.   The QIAGEN-tip 100 was equilibrated by applying 4 mL of Buffer QT allowing the column to empty by gravity flow.   The supernants from the previous step were applied to the QIAGEN-tip and allowing it to enter the resin by gravity flow.   The QIAGEN-tip was washed with 2 × 10 mL of Buffer QC.   The DNA was eluted with 5 mL of Buffer QF.   The DNA was precipitated by adding 3.5 mL of

room-temperature isopropanol and centrifuged at 15,000 rpm for 30 min at 4°C.
The supernants were carefully discarded.   The DNA pellet was washed with 1.5
mL of room-temperature 70% ethanol and centrifuged at 9,000 rpm for 10 min.
The ethanol was carefully discarded without disturbing the pellet.   The wash
and centrifugation steps were repeated once more.   The ethanol was carefully
discarded without disturbing the pellet.   The DNA pellet was washed with 1.5
mL of room-temperature 70% ethanol again and the ethanol was discarded.
The pellet was air-dried for 10 min and redissolved in 50 μL of dH_2O.

A.2   Media Composition

BMGY medium:

> 10 g yeast extract and 20 g peptone in 600 mL H_2O

> 200 mL 0.5 M potassium phosphate pH 6.0 (26.4 mL 0.5 M K_2HPO_4 + 173.6
mL 0.5 M KH_2PO_4)

> 100 mL 10% glycerol (10 g glycerol + 90 mL H_2O)

> Autoclave all above

> 100 mL 10× YNB (17 g YNB without $(NH_4)_2SO_4$ + amino acid) + 50 g
$(NH_4)_2SO_4$ in 500 mL H_2O

> 2 mL of 0.02% (w/v) 500x Biotin

> Filter sterilize the prior two reagents

BMMY medium:

- ➢ 5 g yeast extract and 10 g peptone in 350 mL H_2O

- ➢ 100 mL 0.5 M potassium phosphate pH 6.0 (13.2 mL 0.5 M K_2HPO_4 + 86.8 mL 0.5 M KH_2PO_4)

- ➢ Autoclave

- ➢ 1 mL 500× Biotin

- ➢ 0.5% methanol

- ➢ Filter sterilize

FM22 medium (pH 4.5):

- ➢ Two 4 L flasks with 4 L H_2O in each:

  - ● 214.5 g KH_2PO_4

  - ● 25 g $(NH_4)_2SO_4$

  - ● 71.5 g K_2HPO_4

  - ● 28.6 g $MgSO_4$

  - ● Ca SO_4 ·2 H_2O

- ➢ Autoclave

PTM4 trace metal solution:

- ➢ 1 L H_2O + I mL concentrated H_2SO_4 with:

  - ● 2 g $CuSO_4 \cdot 5H_2O$

  - ● 0.08 g NaI

  - ● 3 g $MnSO_4 \cdot H_2O$

  - ● 0.2 g $Na_2MoO_4 \cdot 2H_2O$

- 0.02 g H_3BO_3

- 0.5 g $CuSO_4 \cdot 2H_2O$

- 0.92 g $CoCl_2 \cdot 6H_2O$

- 7.0 g $ZnCl_2$

- 22 g $FeSO_4 \cdot 7\ H_2O$

➢ Filter sterilize

NZY^+ broth:

➢ Per liter:

- 5 g yeast extract

- 5 g NaCl

- 10 g NZ amine (casein hydrolysate)

- Adjust the pH to 7.5 with NaOH

➢ Autoclave

➢ Add the following supplements before use:

- 12.5 mL of 1 M $MgCl_2$ and 12.5 mL of 1 M $MgSO_4$

- 10 mL of a 2 M filter-sterilized glucose solution or 20 mL of 20%

   (w/v) glucose

➢ Filter sterilize

YPD medium:

➢ 100 mL total volume:

- 1% yeast extract (10 g)

- 2% peptone (20 g)

- 2% glucose (20 g)

- Add dH$_2$O to a final volume of 100 mL

➢ Autoclave

A.3   Agar Plate Contents

Low salt LBZ agar plate:

➢ 500 mL total volume:

- 2.5 g yeast extract

- 5 g trptone

- 5 g NaCl

- 10 g agar

- Adjust pH to 7.0 with 5 N NaOH

- Add dH$_2$O to a final volume of 500 mL

➢ Autoclave

➢ Pour into Petri dishes (25 mL/100 mm plate)

YPDS/Z agar plate:

➢ 300 ml total volume:

- 55.2 g Sorbital (1 M)

- Zeocin (100 mg/mL)

- 3 g yeast extract

- 6 g glucose

- 6 g agar

- 6 g peptone

- 250 mL dH$_2$O

➢ Autoclave

➢ Pour into Petri dishes (25 mL/100 mm plate)

BIOGRAPHICAL SKETCH

Heng-Yen Pan was born on August 6, 1979, in Taipei, Taiwan, R.O.C.   In 2000, she began her college education in the Department of Biology, Southern Oregon University, Ashland, Oregon and graduating in 2004.   She was on the Dean's List from 2001 spring term to 2003 spring term, and received honors from the Vice President for Academic Affairs and Provost of SOU.

Her special interests are in protein expression and purification, and also on the study of salamanders.