RECOMBINANT EXPRESSION AND CHARACTERIZATION OF TWO ISOFORMS OF *ANOPHELES GAMBIAE* LACCASE-2

by

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Abstract

Laccases are multicopper oxidases that catalyze the oxidation of a broad range of substrates, typically phenols and anilines. Research on laccases in fungi, plants, and bacteria has indicated that they have roles in detoxification, pigmentation, wound healing, morphogenesis and lignin synthesis and degradation. However, there has been relatively little investigation on laccases that exist in insects or other invertebrates. Insects have multiple laccase genes, but the function of just one type is known; laccase-2 (Lac2) orthologs are required for tanning of newly synthesized exoskeleton. In the mosquito *Anopheles gambiae* and other insect species whose genomes have been sequenced, alternative exon splicing may generate two isoforms of Lac2. The objective of this study was to characterize the two isoforms of AgLac2. They are identical in their first 500 residues, but the carboxyl-terminal 262 residues derived from alternative exons are 81% identical. Recombinant Lac2A and Lac2B were expressed and purified. They are both glycoproteins of ~81 kDa, and both can oxidize the laccase substrate ABTS as well as the catechols, N-β-alanyldopamine (NBAD) and N-acetyldopamine (NADA). Lac2A and Lac2B with ABTS have pH optima of 5.0-5.5 and 4.5-5.0, respectively. The pH optima with NBAD and NADA are 5.5-6.5. The K_m values (mM) for Lac2A and Lac2B with NBAD are 5.4 ± 2.1 and 5.0 ± 2.6, respectively. The K_m values (mM) for Lac2A and Lac2B with NADA are 0.7 ± 0.2 and 1.4 ± 0.5, respectively. Thus, there is little difference between the isoforms in K_m for these two substrates. The K_m values do indicate that both isoforms have a greater affinity for the substrate NADA. The k_{cat} values (s^{-1}) for Lac2A and Lac2B with NBAD are 14.2 ± 3.5 and 6.0 ± 1.8, respectively. The k_{cat} values (s^{-1}) for Lac2A and Lac2B with NADA are 2.4 ± 0.2 and 0.5 ± 0.04, respectively. The most apparent difference between the two isoforms detected in the study is that Lac2A was four-fold more active than Lac2B when NADA was used as a substrate. Although the two isoforms are very similar in their amino acid sequences, the differences in catalytic properties may indicate different roles in insect physiology.

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Dedication

To my wonderful family, your love and support made it all possible.

CHAPTER 1 - Introduction

One of the first enzymes to ever be described was laccase from the lacquer tree, *Rhus vernicifera* in 1883 (Yoshida, 1883). Laccases, or para-diphenol:dioxygen oxidoreductaases (EC 1.10.3.2), are blue multi-copper enzymes that catalyze the oxidation of a broad range of substrates while simultaneously coordinating a four-electron reduction of molecular oxygen to water (Messerschmidt, 1997). Additional members of the blue oxidase family include ascorbate oxidase and mammalian plasma ceruloplasmin (Mayer and Staples, 2002). Included in the variety of substrates oxidized by laccase are polyphenols, methoxyphenols, anilines, diamines, and several inorganic compounds (Solomon et al., 1996; Piontek et al., 2002). For this reason, laccases have grabbed the attention of scientific researchers. It has been discovered that they have many applications for advancing biotechnological processes in industry. Some of these applications include color modification of food and wine, wood pulp bleaching and delignification, textile bleaching, cosmetic formulation, and biosensor development (Couto and Herrera, 2006). Laccases are also generating interest because of their potential impact of the environment. Currently, fungal laccases are being used in bioremediation for detoxification of industrial effluents as well as degradation of pesticides, herbicides, and some explosives found in soil (Mayer and Staples, 2002; Couto and Herrera, 2006). At present, scientific research is attempting to further progress to expand the applications of laccases.

Molecular structure and properties of laccases

The molecular architecture of laccase is very similar to other multi-copper oxidases, especially ascorbate oxidase. The glycoprotein structure is globular, consisting mainly of three domains all with a similar β-barrel type fold (Messerschmidt, 1997; Ducros et al., 1998; Bertrand et al., 2002; Hakulinen et al., 2002). The structure contains four copper atoms that have been categorized into three main types. The T1 site contains a type I copper that is situated in a trigonal coplanar geometric orientation. This is due to its coordination to several amino acids, specifically two histidines and a cysteine (Piontek

et al., 2002). These particular bonds to copper have very distinct spectroscopic properties. The bond linking type I copper and cysteine results in intense absorption around 600 nm, which is responsible for the blue color that is a common property of proteins in the multi-copper oxidase family. The T2 site contains a type II copper with a characteristic electron paramagnetic resonance (EPR), a technique that detects chemical species that have unpaired electrons. In the T3 site, the pair of type III coppers generates an absorption band at 330 nm, but does not exhibit an EPR signal in the presence of molecular oxygen because of a strong antiferromagnetic coupling interaction between the two type III copper atoms (Solomon et al., 1996; Gray et al., 2000; Enguita et. al, 2003). When a reducing substrate is present, the mononuclear T1 site removes electrons from it and orchestrates their transfer to the trinuclear T2/T3 center where molecular oxygen is reduced to water (Messerschmidt, 1997).

Evolution and function of laccases

The molecular evolution of laccases is still not completely known, but through analysis of the growing collection of sequences and X-ray crystal structures of proteins in the multi-copper oxidase family, it is hypothesized that the origin is a single-domain cupredoxin protein. Duplication and modifications of the single domain are thought to have possibly led to the creation of present day multicopper oxidases (Nakamura and Go, 2005). "They [copper proteins] describe an evolutionary history that spans nearly the entire period of life on Earth and involves prokaryotes, fungi, plants, and animals, whereas most of the other groups studied are confined to evolution of animal life" (Ryden and Hunt, 1993).

Studies of laccases in several organisms have shown that they participate in diverse physiological processes. To date, fungi provide the greatest source of laccases and therefore have been the most widely studied. The ligninolytic activity of white rot fungi, such as *Pycnoporus cinnabarinus* and *Trametes versicolor*, has been attributed to the laccases found in these species, which grow on rotting wood (Bourbonnais et al., 1995; Eggert et al, 1996). In several fungi, laccases are involved in pigmentation and morphogenesis. The fungus, *Aspergillus nidulans* produces a laccase that engages in the formation of conidium green color (Clutterbuck, 1972). In *Schizophyllum commune* and

Agaricus bisporus, laccases have been implicated in aiding fruit body formation (Thurston, 1994). *Cryptococcus neoformans*, a fungal pathogen that infects people with compromised immune systems, produces laccase that is a virulence factor. This fungal laccase is thought to convert host catecholamines into melanin, which protects *C. neoformans*, allowing it to cause more damage to the host (Zhu and Williamson, 2004).

Laccase genes have been identified in a few bacterial species. Laccases are responsible for spore coat formation in *Bacillus subtilis* and melanin synthesis in *Azospirillum lepoferum* (Faure et al., 1994; Claus, 2003; Enguita et al., 2003). In plants, laccases play a part in lignification of cell walls and wound response (Bao et al., 1993; Mayer and Staples, 2002). There has been little biochemical study on laccases that exist in insects or other invertebrates. However, laccase has been demonstrated to function in sclerotization and tanning of the new exoskeleton that occurs after insect molting (Dittmer et al., 2004; Arakane et al., 2005; Anderson, 2007).

Insect laccases

Analysis of several insect species whose genomes have been sequenced show the existence of two laccase genes in *Bombyx mori* and *Tribolium castaneum*, four in *Drosophila melanogaster*, and five in *Anopheles gambiae* and *Aedes aegypti* (Gorman et al., 2008). Although its genome has yet to be sequenced, *Manduca sexta* appears to have two laccases as well. In *A. gambiae* and these other insect species, there are apparently two isoforms of laccase-2 (Lac2A and Lac2B), arising from alternative exon splicing (Dittmer et al., 2004; Gorman et al., 2008) (Figure 1). Recently, through the use of the RNA interference technique, the laccase-2 transcript was knocked down in *T. castaneum*. As a result, beetles injected with TcLac2A dsRNA had soft cuticles that displayed little or no tanning in the pupal or adult stages, and beetles injected with TcLac2B dsRNA showed a delay in adult cuticle tanning. Both dsRNA treatments were lethal, showing that even though the two laccase-2 isoforms may have different roles, they are critical to the life of the insect (Arakane et al., 2005).

Comparison of *A. gambiae* laccase-2 with orthologous sequences available in GenBank show greater than 84% sequence similarity. Upon closer examination, specifically of the two isoforms, there exists high sequence similarity between the

isoforms within each species and an even greater sequence similarity among A isoforms and among B isoforms from the various insect species (Arakane et al., 2005).

Anopheles gambiae

A. gambiae mosquitoes are insects that belong to the family Culicidae in the order Diptera. They have a life cycle that proceeds through four stages, which are egg, larva, pupa, and adult. The adults can be distinguished from other species by the palps, which are as long as the proboscis, and by the presence of black and white scales on the wings. These mosquitoes are classified as anthropophilic, since the females prefer to feed on humans for blood meals (www.cdc.gov/malaria/biology/mosquito/). They are found in Sub-Saharan Africa where they are the primary vector of the malaria parasite, *Plasmodium* (Kiszewski et al., 2004). For this reason, biochemical studies of *Anopheles* mosquito enzymes, such as laccase, are essential for aiding in the development of new insecticides or other control strategies.

Specific aims

It has been determined that the AgLac2 isoforms are identical in their first 500 residues, but the carboxyl-terminal 262 residues derived from alternative exons are 81% identical. They have been largely conserved through evolution leading to the hypothesis that both have very important, but different physiological roles in the *Anopheles gambiae* mosquito.

The first aim of this research was to express the two isoforms of *Anopheles gambiae* laccase-2 as recombinant proteins and purify them.

The second aim was to determine substrate specificity of the two isoforms of laccase-2 by analyzing the activity of these enzymes in oxidizing the substrate 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), and with the catechols *N*-β-alanyldopamine (NBAD) and *N*-acetyldopamine (NADA) (Figure 2), which are likely natural substrates for laccase-2.

Based on homology modeling results, I developed a hypothesis that the catechol, NADA, may have a greater affinity as a substrate for the recombinant A isoform of *A.*

gambiae laccase-2 (rAgLac2A), while the catechol, NBAD, may have a greater affinity as a substrate for the recombinant B isoform of *A. gambiae* laccase-2 (rAgLac2B).

CHAPTER 2 - Materials and Methods

Homology modeling

AgLac2 protein target sequences were used as input into an automated web-based homology modeling program known as SWISS-MODEL. SWISS-MODEL performed a BLAST search for a protein of known structure to be used as the template (Peitcsh, 1995; Guex and Peitcsh, 1997; Arnold et al., 2006). The template and target sequences were next submitted to the web-based sequence alignment program, ClustalW (Larkin et al., 2007). The aligned sequences were returned to SWISS-MODEL for production of a homology model of each AgLac2 isoform. Each model data file was then uploaded into the Protein Explorer website for viewing and manipulation (http://molvis.sdsc.edu/protexpl/frntdoor.htm).

Construction of recombinant baculoviruses

Recombinant AgLac2A and AgLac2B baculoviruses were generated with the BAC-TO-BAC Expression System (Invitrogen). cDNAs containing the full coding regions of AgLac2A and AgLac2B cloned into the pCR4 vector were obtained from Maureen Gorman in the Department of Biochemistry, Kansas State University. AgLac2A and AgLac2B plasmid DNA was used to transform *E. coli* strain TOP10 (QIAGEN). Common molecular biology techniques were carried out as described in Molecular Cloning: A Laboratory Manual – Volume 1 (Sambrook and Russell, 2001). AgLac2A and AgLac2B plasmid DNA purified from the *E. coli* cells using a QIAprep Spin Miniprep Kit (QIAGEN) was quantitated by measuring absorbance at 260 nm. Identity of the plasmids was verified by restriction endonuclease digestion (EcoRI, Not I, Cla I). The AgLac2A and AgLac2B plasmid DNA and a pFASTBAC vector were then subjected to digestion by EcoRI, and the fragments were isolated by agarose gel electrophoresis, then purified from agarose using a QIAquick Gel Extraction Kit (QIAGEN). AgLac2A or AgLac2B DNA was ligated into the pFASTBAC vector and used to transform *E. coli* strain TOP10. Eight AgLac2A colonies and eight AgLac2B colonies were selected and cultured overnight at 37ºC in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/mL) for verification of the correct orientation of

insertions of the cDNAs by restriction endonuclease digestion (EcoRI, Not I, Kpn I). The recombinant plasmids with desired orientation of cDNA insertions were used to transform *E. coli* strain DH10BAC, which contains a bacmid (a plasmid containing a baculovirus genome) with a mini-*att*Tn7 target site and a helper plasmid encoding transposase. The transformed bacteria were streaked onto LB agar plates supplemented with kanamycin (50 μg/mL), gentamicin (7 μg/mL), tetracycline (10 μg/mL), X-gal (100 μg/mL), and IPTG (40 μg/mL). The plates were incubated at 37ºC for 24-48 hours. Colonies containing recombinant bacmids were identified by disruption of the lacZα gene and resulting white color. From a single AgLac2A or AgLac2B colony confirmed as having a white phenotype on agar plates containing X-gal and IPTG, a liquid culture (3 mL) contained antibiotics (kanamycin, gentamicin, and tetracycline) was incubated at 37ºC with shaking overnight. The High Purity Miniprep System (Marligen Biosciences) was used for isolating bacmid DNA from the selected *E. coli* clones. PCR analysis was used to verify successful transposition of the laccase constructs to the bacmid.

