

MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF WHEAT

(*Triticum aestivum*. L) POLYPHENOL OXIDASES (PPOS)

by

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ABSTRACT

Polyphenol oxidases (PPOs) from several plant species, including wheat, have been implicated in the undesirable brown discoloration of food products. Wheat (*Triticum aestivum* L.) represents an interesting system to advance our understanding of plant PPO function for two important reasons, namely (1) the large size and complexity of its (allohexaploid) genome, and (2) its economic importance. Prior to this study, the molecular and biochemical properties of wheat PPOs were largely unknown. To remedy this situation, we have performed several BLAST searches of expressed sequence tag (EST) databases, using a known wheat PPO sequence as a search tool. This study suggested the presence of at least six PPO genes in hexaploid wheat, falling into two different phylogenetic clusters of three genes each. Presence of a wheat PPO multigene family was confirmed by Southern blotting. A combination of biochemical (enzyme purification and mass spectrometric analysis) and molecular (Northern) approaches confirmed that members of one cluster are not expressed in the developing kernels and senescing flag leaves, while regulation of one or several members of the other gene cluster controls PPO activity in these tissues. Our data, including immunoblotting and enzyme activity studies, further indicated that wheat PPOs are synthesized as inactive precursor (early kernel development) which are proteolytically processed and activated as the kernels mature. Activation of PPO precursor proteins was also demonstrated *in vitro*, in presence of purified trypsin. In these experiments, PPO activity increased during the first four hours and remained stable thereafter, indicating that the protein domains responsible for catalytic activity are quite stable. Research performed as a part of this dissertation has also demonstrated that wheat PPO activity is influenced by strong anionic detergents such as SDS and N-lauroylsarcosine. The corresponding experiments indicated that these detergents influenced both enzyme extraction and activity, at least in high-PPO wheat varieties. This work also has practical aspects, making PPO assays (as used by breeders for germplasm screening) more robust. In conclusion, as a result of this dissertation, wheat PPOs have emerged as a fascinating example of a plant multigene family with complex transcriptional and posttranscriptional regulation.

CHAPTER 1

DISSERTATION OVERVIEW

IntroductionOverview

Polyphenol oxidases (PPOs) have been implicated in browning of vegetables, fruits and cereal products (Feillet et al., 2000). Bhattacharya et al., (1999) have demonstrated that PPOs play a major role in the time-dependent darkening of noodles and other wheat products. The cause of undesirable browning of wheat based products (especially noodles and pastas) is complex. The color of wheat based products results from a desirable bright/yellow component, an undesirable brown component and sometimes, a red component (Feillet et al., 2000). In addition to color, texture and flavor are critical attributes governing the quality of Asian noodles (Bhattacharya et al., 1999). Color is very critical especially for raw noodles. Discoloration of steamed or boiled noodles is not a major concern because PPOs are denatured by heat treatment, and the darkening components get leached out during boiling (Kruger et al., 1992).

Darkening/browning of wheat products is a major hindrance in consumer acceptance as they prefer bright colored noodles. U.S. wheat exports during 2004/05 were 28 million tons, one third of which is imported by only four pacific-rim countries (Japan, China, Philippines and South Korea; Wheat Situation and Outlook Yearbook, 2005). Wheat is mostly consumed in the form of noodles in these and other east Asian nations which

import U.S. wheat. Therefore, it has become one of the goals of many wheat breeding programs to develop wheat varieties with reasonably low-PPO levels in mature grains. Polyphenol oxidases have also been demonstrated to be involved in plant defense (Thipyapong et al., 2004) and pigment formation in plants (Strack and Schliemann 2001).

We have initiated the biochemical and molecular characterization of wheat PPOs. Our efforts have added to an understanding of central aspects of the biology of these enzymes, including their regulation at the transcriptional and post-transcriptional levels. Obtained results are directly applicable for both basic (PPO function in wheat) and practical aspects (breeding for low-PPO germplasm).