Sf9 cells (1 x 10^6 cells/mL serum-free medium [Sf-900 II SFM]) were transfected with the recombinant bacmid DNA using CELLFECTIN Reagent (Invitrogen). After amplification of virus stocks, plaque assays were implemented to determine titers (BAC-TO-BAC Baculovirus Expression Systems Instruction Manual – Invitrogen). Successful transposition of AgLac2A to the bacmid was verified by PCR, using forward primer specific for a sequence in exon 8A 5' – CAC CGG ACT CGA ACG TCA – 3', and a reverse primer (RP 014 for pBK-CMV and pBluescript) that anneals to the vector 5' – CAC ACA GGA AAC AGC TAT GAC C – 3'. The final product size was 900 base pairs. To verify successful transposition of AgLac2B to the bacmid, PCR was done using 5' – CGC TGG AGA TCT TCA CCA TT – 3', which is a forward primer in exon 4 of the AgLac2 sequence and 5' – TGA TCT TCT TCA CCG TGC T – 3', which is a reverse primer specific for a sequence in exon 8B. The final product size was 867 base pairs.

DNA electrophoresis

For electrophoretic analysis of DNA, 0.8% w/v agarose gels with 2 μL ethidium bromide (10 μg/μL) were submerged in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) and run at a constant voltage (100 V). DNA bands were visualized

by exposure to ultra-violet light using the Electrophoresis Documentation and Analysis System 120 equipment and recorded with KDS1D 2.0 software.

Expression and purification of recombinant proteins

For expression, 1.5 liters of Sf9 cells (2 x 10^6 cells/mL serum-free medium [Sf-900 II SFM]) were infected with baculovirus at a multiplicity of infection of 2, and cells were incubated at 28ºC with shaking (150 rpm) for 48 hours. Cells were removed by centrifugation (600 rpm for 15 min), followed by vacuum filtration of the supernatant using a sterile 0.20 µm membrane filter. To help inhibit proteases in the clarified medium, *p*-aminobenzamidine (Sigma) was added to a concentration of 0.5 mM.

Purification of rAgLac2A and rAgLac2B began with binding of glycoproteins in the culture medium to Concanavalin-A Sepharose (GE Healthcare) (34 mL at 60% slurry for rAgLac2A, 46 mL at 60% for rAgLac2B) in a shaking incubator (200 rpm, 4ºC) for approximately eight hours. The resin, after separation from the culture medium, was poured into a 2.5 cm diameter column at 4ºC. The column was washed and packed by gravity flow with binding buffer (20 mM Tris-HCl, pH 7.5 (4ºC), 500 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$) until the flow through had an absorbance reading of <0.01 at 280 nm. Laccase was eluted with 0.5 M methyl-α-D-mannopyranoside in 80 mL of 20 mM Tris-HCl, pH 7.5 (4ºC), 0.5 M NaCl, and protease inhibitor cocktail diluted 1:200 (for tissue culture media, DMSO solution – Sigma P1860). Pooled fractions containing recombinant laccase were dialyzed twice against 4 liters of 20 mM Tris-HCl, pH 8.0 (4ºC), 0.5 mM *p*-aminobenzamidine. A 1.0 cm diameter (3.0 cm packed height) Q-Sepharose (GE Healthcare) chromatography column was prepared by packing the resin with 20 mM Tris-HCl, pH 8.0 (4ºC) using a flow rate of 4.0 mL/min. The dialyzed pooled fractions containing recombinant laccase were loaded onto the Q-Sepharose column using a flow rate of 1.0 mL/min. Next the column was washed with 20 mM Tris-HCl, pH 8.0 (4ºC) until the wash fractions had an absorbance reading of <0.01 at 280 nm. Proteins were eluted as 1 mL fractions with an 80 mL linear gradient of NaCl in 20 mM Tris-HCl (0-300 mM NaCl), pH 8.0 (4ºC), protease inhibitor cocktail diluted 1:200 (for tissue culture media, DMSO solution – Sigma). Finally, fractions containing rAgLac2A

protein were pooled. The same was performed with fractions containing rAgLac2B protein.

Protein Analysis

The presence of protein throughout the purification process was detected using a spectrophotometer to measure absorbance at 280 nm, while protein concentration was determined as follows. For the protein concentration assay, a bovine serum albumin (BSA) stock solution (0.2 mg/mL) was used to set up 50 µL of each of the following standard concentrations: 0.2 µg/µL, 0.4 µg/µL, 0.6 µg/µL, 0.8 µg/µL, 1.0 µg/µL, and 1.2 µg/µL. Each standard was loaded onto a 96-well plate along with samples collected throughout the purification process. To each well, 50 µL of Coomassie Plus Protein Reagent (Pierce) was added and mixed using a pipette. After incubation for 10 minutes at room temperature, absorbance was measured at 595 nm (Bio-Tek Instruments Power Wave$_x$). The standard values were utilized to create a standard curve for determination of unknown protein concentration by a computer program called KC Junior.

For SDS-PAGE, samples from fractions collected during the purification process were combined with 6X SDS sample loading buffer (350 mM Tris-HCl, pH 6.8, 10% SDS, 30% glycerol, 0.125% bromophenol blue) and heated at 95ºC for five minutes. The samples were then loaded onto a NuPAGE 4-12% Bis-Tris SDS polyacrylamide gel (Invitrogen) in 1X 3-[N-morpholiono] propanesulfonic acid (MOPS) buffer (Invitrogen) and electrophoresed at a constant voltage (200 V) until the bromophenol blue dye reached the bottom of the gel (approximately one hour). The gel was then transferred to Coomassie blue staining solution for at least one hour before destaining in 10% acetic acid, 30% methanol. The destaining solution was changed approximately every hour until blue bands of protein were visualized against a clear gel background.

The same SDS-PAGE procedure was followed for Western blot analysis, except that when electrophoresis was finished, the gel was equilibrated twice in Western transfer buffer (48 mM Tris, pH 9.2, 39 mM glycine, 1.3 mM SDS, 20% methanol) for 10 minutes each time and then put onto a semi-dry electrophoretic transfer cell (Bio-Rad), where proteins were transferred onto a 0.45 µm nitrocellulose membrane (GE Water and Process Technologies) for 1.5 hours at a constant voltage (10 V). The membrane was

then stained with Ponceau S for two minutes to visualize the protein size marker, Mark 12 (Invitrogen) and rinsed with distilled deionized water (ddH_2O) until clear again. The membrane was subsequently washed three times with 1X TBST (25 mM Tris, pH 7.4, 137 mM NaCl, 2 mM KCl, 0.05% (v/v) Tween 20) for five minutes each and then incubated in 3% dry milk in 1X TBST for one hour. The membrane was washed again as before and incubated for one hour in affinity-purified *Manduca sexta* laccase-2 antibody (provided by Neal Dittmer, Department of Biochemistry, Kansas State University) at a dilution 1:1000 in 3% dry milk in 1X TBST. The membrane was washed again as previously described and then incubated for one hour in alkaline phosphatase (AP) conjugated goat anti-rabbit secondary antibody (Bio-Rad) at a dilution of 1:3000 in 3% dry milk in 1X TBST. The membrane was washed again as before, developed using an AP conjugate substrate kit (Bio-Rad), and rinsed twice with water for five minutes.

Enzyme assays and kinetics

To detect the presence of recombinant laccase in column fractions, an assay was performed in 100 mM citric acid-sodium citrate buffer (pH 4.5). The assay used 50 µL of collected fraction sample in a 200 µL total volume containing 0.5 mM ABTS in a 96-well plate. The plate was wrapped in aluminum foil and left undisturbed for 6-8 hours. An endpoint absorbance reading at 414 nm was then measured to detect the oxidized product.

To determine optimum pH, assays were performed in 100 mM citric acid-sodium citrate buffer. Each assay contained 250 ng protein and 0.5 mM substrate in a 200 µL total volume. Assays were set up in a 96-well plate, and production of oxidized product was monitored at 414 nm for ABTS and 390 nm for NADA and NBAD (National Institute of Mental Health). The molar absorption coefficient of ABTS stable radical is 64,000 cm^{-1} M^{-1} and the absorption coefficient of NADA and NBAD quinones is 1100 cm^{-1} M^{-1} (Thomas et al., 1989). For the substrate ABTS, one unit of activity was defined as 0.001 ΔA_{414}/min/mg protein. For NADA and NBAD, one unit of activity was defined as 0.001 ΔA_{390}/min/mg protein.

To characterize substrate specificity, assays were performed in 100 mM citric acid-sodium citrate buffer (pH 6.0). Each assay contained 250 ng protein and a select substrate concentration (0.1 mM, 0.2 mM, 0.4 mM, 0.6 mM, 1 mM, 2 mM, 4 mM, 6 mM)

in a 200 μL total volume. Assays were set up in a 96-well plate and production of the oxidized quinones was monitored for 30 minutes at 390 nm for NADA and NBAD. One unit of activity was defined as 0.001 ΔA_{390}/min/mg protein.

CHAPTER 3 - Results

Homology modeling

Of the known crystal structures of laccases, SWISS-MODEL identified *Trametes versicolor* laccase (TvLacIIIb) (PDB accession number: 1KYA) as most similar in sequence with AgLac2. ClustalW was used to create an alignment of TvLacIIIb and the AgLac2 isoforms and determine the extent of their sequence identity. AgLac2A and TvLacIIIb are 32% identical, while AgLac2B and TvLacIIIb are 33% identical (Figure 3). The isoforms of AgLac2 are 100% identical in their first 500 residues, but the carboxyl-terminal 262 residues derived from alternate exons are 81% identical. Based upon this sequence alignment input, SWISS-MODEL produced a homology model for each of the isoforms of AgLac2, but without the first 240 N-terminal residues, since they do not align with the fungal enzyme (Figure 4). The models predict that each isoform has three domains. Both AgLac2 isoforms also have four additional surface loops not present in TvLacIIIb, due to insertions that are not in the fungal sequence. These insertions are at residues 501-518, 542-562, 588-595, and 672-687. Comparison of the substrate-binding site in the AgLac2 isoforms with that of TvLacIIIb shows a similar hydrophobic pocket and conservation of the histidine residue 458 in TvLacIIIb and 718 in the AgLac2 isoforms that interacts with the single copper and the substrate (Figure 5). Most of the residues in AgLac2A and 2B that align with the substrate-binding residues of TvLacIIIb do not differ between isoforms. Previous structural analysis of TvLacIIIb with other multicopper oxidases has revealed a loop region possibly responsible for substrate-binding specificity (Larrondo et al., 2003). Analysis of this region found in domain 3 in the two isoforms suggests it as a putative substrate-binding loop. Looking at this loop shows a difference at residue 633, a proline in AgLac2A and glutamic acid in AgLac2B (Figure 6).

Upon further examination of this difference, a ClustalW sequence alignment of the alternate exon 6-9 regions for laccase in *Anopheles gambiae*, *Aedes aegypti*, *Drosophila melanogaster*, *Bombyx mori*, *Tribolium casteneum*, and *Manduca sexta* revealed that the difference between Lac2 isoforms at residue 633 is not conserved among the different insect species. However this alignment does show there are

differences between isoforms at residue 544 and residue 728 that are conserved between insect species. At residue 544, this difference is tyrosine in Lac2A and isoleucine in Lac2B, with the exception of BmLac2B having a substitution of methionine instead. At residue 728, the conserved difference is glutamine in Lac2A and histidine in Lac2B (Figure 7).

Expression and purification of rAgLac2 isoforms

In order to characterize AgLac2, the recombinant proteins AgLac2A and AgLac2B were expressed in insect cells using a baculovirus expression system. Recombinant AgLac2A and AgLac2B were secreted into the cell culture medium, an indication that their own signal peptides were functional in this system. The molecular mass of both isoforms determined by SDS-PAGE analysis was ~81 kDa. rAgLac2A and rAgLac2B bound to a Conconavalin A chromatography column, indicating that they are glycosylated (Figures 8 and 10). The Con A chromatography step yielded a 15-fold increase in specific activity for rAgLac2A and 25-fold increase for rAgLac2B.

The predicted isoelectric point of AgLac2A is 6.25 and 6.72 for AgLac2B (ExPasy). The combined Con A fractions were applied to a Q-Sepharose chromatography column at pH 8.0, and the enzymes were eluted using a NaCl gradient (Figures 9 and 11). The combined fractions yielded an additional three-fold increase in specific activity for rAgLac2A and rAgLac2B (Table 1). A total of ~120 μg rAgLac2A (15 ng/μL) and ~40 μg rAgLac2B (5 ng/μL) was purified from 1500 mL medium (Figure 12).

rAgLac2 enzyme activity

When assayed with ABTS as a substrate, rAgLac2A had a pH optimum of 5.0-5.5 and rAgLac2B had a pH optimum of 4.5-5.0 (Figure 13A). When assayed with NADA or NBAD as a substrate, Lac2A and Lac2B have pH optima of 5.5-6.5 (Figures 13B and 13C).

NBAD and NADA are naturally occurring compounds found in mosquitoes and potential physiological substrates for Lac2 (Munkirs et al., 1990; Kerwin et al., 1999). Michaelis-Menten kinetics were observed with NBAD and NADA as substrates for both

of the recombinant enzymes (Figure 14). The K_m values (mM) for Lac2A and Lac2B with NBAD are 5.4 ± 2.1 and 5.0 ± 2.6, respectively. The K_m values (mM) for Lac2A and Lac2B with NADA are 0.7 ± 0.2 and 1.4 ± 0.5, respectively. Thus, there is little difference between the isoforms in K_m for each substrate. The K_m values do indicate that both isoforms have a greater affinity for the substrate NADA. The k_{cat} values (s^{-1}) for Lac2A and Lac2B with NBAD are 14.2 ± 3.5 and 6.0 ± 1.8, respectively. The k_{cat} values (s^{-1}) for Lac2A and Lac2B with NADA are 2.4 ± 0.2 and 0.5 ± 0.04, respectively. The most apparent difference between the two isoforms detected in this study is that Lac2A was four-fold more active than Lac2B when NADA was used as a substrate (Table 2).

CHAPTER 4 - Discussion

The discovery of laccase in insects has brought even more intrigue to this very diverse and long studied enzyme. RNAi experiments in the red flour beetle, *T. castaneum*, establishing that cuticle sclerotization can be added to the repertoire of roles to which laccase is involved (Arakane et al., 2005), has fueled the growing intention to ascertain more information about the enzyme. The identification of laccase genes in the *A. gambiae* mosquito, an insect with such an impact on the health of the world's populace today, has helped lead to this exploration of new possible hypotheses regarding insect laccases. The goals of this research were to generate homology models of the *A. gambiae* laccase-2 isoform structures, express a recombinant form of each AgLac2 isoform and compare their biochemical properties.

The alternative method of homology modeling was applied to generate models of the AgLac2 isoform structures since the three-dimensional structures of AgLac2 are not yet available. SWISS-MODEL in combination with alignment information provided by ClustalW was used to produce a model of each isoform based upon the known crystal structure of the fungal laccase, TvLacIIIb. With the sequence identity between the AgLac2 targets and TvLacIIIb template being in the 30-50% range, the models can be considered reliable, with majority of possible inaccuracies being located in the loops generated from the four sequence insertions not found in the fungal sequence (Baker and Sali, 2001).

A closer look at the homology models provides some ideas about the different substrates that could be accommodated by the enzymes. In the substrate cavity, the residues in AgLac2A and 2B that align with the substrate-binding residues of TvLacIIIb do not differ between isoforms. Several residues could have hydrophobic interactions with a substrate. The charged residue Asp 206 that is conserved among fungal laccases has been replaced by the corresponding hydrophobic residue (Val 397) in the AgLac2 isoforms. However, two polar residues Thr 347 and Ser 568 replace the corresponding hydrophobic residues (Leu 164 and Phe 337) of TvLacIIIb. The charged residue (His 458) of the fungal laccase that coordinates with the copper that acts as the primary

electron acceptor is conserved (His 718) in the AgLac2 isoforms (Bertrand et al, 2002). Pairing this information with the knowledge that laccases typically have a low substrate specificity, a wide variety of phenols and aromatic amines could be suggested for oxidation by the AgLac2 enzymes (Messerschmidt, 1997). Also, a number of various sized molecules could fit if the substrate cavity is as wide as that of TvLacIIIb. Substrate selectivity of the AgLac2 isoforms, however, may be increased by the presence of the two surface loops positioned near the substrate-binding cavity. These could also be involved with the previous suggestion that there is a ligand-induced fit of laccases (Bertrand, 2002). Currently, there is no explanation for the presence of the other two surface loops on the opposite side of the AgLac2 isoforms.

Redox potential of the compounds that might be possible substrates needs to be considered as well. Studies have recently shown evidence to support the suggestion that laccases better oxidize compounds with lower redox potentials (Tadesse et al., 2007). Examination of the T1 active site in laccases has found that higher redox potential is correlated with the presence of more hydrophobic residues (Matera et al., 2008).

Leading to the development of the hypothesis for this research was the examination of the putative substrate-binding loop in the AgLac2 isoforms. Its location can be considered reliable due to its position near the substrate-binding cavity in the models as well as being near well conserved regions of sequence containing histidine residues that interact with the different coppers. The only difference between the two isoforms found in the putative substrate-binding loop was residue 633, which is proline (a hydrophobic residue) in AgLac2A and glutamic acid (a charged polar residue) in AgLac2B. Although this difference is not conserved between Lac2 isoforms among the different insect species, it is the only major variation located near the substrate-binding site. Compounds NADA and NBAD are not only diphenols, but also naturally occurring in *Aedes aegypti* and *Toxorhynchites amboinensis* mosquitoes (Munkirs et al., 1990; Kerwin et al., 1999). Combining this information with that provided by the homology models, a conclusion can be draw that these compounds hold high potential as substrates of the AgLac2 isoforms. Thus, next the hypothesis that NADA may have a greater affinity as a substrate for the recombinant A isoform of *A. gambiae* laccase-2 (rAgLac2A), while NBAD may have a greater affinity as a substrate for the recombinant

B isoform of *A. gambiae* laccase-2 (rAgLac2B). It is possible that the uncharged compound NADA will interact with the hydrophobic Pro 633 residue in rAgLac2A and the positive charge on NBAD will interact with the negative charge of Glu 633.

As homology models can provide only a limited amount of information, it was necessary to express the two isoforms of laccase-2 in *A. gambiae* as recombinant proteins and purify them to further explore this hypothesis. The recombinant AgLac2 proteins were produced by means of a baculovirus expression system in Sf9 insect cells. As determined by SDS-PAGE, both recombinant isoforms had a molecular mass of ~81 kDa. However, three bands consistently appeared on Western blots of purified fractions of each isoform. This leads to the question, why are there three bands? It has already been indicated that rAgLac2A and 2B are glycosylated due to their ability to bind to a Conconavalin A column. Therefore, it is possible that the different bands are from various levels of glycosylation. Another prospect to consider is degradation, despite the various protease inhibitors used throughout the purification process. To determine if this is indeed the case, Edman degradation would need to be performed on each of the bands so that the N-terminus can be examined.

Having partially purified preparations of each recombinant isoform, it was time to delve into the investigation of their specific biochemical properties. The pH profile with ABTS as a substrate indicates that rAgLac2A has optimal activity at pH 5.0-5.5 and rAgLac2B has optimal activity at pH 4.5-5.0. The pH profile with NADA and NBAD as substrates indicates that Lac2A and Lac2B have optimal activity at pH 5.5-6.5. These results are consistent with previous studies on insect laccases, such as the fruit fly, *Drosophila melanogaster* (pH 6.5), silkworm, *Bombyx mori* (pH 5.5), desert locust, *Schistocerca gregaria* (pH 5.0), blowfly, *Calliphora vicina* (pH 4.5), sheep blowfly, *Lucilia cuprina* (pH 4.5), green rice leafhopper, *Nephotettix cincticeps* (pH 4.75), and tobacco hornworm, *Manduca sexta*, (pH 5.0) (Yamazaki, 1969; Yamazaki, 1972; Andersen, 1978; Barrett and Andersen, 1981; Barrett, 1987; Sugumaran et al., 1992; Hattori et al., 2005; Dittmer et al., 2009).

Michaelis-Menten kinetics were observed with NADA and NBAD as substrates for both of the recombinant enzymes. The K_m values for Lac2A and Lac2B with NADA are 0.7 mM and 1.4 mM, respectively. The results for rAgLac2A are similar to the

observed values for the laccases from the blowfly, *C. vicina* (K_m = 0.53 mM) and tobacco hornworm, *M. sexta* (K_m = 0.43 – 0.63 mM) (Barrett and Andersen, 1981; Dittmer et al., 2009). The results for rAgLac2B are similar to the value reported for the laccase from the desert locust, *S. gregaria* (K_m = 1.3 mM) (Andersen, 1978). The K_m values for Lac2A and Lac2B with NBAD are 5.4 mM and 5.0 mM, respectively. The results for both isoforms are higher than the values detected for both the recombinant and endogenous forms of MsLac2 (K_m = 1.90 mM and K_m = 0.47 mM, respectively). However, the substrate inhibition with NBAD observed with MsLac2 (Neal Dittmer – personal communication) did not occur with the rAgLac2 isoforms. A comparison shows there is little difference between the isoforms in K_m for each substrate, but that both Lac2 isoforms have a greater affinity for the substrate NADA than for NBAD.

The k_{cat} values for Lac2A and Lac2B with NADA are 2.4 s^{-1} and 0.5 s^{-1}, respectively. The k_{cat} values for Lac2A and Lac2B with NBAD are 14.2 s^{-1} and 6.0 s^{-1}, respectively. This kinetic parameter points to Lac2A being four-fold more active than Lac2B when NADA was used as a substrate and two-fold more active than Lac2B when NBAD was used as a substrate. This could suggest that the AgLac2A is possibly the dominant isoform, as was found to be the case in the RNA interference studies of TcLac2 (Arakane et al., 2005). On the other hand, this difference may still point toward the two isoforms each playing different roles in the mosquito's physiology. Analysis of k_{cat}/K_m indicates that neither isoform can oxidize NADA or NBAD with a very high catalytic efficiency.

When merged together, it can be concluded that these results do not support the hypothesis that NADA has a greater affinity as a substrate for rAgLac2A, while NBAD has a greater affinity as a substrate for rAgLac2B. So what paths can be taken from here? Other than its role in cuticle sclerotization, little is really known about what other functions laccase may have in insects. There are still a number of different compounds that can be looked at in further substrate specificity studies for *A. gambiae* laccase-2. Also, this research only looked at each isoform individually. When performing assays on other compounds, the two isoforms could be combined to determine whether or not they can act synergistically. The purpose of two isoforms of laccase-2 being present in the *A. gambiae* mosquito as well as several other insect species is still unknown. The more data

that can be accumulated about the biochemical properties of insect laccases, the likelihood of being able to design insecticides specific to these insect laccases will significantly increase.

	Activity (U/µL) (U=mOD/min)	**Total Volume** (mL)	**Total Activity** (U)	**Protein Concentration** (mg/mL)	**Total Protein** (mg)	**Specific Activity** (U/mg)	**% Yield Activity**	**Fold Purification**
rAgLac2A Media	0.023	1450	33988	0.30	436	78	100	1
rAgLac2A Con A	0.205	75	15363	0.17	12.5	1230	45	16
rAgLac2A Q column	0.459	8	3668	0.10	0.81	4540	24	58
rAgLac2B Media	0.006	1475	8437	0.39	580	15	100	1
rAgLac2B Con A	0.046	80	3658	0.12	9.6	381	43	25
rAgLac2B Q column	0.127	8	1020	0.08	0.67	1517	28	101

Table 1 – Summary for purification of recombinant AgLac2 isoforms. rAgLac2 isoforms were expressed using a baculovirus vector in Sf9 cells infected at a MOI = 2 for 2 days. rLac2 secreted from the cells was purified from media by affinity chromatography on Concanavalin A Sepharose, followed by anion exchange chromatography (Q Sepharose). Specific activity significantly increased with each purification step yielding relatively pure rAgLac2A and 2B.

Enzyme	Substrate	V_{max} (mM min^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
rAgLac2A	NBAD	28.2 ± 6.3	5.4 ± 2.1	14.2 ± 3.5	2.6
rAgLac2B	NBAD	11.8 ± 3.5	5.0 ± 2.6	6.0 ± 1.8	1.2
rAgLac2A	NADA	4.7 ± 0.3	0.7 ± 0.2	2.4 ± 0.2	3.4
rAgLac2B	NADA	0.9 ± 0.08	1.4 ± 0.5	0.5 ± 0.04	0.4

Table 2 – **Summary of enzyme kinetics**. Enzyme assays were performed with 250 ng of purified laccase-2 and various concentration of NBAD or NADA to provide the measured kinetic values of maximal velocity (V_{max}), the Michaelis constant (K_m), catalytic constant (k_{cat}), and catalytic efficiency (k_{cat}/K_m).

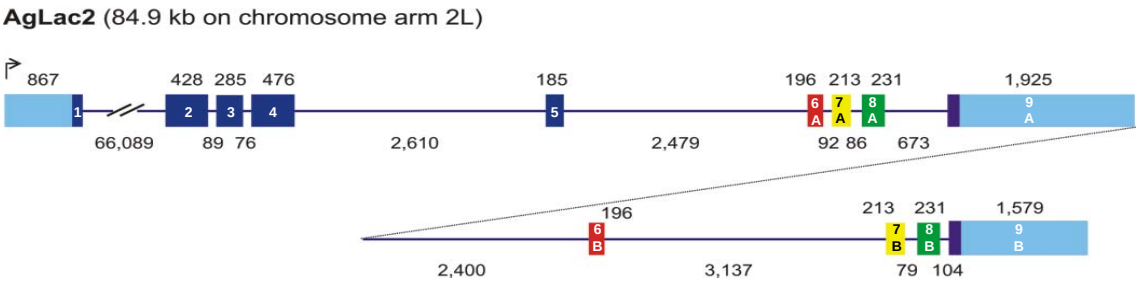
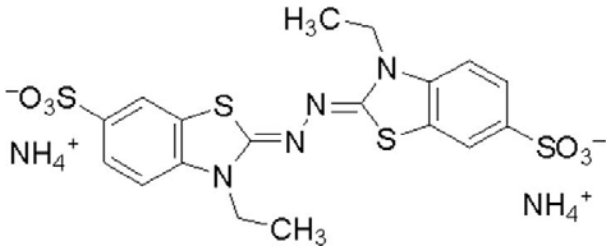


Figure 1 – *Anopheles gambiae* laccase gene structure. Exons are indicated by rectangles with the length shown above them. Introns are indicated by lines with the length shown below them. Light blue regions correspond to untranslated sequences. Color coding for Lac2 indicates alternate exons 6-9. (Figure courtesy of Maureen Gorman, Department of Biochemistry, Kansas State University).

A



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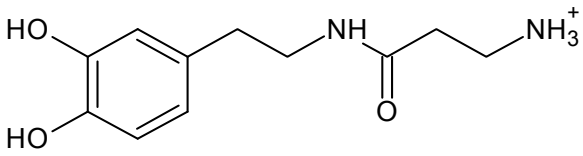

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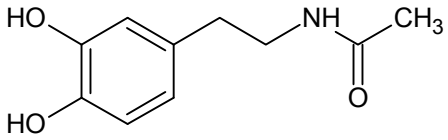
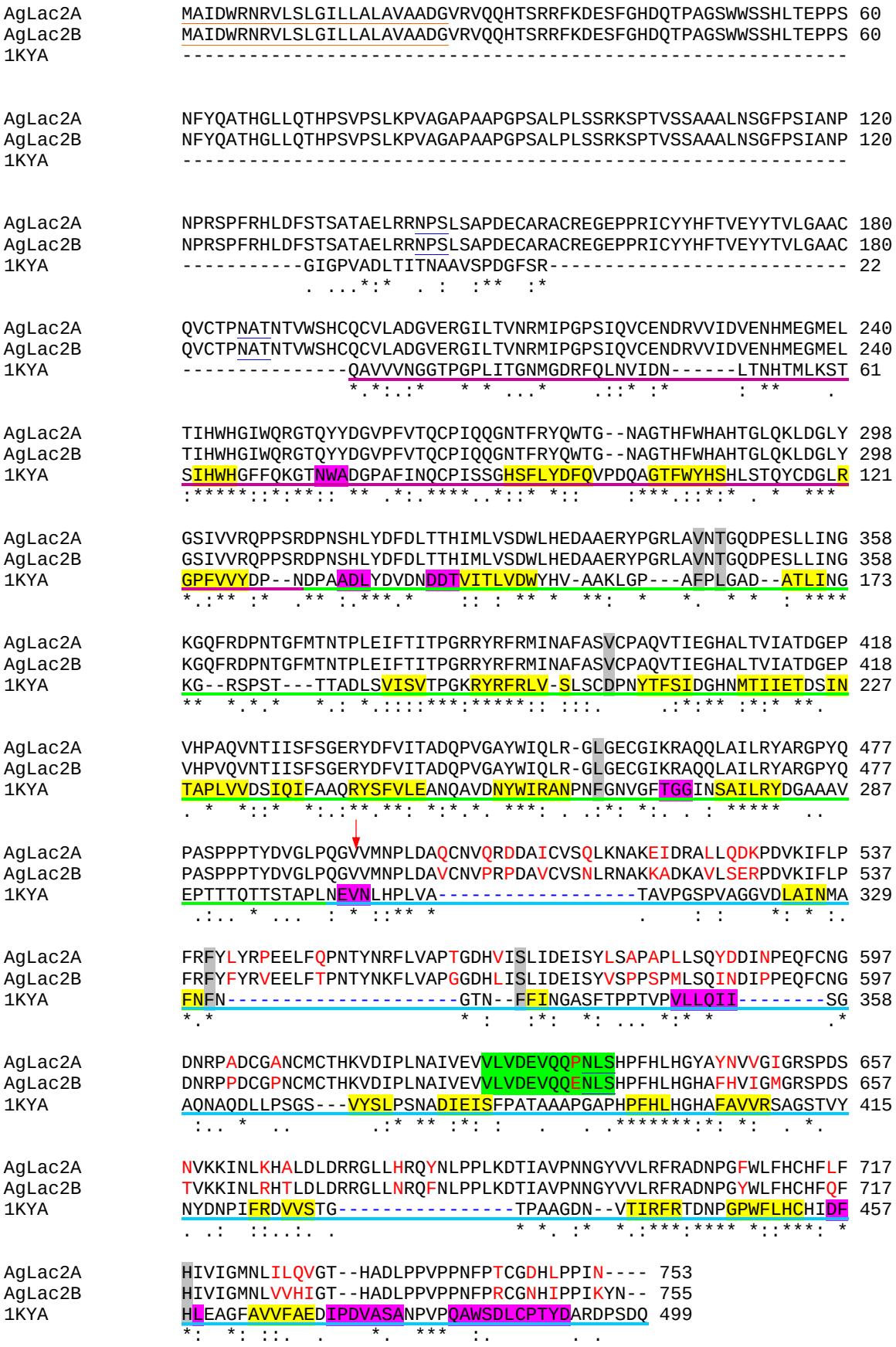


Figure 2 – Substrate chemical structures. (A) 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (http://www.sigmaaldrich.com/united-states.html) **(B)** *N*-β-alanyldopamine (NBAD) (C) *N*-acetyldopamine (NADA).

Figure 3 - Sequence alignment of AgLac2 isoforms and TvLacIIIb. Predicted signal sequences are underlined in orange. Putative N-linked glycosylation sites are underlined in blue. Alpha helical residues are highlighted in yellow and beta sheet residues are highlighted in pink. Domain 1 of the proteins is underlined in purple, domain 2 is underlined in green, and domain 3 is underlined in light blue. Residue differences between AgLac2 isoforms are colored red. Substrate binding residues are highlighted in gray. Residues of a putative substrate-binding loop are highlighted in green. The arrow (↓) indicates the exon boundary (the start of alternative exons).


AgLac2A MAIDWRNRVLSLGILLALAVAADGVRVQQHTSRRFKDESFGHDQTPAGSWWSSHLTEPPS 60
AgLac2B MAIDWRNRVLSLGILLALAVAADGVRVQQHTSRRFKDESFGHDQTPAGSWWSSHLTEPPS 60
1KYA ----------
AgLac2A NFYQATHGLLQTHPSVPSLKPVAGAPAAPGPSALPLSSRKSPTVSSAAALNSGFPSIANP 120
AgLac2B NFYQATHGLLQTHPSVPSLKPVAGAPAAPGPSALPLSSRKSPTVSSAAALNSGFPSIANP 120
1KYA ----------
AgLac2A NPRSPFRHLDFSTSATAELRRNPSLSAPDECARACREGEPPRICYYHFTVEYYTVLGAAC 180
AgLac2B NPRSPFRHLDFSTSATAELRRNPSLSAPDECARACREGEPPRICYYHFTVEYYTVLGAAC 180
1KYA ----------GIGPVADLTITNAAVSPDGFSR---------- 22
AgLac2A QVCTPNATNTVWSHCQCVLADGVERGILTVNRMIPGPSIQVCENDRVVIDVENHMEGMEL 240
AgLac2B QVCTPNATNTVWSHCQCVLADGVERGILTVNRMIPGPSIQVCENDRVVIDVENHMEGMEL 240
1KYA --------------QAVVVNGGTPGPLITGNMGDRFQLNVIDN------LTNHTMLKST 61
AgLac2A TIHWHGIWQRGTQYYDGVPFVTQCPIQQGNTFRYQWTG--NAGTHFWHAHTGLQKLDGLY 298
AgLac2B TIHWHGIWQRGTQYYDGVPFVTQCPIQQGNTFRYQWTG--NAGTHFWHAHTGLQKLDGLY 298
1KYA SIHWHGFFQKGTNWADGPAFINQCPISSGHSFLYDFQVPDQAGTFWYHSHLSTQYCDGLR 121
AgLac2A GSIVVRQPPSRDPNSHLYDFDLTTHIMLVSDWLHEDAAERYPGRLAVNTGQDPESLLING 358
AgLac2B GSIVVRQPPSRDPNSHLYDFDLTTHIMLVSDWLHEDAAERYPGRLAVNTGQDPESLLING 358
1KYA GPFVVYDP--NDPAADLYDVDNDDTVITLVDWYHV-AAKLGP---AFPLGAD--ATLING 173
AgLac2A KGQFRDPNTGFMTNTPLEIFTITPGRRYRFRMINAFASVCPAQVTIEGHALTVIATDGEP 418
AgLac2B KGQFRDPNTGFMTNTPLEIFTITPGRRYRFRMINAFASVCPAQVTIEGHALTVIATDGEP 418
1KYA KG--RSPST---TTADLSVISVTPGKRYRFRLV-SLSCDPNYTFSIDGHNMTIIETDSIN 227
AgLac2A VHPAQVNTIISFSGERYDFVITADQPVGAYWIQLR-GLGECGIKRAQQLAILRYARGPYQ 477
AgLac2B VHPVQVNTIISFSGERYDFVITADQPVGAYWIQLR-GLGECGIKRAQQLAILRYARGPYQ 477
1KYA TAPLVVDSIQIFAAQRYSFVLEANQAVDNYWIRANPNFGNVGFTGGINSAILRYDGAAAV 287
AgLac2A PASPPPTYDVGLPQGVVMNPLDAQCNVQRDDAICVSQLKNAKEIDRALLQDKPDVKIFLP 537
AgLac2B PASPPPTYDVGLPQGVVMNPLDAVCNVPRPDAVCVSNLRNAKKADKAVLSERPDVKIFLP 537
1KYA EPTTTQTTSTAPLNEVNLHPLVA------------------TAVPGSPVAGGVDLAINMA 329
AgLac2A FRFYLYRPEELFQPNTYNRFLVAPTGDHVISLIDEISYLSAPAPLLSQYDDINPEQFCNG 597
AgLac2B FRFYFYRVEELFTPNTYNKFLVAPGGDHLISLIDEISYVSPPSPMLSQINDIPPEQFCNG 597
1KYA FNFN----------GTN--FFINGASFTPPTVPVLLQII--------SG 358
AgLac2A DNRPADCGANCMCTHKVDIPLNAIVEVVLVDEVQQPNLSHPFHLHGYAYNVVGIGRSPDS 657
AgLac2B DNRPPDCGPNCMCTHKVDIPLNAIVEVVLVDEVQQENLSHPFHLHGHAFHVIGMGRSPDS 657
1KYA AQNAQDLLPSGS---VYSLPSNADIEISFPATAAAPGAPHPFHLHGHAFAVVRSAGSTVY 415
AgLac2A NVKKINLKHALDLDRRGLLHRQYNLPPLKDTIAVPNNGYVVLRFRADNPGFWLFHCHFLF 717
AgLac2B TVKKINLRHTLDLDRRGLLNRQFNLPPLKDTIAVPNNGYVVLRFRADNPGYWLFHCHFQF 717
1KYA NYDNPIFRDVVSTG----------------TPAAGDN--VTIRFRTDNPGPWFLHCHIDF 457
AgLac2A HIVIGMNLILQVGT--HADLPPVPPNFPTCGDHLPPIN---- 753
AgLac2B HIVIGMNLVVHIGT--HADLPPVPPNFPRCGNHIPPIKYN-- 755
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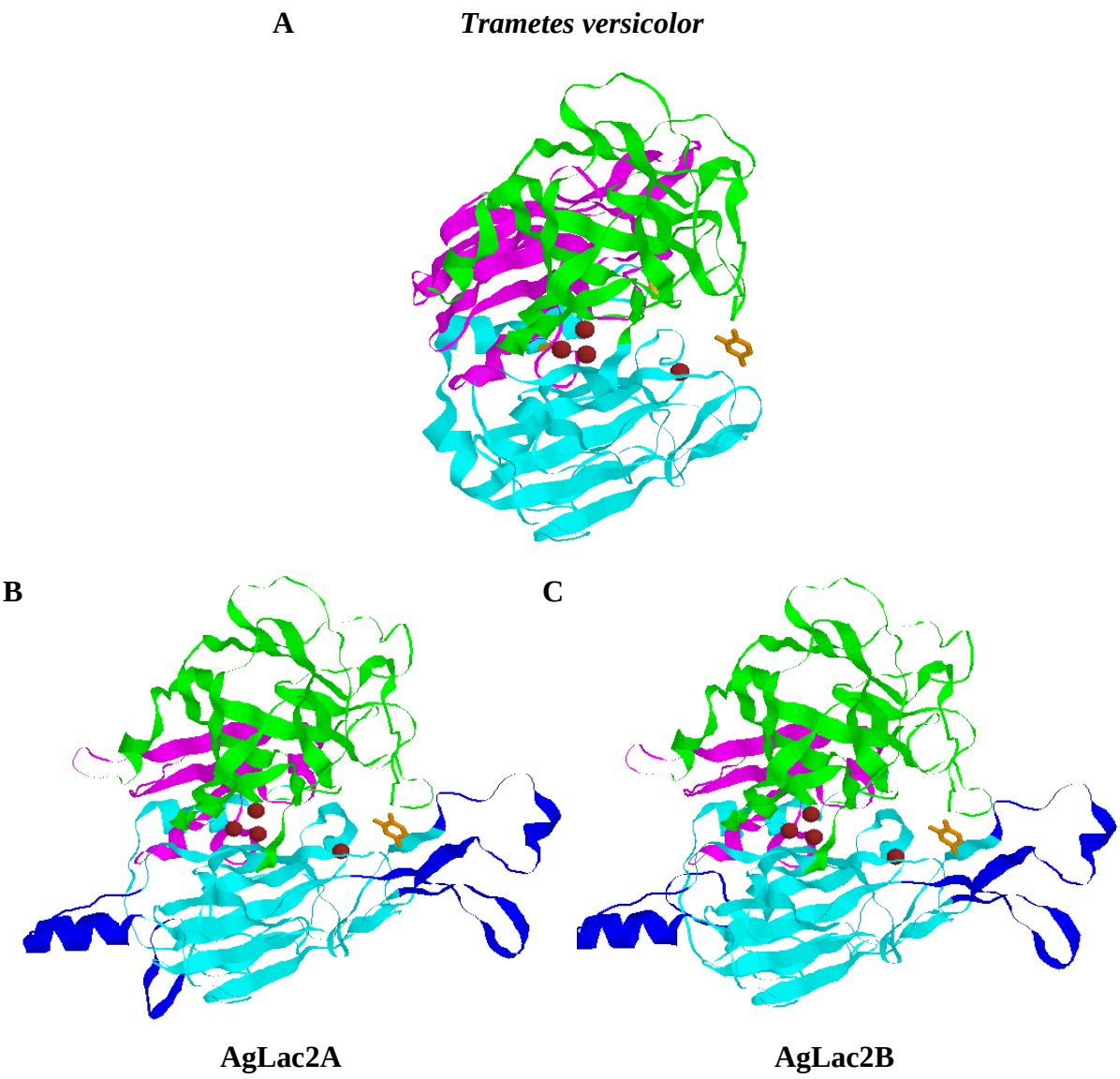


Figure 4 - Homology models of AgLac2 isoforms. (A) *Trametes versicolor* laccase (pdb number – 1KYA) was aligned with **(B)** AgLac2A and **(C)** AgLac2B isoform sequences using ClustalW and were modeled with SWISS-MODEL. Shown are Domain 1 (magenta), Domain 2 (green), and Domain 3 (cyan). Insertions not present in the fungal sequence are colored blue. Copper atoms are indicated as red spheres, and 2,5-xylidine in the substrate-binding site is represented in orange sticks.

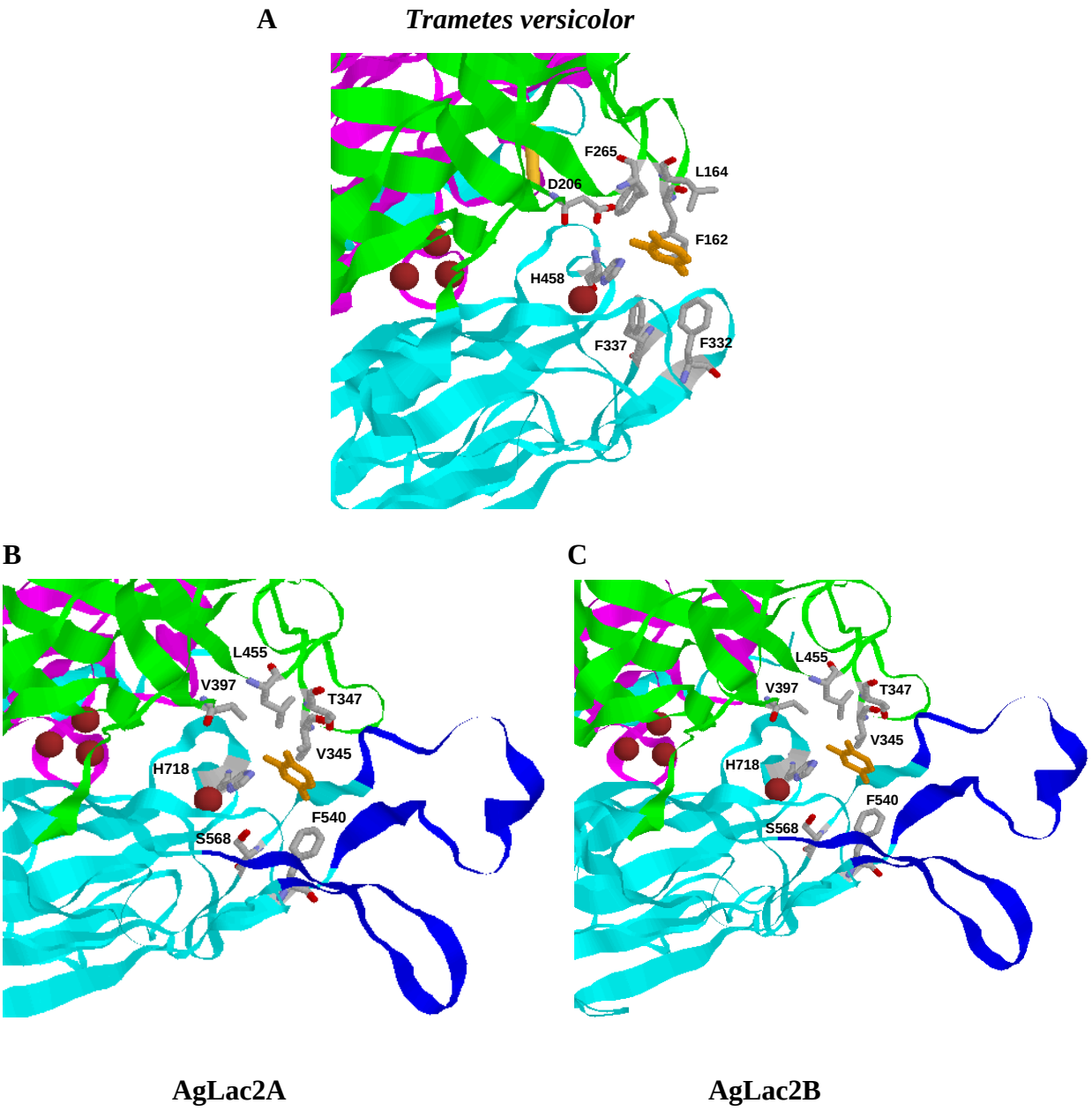
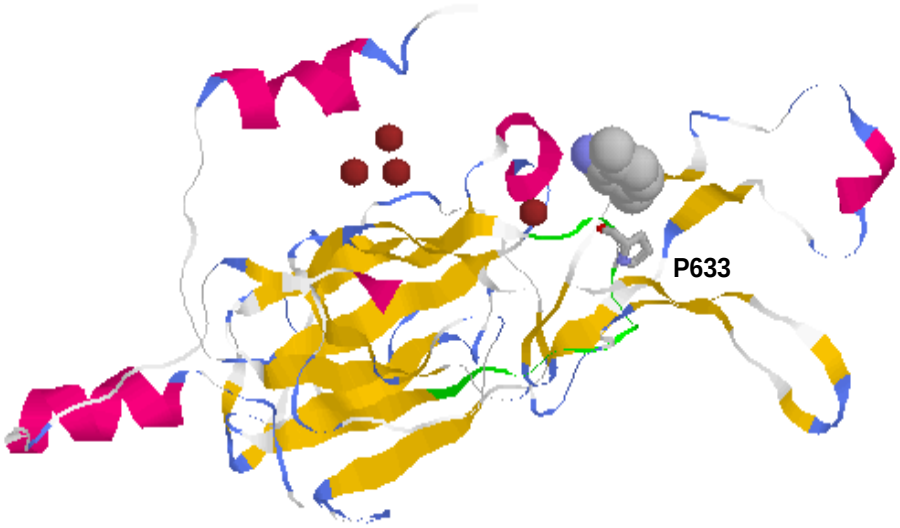


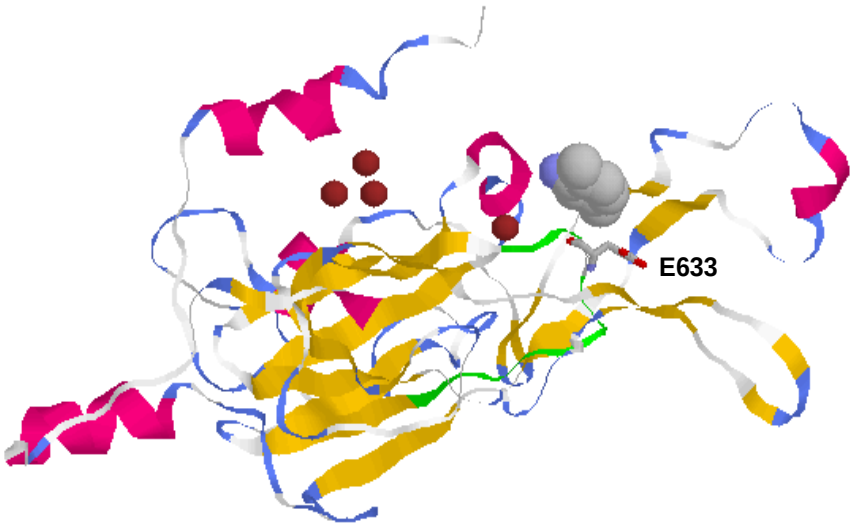
Figure 5 - Comparison of AgLac2 substrate-binding site. Comparison of the substrate-binding site in the AgLac2 isoforms with that of **(A)** TvLacIIIb shows a similar hydrophobic pocket and conservation of a His residue that interacts with the single copper and the substrate. The residues in **(B)** AgLac2A and **(C)** AgLac2B that align with substrate-binding residues of TvLacIIIb do not differ between isoforms.

A



AgLac2A

B



AgLac2B

Figure 6 - Comparison of a putative AgLac2 substrate-binding loop. Comparison of an AgLac2 putative substrate-binding loop (shown in green) in Domain 3 found specifically in alternate exons 7-8, shows a difference that might affect substrate-binding specificity, particularly residue 633, which is Pro in **(A)** AgLac2A and Glu in **(B)** AgLac2B.

Figure 7 - Sequence alignment of domain 3 of insect laccase-2 orthologs. *Anopheles gambiae* (AgLac2), *Aedes aegypti* (AeLac2), *Drosophila melanogaster* (DmLac2), *Bombyx mori* (BmLac2), *Tribolium castaneum* (TcLac2), and *Manduca sexta* (MsLac2) are the laccase-2 orthologs shown in the sequence alignment. Highlighted in yellow is the difference between Lac2 isoforms at residue 633, which is not conserved among the different insect species. Highlighted in green are the differences between isoforms at residue 544 and residue 728 that are conserved between insect species. At residue 544, this difference is tyrosine in Lac2A and isoleucine in Lac2B, with the exception of BmLac2B having a substitution of methionine instead (colored red). At residue 728, the conserved difference is glutamine in Lac2A and histidine in Lac2B. The arrow (↓) indicates the exon boundary (the start of alternative exons).


AgLac2A YWIQLRGLGECGIKRAQQLAILRYARGPYQPASPPPTYDVGLPQGVVMNPLDAQCNVQRD 507
AeLac2A YWIQLRGLGECGIKRAQQLAILRYARGPYQPASPPPTYDVGLPQGVVLNPLDAQCNVQRD 501
DmLac2A YWIQLRGLGECGIRRAQQLAILRYARGPYQPASSPPTYDVGIPQGVVMNPLDAQCNRQRN 536
BmLac2A YWIQVRGLGECGIKRAQQLGILRYARGPYQPSSLAPTYDVGIPQGVVMNPLDARCNISRN 513
TcLac2A YWIQLRGLGECGIRRVQQLGILRYAKGPYQPSQAPPTYDYGIPQGVVLNPLDARCNEIRP 465
MsLac2 YWIQVRGLGECGIKRAQQLGILRYARGPYQPSLPAPTYDIGIPQGVVMNPLDARCNILRN 509
AgLac2B YWIQLRGLGECGIKRAQQLAILRYARGPYQPASPPPTYDVGLPQGVVMNPLDAVCNVPRP 507
AeLac2B YWIQLRGLGECGIKRAQQLAILRYARGPYQPASPPPTYDVGLPQGVVLNPLDAVCNVPRP 504
DmLac2B YWIQLRGLGECGIRRAQQLAILRYARGPYQPASSPPTYDVGIPQGVILNPLDAICDRKRA 536
BmLac2B YWIQVRGLGECGIKRAQQLGILRYARGPYQPSSLAPTYDVGIPQGVVMNPLDAICDVKRN 512
TcLac2B YWIQLRGLGECGIRRVQQLGILRYAKGPYQPSQAPPTYDYGIPQGVVLNPLDAICNKPRK 465
AgLac2A DAICVSQLKNAKEIDRALLQDKPDVKIFLPFRFYLYRPEELFQPNTYNRFLVAPTGDHVI 567
AeLac2A DAICVSQLKNAKDIDRALLQEKPDVKIFLPFRFYLYRPEELFQPNTYNRFLVAPTGDHVI 561
DmLac2A DAICVSQLKNALEIDRGILAEKPDVKIFLPFRFFVYRAEDLFQPNTYNRFLVAPTGDHVI 596
BmLac2A DAICVSQLKNAQNIDPAILQERPDVKIFLPFRFFVYRPEMLFQPNTYNRYLVAPGGDHVI 573
TcLac2A DAICVSQLKNALSIDKGILREKPDVKIFLPFRFHIYTPEDLFAPNTYNRHLVAPNGDHVI 525
MsLac2 DAICVSQLKNAKHIDPAILQERPDIKIFLPFRFFVYGPETLFQPNTYNRYLVAPSGDHVI 569
AgLac2B DAVCVSNLRNAKKADKAVLSERPDVKIFLPFRFYFYRVEELFTPNTYNKFLVAPGGDHLI 567
AeLac2B DAICVSNLRSAKKLDKAVLTERPDVKIFLPFRFYFYRVEELFTPNTYNKFLVAPGGDHLI 564
DmLac2B DAVCVSNLKNAKKVDKGVLVERPDVKIFLPFRFFVYEPKALFIPNTYNRFLVVPSGDHLT 596
BmLac2B DAVCVSNLKSARPVDKALLQDRPDVKIFLPFRFHFYKPKDLFKENTYRNFLVAPGGDHVL 572
TcLac2B DAVCVSQLRNAKKVDEAILEERPDVKIFLPFRFLFYKPEDIFRPNTYNRFLAATGGDHVI 525
AgLac2A SLIDEISYLSAPAPLLSQYDDINPEQFCNGDNRPADCGANCMCTHKVDIPLNAIVEVVLV 627
AeLac2A SLIDEISYLSAPAPLLSQYDDINPEQFCNGDNRPADCGANCMCTHKVDIPLNAIVEVVLV 621
DmLac2A SLIDEISYLSAPAPLTSQYNDINPDQFCNGDNRPADCGPNCMCTHKIDIPLNAIVEVVLV 656
BmLac2A SLIDEISYMSPPAPLISQYDDINPDQFCNGDNRPANCGQNCMCTHKVDIPLNAVVEIVLV 633
TcLac2A SLIDEISYMAPPAPLISQYDDIDPQQFCNGDNRPADCQQNCMCTHKVDIPLNAIVEIVLV 585
MsLac2 SLIDEISYMSPPAPLLSQYDDINPEQFCNGDNRPANCGQNCMCTHKVDIPLNAVVEIVLV 629
AgLac2B SLIDEISYVSPPSPMLSQINDIPPEQFCNGDNRPPDCGPNCMCTHKVDIPLNAIVEVVLV 627
AeLac2B SLIDEISYVSPPSPMLSQIEDIPPEQFCNGDNRPPDCGPNCMCTHTVDIPLNAIVEVVLV 624
DmLac2B SLVDEISYISPPAPPLSQIDDIPPEYFCNGDNRPPNCGPNCECTHMVDIPLGAIVEVVLV 656
BmLac2B SLVDEISYSAPPAPPLSQMHELSPDLFCNGDNRPPNCAVDCRCTHMIDVPLNSIVEIVLV 632
TcLac2B SLIDEISFTFPPSPPLSQIHDLSPDQFCNGDNRPPDCGQNCMCTHQVDIPLNAIVEVVLV 585
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DmLac2A DEVQQPNLSHPFHLHGYGFSVIGIGRSPDSSVKKINLKHALDLDRRGLLHRQYN---LPP 713
BmLac2A DEVQIANLSHPFHLHGYSYNVIGIGRSPDQNVKKINLKHALDLDRRGLLERHLKQGDLPP 693
TcLac2A DEVQQPNLSHPFHLHGYAFNVIGIGRSPDQNVKKINLKHALDLDRQGLLHRQFN---LPP 642
MsLac2 DEVQITNLSHPFHLHGYAYNVIGIGRSPDQNVKKINLKHALDLDRRGLLERHLKQGDLPP 689
AgLac2B DEVQQENLSHPFHLHGHAFHVIGMGRSPDSTVKKINLRHTLDLDRRGLLNRQFN---LPP 684
AeLac2B DEVQQDNLSHPFHLHGHSFNVIGMGRSPDTTVKKINLRHALDLDRRGLLNRQFN---LPP 681
DmLac2B DEVQQVNLSHPFHLHGTAFYVVGLGRSPDKSIKKINLKHALELDQMGMLERHFS---KPP 713
BmLac2B DEVQSPNLSHPFHLHGTSYNVIGMGRSPDKNIKKINLKHALDLDRKGLLHRQYN---LPP 689
TcLac2B DEVQSPNLSHPFHLHGYAFNVVGIGRSPDQNVKKINLKHALDLDRRGLLHRQFN---LPP 642

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AgLac2A         LKDTIAVPNNGYVVLRFRADNPGFWLFHCHFLFHIVIGMNLILQVGTHADLPPVPPNFPT 744
AeLac2A         LKDTIAVPNNGYVVLRFRADNPGFWLFHCHFLFHIVIGMNLILQVGTLQDLPPVPPNFPT 738
DmLac2A         TKDTIAVPNNGYVVLRFRADNPGFWLFHCHFLFHIVIGMNLILQVGTNADLPPVPPGFPT 773
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TcLac2A         AKDTIAVPNNGYVVLRLRANNPGFWLFHCHFLFHIVIGMNLVLQVGTHADLPPVPPNFPT 702
MsLac2          AKDTIAVPNSGYVILRFRATNPGFWLLHCHFLFHIVIGMSLVLQVGTQADLPPVPPGFPT 749

AgLac2B         LKDTIAVPNNGYVVLRFRADNPGYWLFHCHFQFHIVIGMNLVVHIGTHADLPPVPPNFPR 744
AeLac2B         LKDTVAVPNNGYVVMRFRANNPGYWLFHCHFQFHIVIGMNLVVHVGSKADLPPVPPNFPR 741
DmLac2B         LKDTIAVPNNGYVVIRFRADNPGYWLFHCHFLFHIVIGMNLIFHIGTTADLPPVPPRFPT 773
BmLac2B         HKDTLAVPNNGYVVLRLKADNPGYWLFHCHFIYHIVIGMSLILHIGTQGDLPPVPPNFPR 749
TcLac2B         SKDTIAVPNNGYVIFRFRADNPGYWLFHCHFLFHIVIGMNLIIHVGTQLIYRPFS-HFPR 701
                 ***:****.***::*::* ***:**:**** :******.*:.::*:     *..  **

AgLac2A         CGDHLPPIN---------- 753
AeLac2A         CGDHLPPIQ---------- 747
DmLac2A         CGDHTPSIPIN-------- 784
BmLac2A         CGDHLPAI-----PLH---- 764
TcLac2A         CGDHVPEIN-----SNPNLV 717
MsLac2          CGDHLPPI-----PLH---- 760

AgLac2B         CGNHIPPIKYN-------- 755
AeLac2B         CGNHIPPIRFN-------- 752
DmLac2B         CGDHVPPVTWY-------- 784
BmLac2B         CGHHLPTISPPFYPIH---- 765
TcLac2B         CGNHLPPIS-----LH---- 712
                **.* * :
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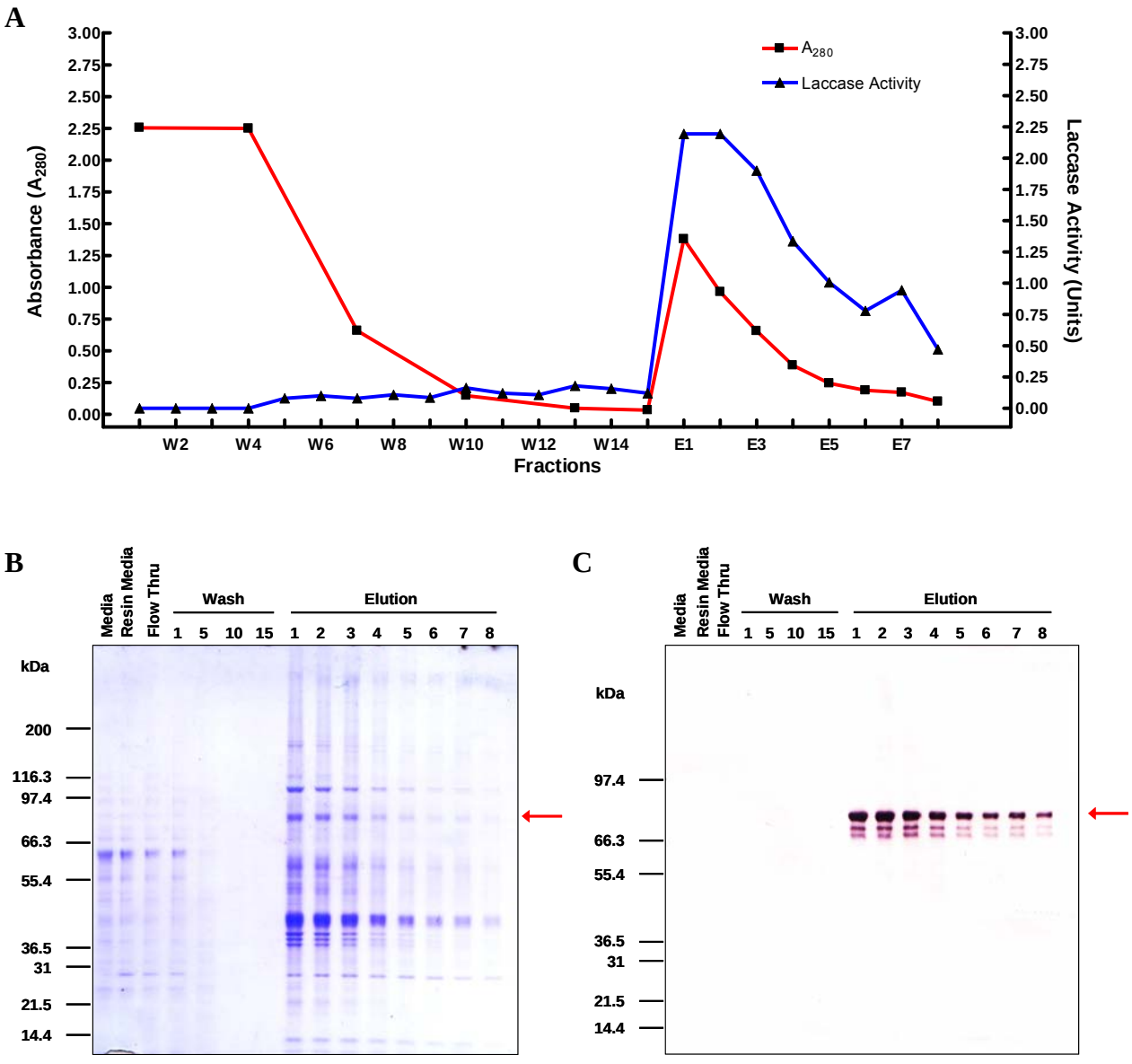
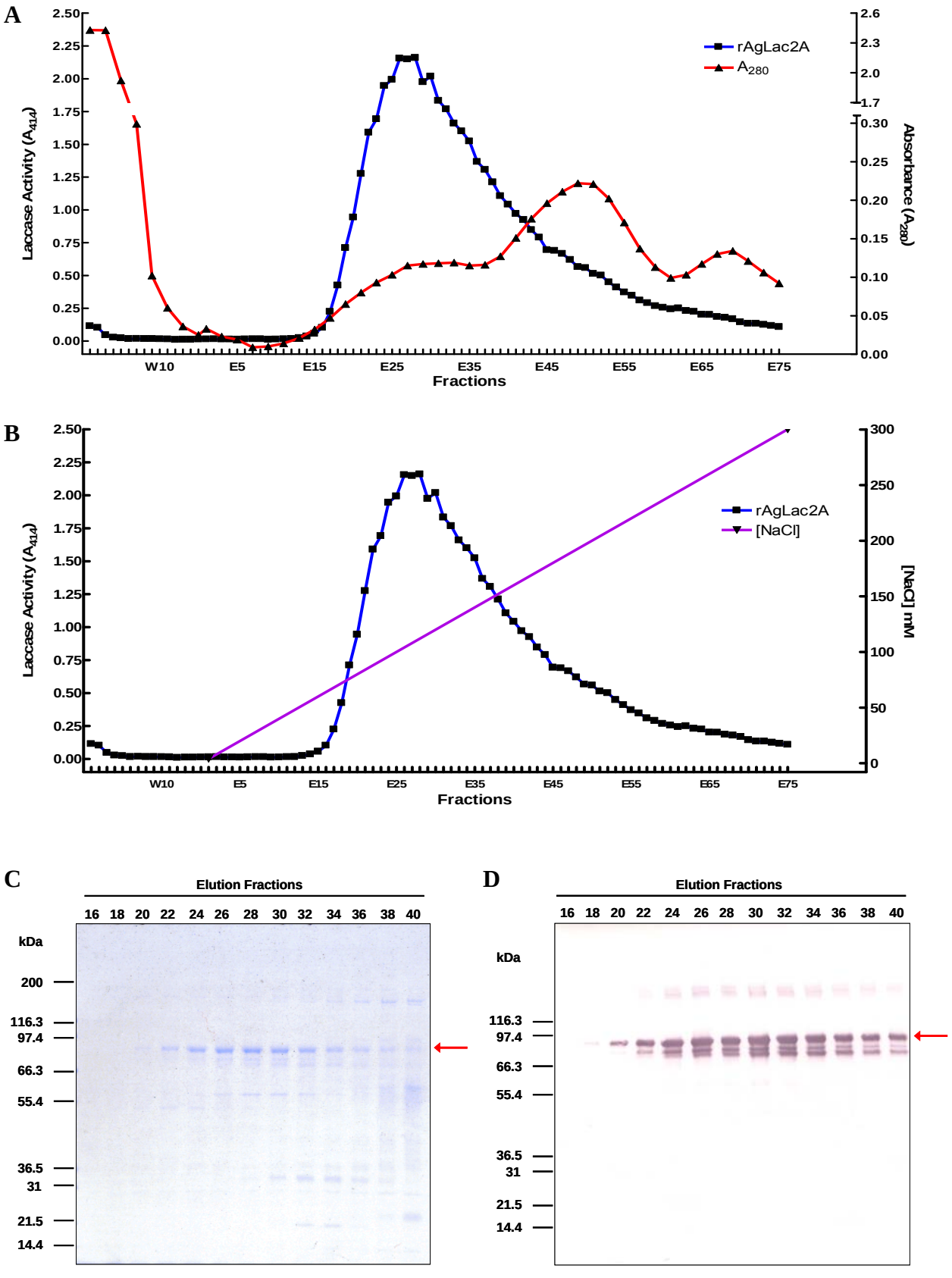


Figure 8 – Purification of rAgLac2A by affinity chromatography (Con A). (A) Graph showing elution profile from column. rAgLac2A activity was assayed using ABTS as a substrate. Elution fractions 1-8 were combined for further purification by anion exchange chromatography. **(B)** SDS-PAGE analysis of collected fractions on a NuPAGE 4-12% Bis-Tris SDS polyacrylamide gel stained with Coomassie blue. **(C)** Western blot analysis of collected fractions using *Manduca sexta* Laccase-2 antibody. The red arrow indicates rAgLac2A. Mark 12 protein standard was used for estimation of molecular masses.

Figure 9 – Purification of rAgLac2A by anion exchange chromatography (Q Sepharose). Graphs showing elution profile from column with **(A)** absorbance at 280 nm and **(B)** linear 0-300 mM NaCl gradient. rAgLac2A activity was assayed using ABTS as a substrate. Elution fractions 23-30 were combined for use in experiments. **(C)** SDS-PAGE analysis of collected fractions on a NuPAGE 4-12% Bis-Tris SDS polyacrylamide gel stained with Coomassie blue. **(D)** Western blot analysis of collected fractions using *Manduca sexta* Laccase-2 antibody. The red arrow indicates rAgLac2A. Mark 12 protein standard was used for estimation of molecular masses.


A
Laccase Activity (A_{414})
Absorbance (A_{280})
rAgLac2A
A_{280}
Fractions
W10
E5
E15
E25
E35
E45
E55
E65
E75
B
Laccase Activity (A_{414})
[NaCl] mM
rAgLac2A
[NaCl]
Fractions
C
Elution Fractions
16 18 20 22 24 26 28 30 32 34 36 38 40
kDa
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4
D
Elution Fractions
16 18 20 22 24 26 28 30 32 34 36 38 40
kDa
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4

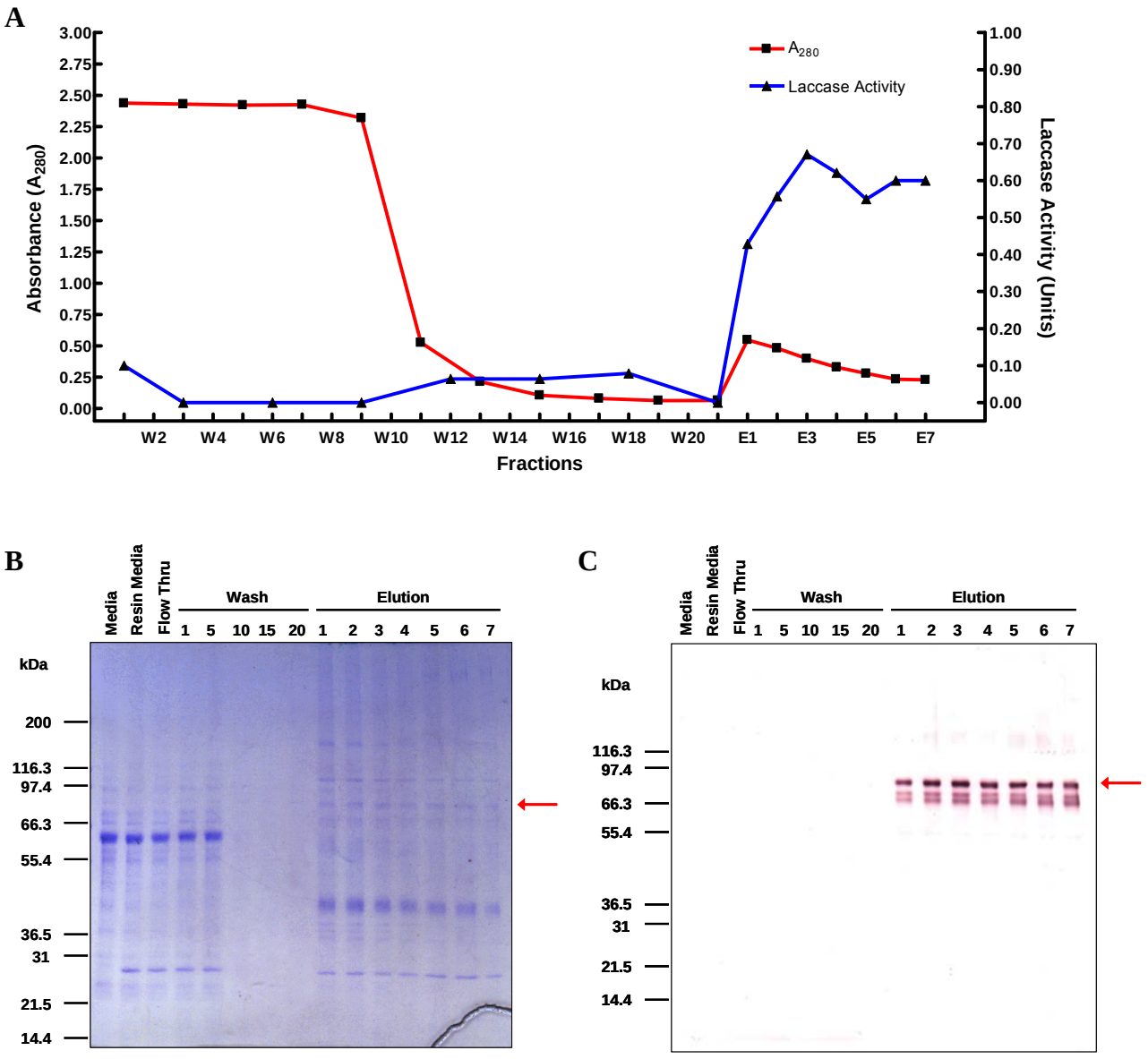
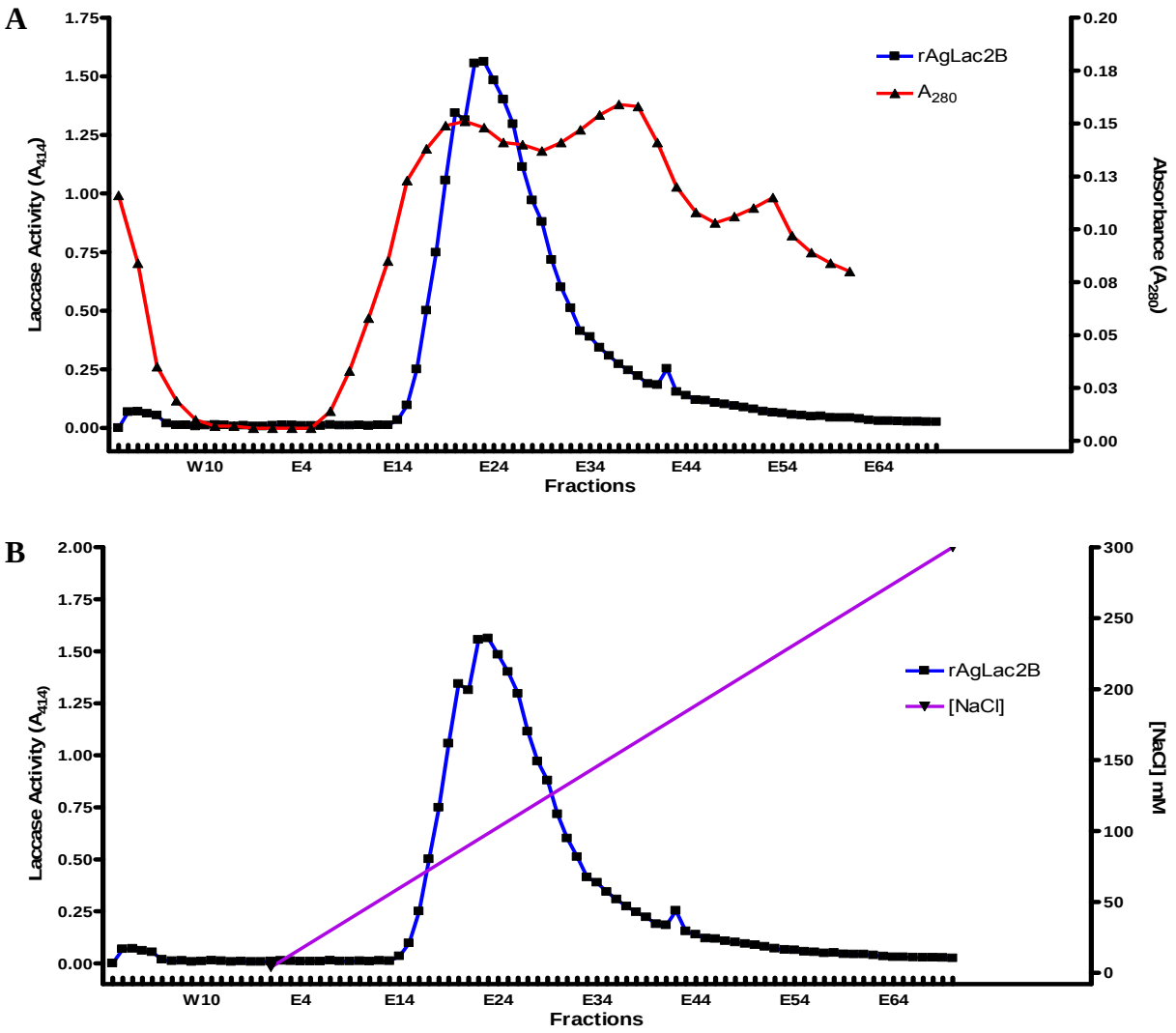
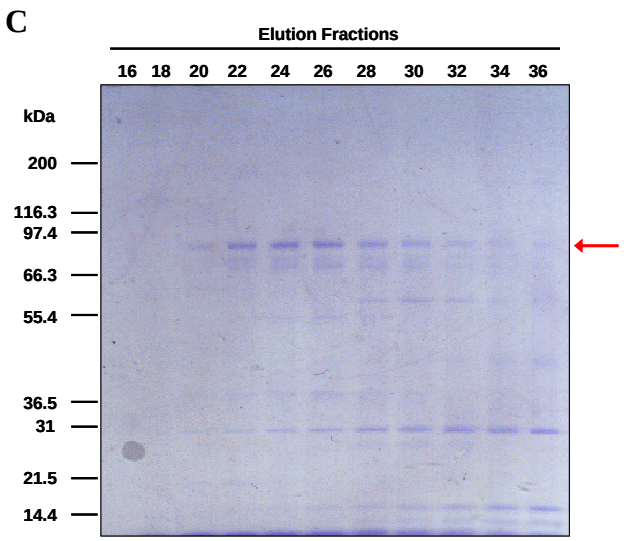
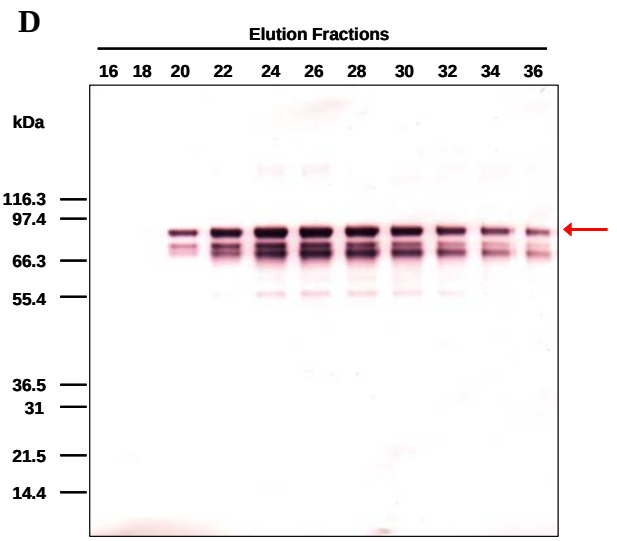


Figure 10 – Purification of rAgLac2B by affinity chromatography (Con A). (A) Graph showing elution profile from column. rAgLac2B activity was assayed using ABTS as a substrate. Elution fractions 1-7 were combined for further purification by anion exchange chromatography. **(B)** SDS-PAGE analysis of collected fractions on a NuPAGE 4-12% Bis-Tris SDS polyacrylamide gel stained with Coomassie blue. **(C)** Western blot analysis of collected fractions using *Manduca sexta* Laccase-2 antibody. The red arrow indicates rAgLac2B. Mark 12 protein standard was used for estimation of molecular masses.

Figure 11 – Purification of rAgLac2B by anion exchange chromatography (Q Sepharose). Graphs showing elution profile from column with **(A)** absorbance at 280 nm and **(B)** linear 0-300 mM NaCl gradient. rAgLac2B activity was assayed using ABTS as a substrate. Elution fractions 22-29 were combined for use in experiments. **(C)** SDS-PAGE analysis of collected fractions on a NuPAGE 4-12% Bis-Tris SDS polyacrylamide gel stained with Coomassie blue. **(D)** Western blot analysis of collected fractions using *Manduca sexta* Laccase-2 antibody. The red arrow indicates rAgLac2B. Mark 12 protein standard was used for estimation of molecular masses.


A
Laccase Activity (A414)
Absorbance (A280)
rAgLac2B
A280
W10
E4
E14
E24
E34
E44
E54
E64
Fractions
B
Laccase Activity (A414)
[NaCl] mM
rAgLac2B
[NaCl]
Fractions


C
Elution Fractions
16 18 20 22 24 26 28 30 32 34 36
kDa
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4


D
Elution Fractions
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kDa
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97.4
66.3
55.4
36.5
31
21.5
14.4

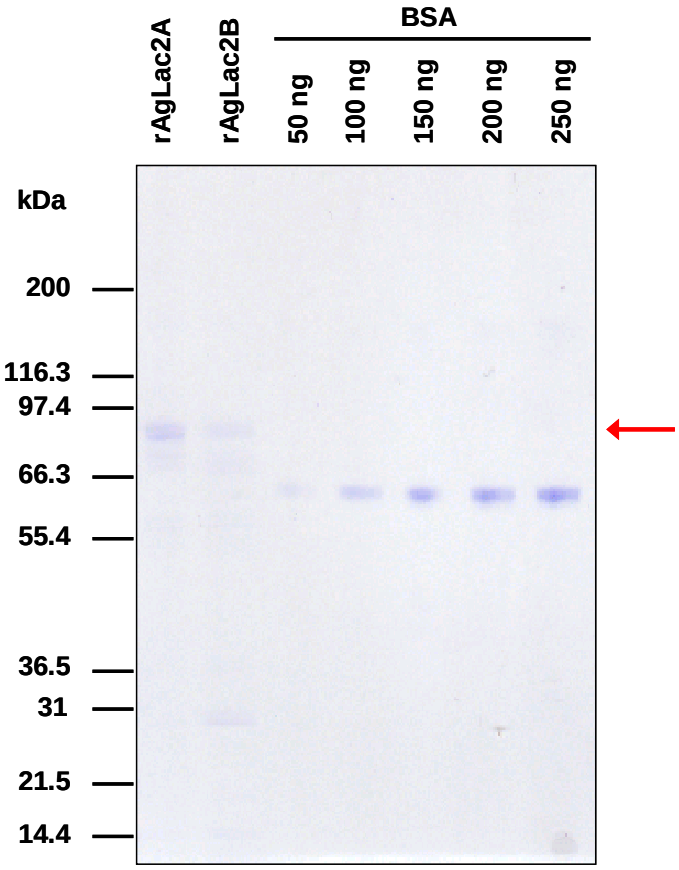
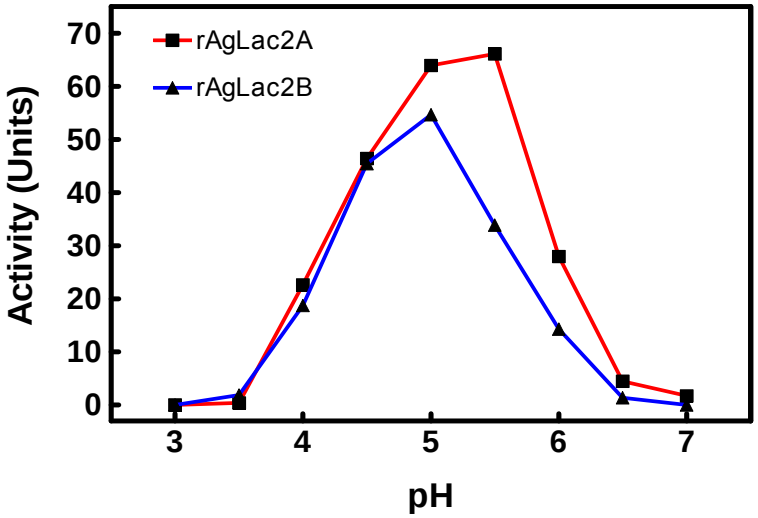


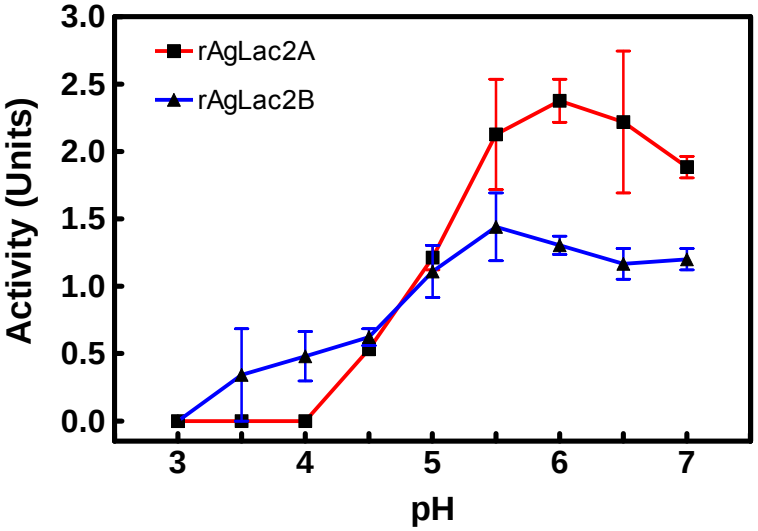
Figure 12 – SDS-PAGE analysis of purified rAgLac2. Purified rAgLac2 (10 µL) and known concentrations of bovine serum albumin were treated with 6X SDS loading buffer and separated on a NuPAGE 4-12% Bis-Tris SDS polyacrylamide gel, followed by staining with Coomassie blue. The red arrow indicates rAgLac2. Mark 12 protein standard was used for estimation of molecular masses.

Figure 13 - pH profile analysis of recombinant AgLac2 isoforms. Assays were performed with 250 ng of purified laccase-2 and 0.5 mM ABTS **(A)** or NBAD **(B)** or NADA **(C)** in 100 mM citric acid-sodium citrate buffer of various pH. Activity was determined by monitoring production of oxidized product at 414 nm for ABTS and 390 nm for NBAD and NADA. Each data point for NBAD and NADA indicates the mean of 2 replicates ± range.

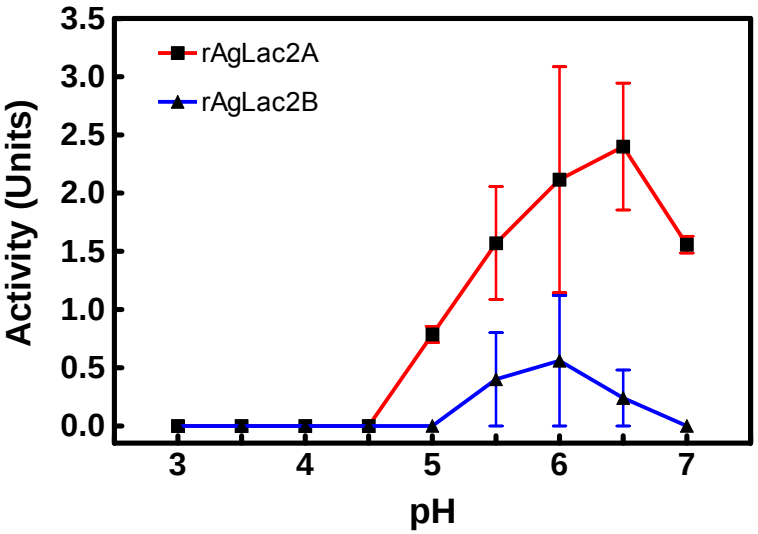
A


rAgLac2A
rAgLac2B
Activity (Units)
pH
0
10
20
30
40
50
60
70
3
4
5
6
7

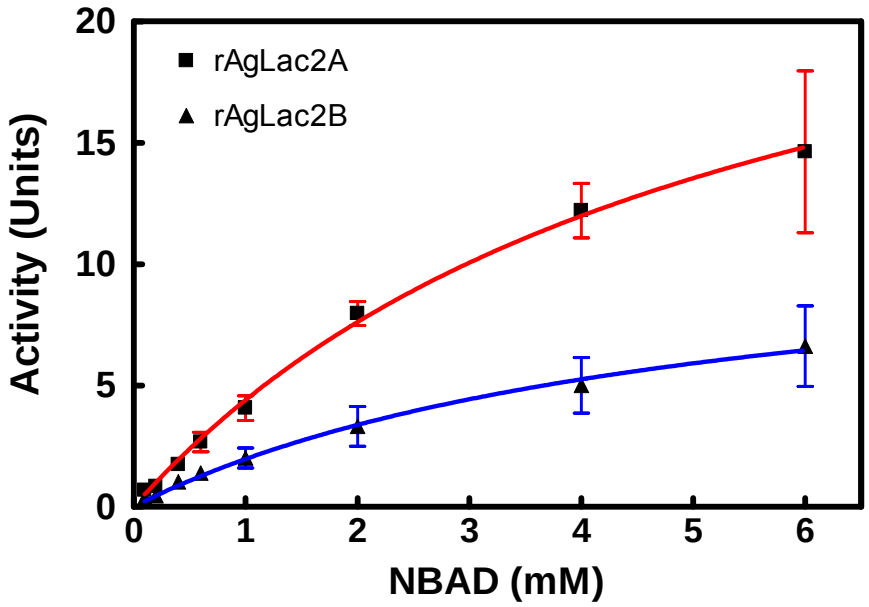
B


rAgLac2A
rAgLac2B
Activity (Units)
pH
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3
4
5
6
7

C


rAgLac2A
rAgLac2B
Activity (Units)
pH
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
3
4
5
6
7

A



B

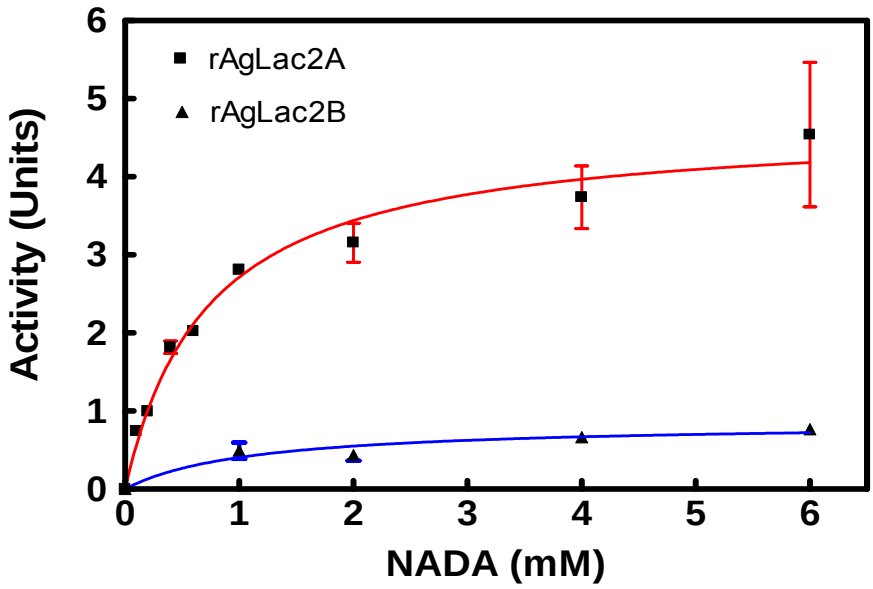


Figure 14 – rAgLac2 kinetics. Assays were performed with 250 ng of purified laccase-2 and various concentration of **(A)** NBAD **(B)** or NADA in 100 mM citric acid-sodium citrate buffer (pH 6). Activity was determined by monitoring production of oxidized product at 390 nm for each quinone. Each data point for NBAD and NADA indicates the mean of 2 replicates ± range. Curves were fitted to the data by non-linear regression using the GraphPad Prism software.

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