Wheat Genetics

Wheat is arguably the most important food crop in the world. Botanically, wheat is a diverse family of related species. Over two dozen individual species have been characterized as members of the genus *Triticum*. Of these, only four (*T. monococcum* L., *T. turgidum* L., *T. timopheevii* Zhuk. and *T. aestivum* L. em Thell.) are widely cultivated. Common wheats include varieties that may have soft or hard endosperm, red or white bran color, winter or spring habit. Durum and common wheats are allotetraploids (AABB; $2n = 4x = 28$) and allohexaploids (AABBDD; $2n = 6x = 42$), respectively (Morris and Rose 1996). Hexaploid wheat contains three closely related genomes designated A, B and D, which are derived from different progenitors. Gene redundancy is therefore the norm, with at least a triplicate homoeoallelic set for most of the genes (some genes show deletions for one or more genome). Unlike the smaller genome of the model cereal rice (400 Mb), wheat has a large genome estimated at 16000 Mb (Lagudah et al.,

2001). The genetic constitution of wheat is important because most quality traits (for example polyphenol oxidase [PPO] levels) result from the interaction between genotype and the environment (Morris and Rose 1996). It has been observed that the PPO trait (high PPO activity in mature kernels) exhibits genetic variability and sufficient heritability (Demeke et al., 2001) to be manipulated through breeding efforts. Selection for low PPO activity may result in correlated changes in other traits (e.g., plant defense), depending on heritability and degree of genetic association of the trait (Falconer and Mackay 1996).

Economics of Wheat

Wheat plays a pivotal role for the agricultural exports of the United States of America. Wheat acreage in the U.S. in 2005 is expected to reach 58 million acres (winter wheat 41.6 m. acres and spring wheat 16.4 m. acres), with a projected production of 2,085 million bushels (Wheat Situation and Outlook Yearbook, 2005). Wheat exports are projected at 1,050 million bushels (28 million tons) for 2004-05 and food-use for the same period is projected at 890 million bushels. During recent years, the top five purchasers of U.S. wheat have been Egypt, Japan, Mexico, the Philippines and South Korea. China emerged early in 2004/05 as a major buyer of U.S. wheat (Wheat Situation and Outlook Yearbook 2005). Wheat produced in the U.S. Northern Great Plains has traditionally been used for bread and bread products, but changing market conditions have led to a situation where a considerable fraction of this wheat is now being used for Asian noodles. On an average over the last 30 years, 10 major Asian countries have annually imported approximately 107 metric tons of U.S. wheat (Jin and Koo 2003).

Oriental noodles are one of the oldest forms of processed foods consumed and have been a major dietary component for many Asians for thousands of years (Hou 2001). Hard wheat grown in the Northern Great Plains is suitable for making Chinese raw noodles where consumers prefer firmer texture and bite characteristics. Both color and texture of the noodles play a role in consumer acceptance (Martin et al., 2005). In this context, while the texture of noodles is affected by several factors (Hou 2001) and also varies by region and with individual preferences, it is universally accepted that consumers prefer bright noodles that conserve their color over time (Hou and Kruk 1998).

Polyphenol oxidases (PPOs) have been implicated in enzymatic browning of vegetables, fruits and cereal products (Feillet et al., 2000). Specifically, PPOs have been demonstrated to play a major role in the time-dependent darkening of noodles and other wheat products (Baik et al., 1995; Park et al., 1997; Bhattacharya et al., 1999; Feillet et al., 2000). Therefore, it is one of the goals of many U.S. wheat breeding programs to develop lines with reasonably low PPO levels in mature grains. PPOs are mostly located in the aleurone layer of the cereal kernel, which is removed during the milling process; however, the remaining activity appears to be high enough to cause problems during food processing (Sullivan 1946; Rani et al., 2001). Additionally, enzyme activity increases from bran contamination with increasing flour extraction rate (Hatcher and Kruger 1993). Growing environment was also shown to influence PPO activity in wheat grain for cultivars grown in USA and Australia (Baik et al., 1994) and hard whites grown in southern Plains (Park et al., 1997).

Polyphenol Oxidases

Reaction Catalyzed

Polyphenol oxidases (PPOs) are ubiquitous, copper-containing metalloproteins that have been found or characterized in several higher plant species (Flurkey 1989; Cary et al., 1992; Kwon and Kim 1996; Constabel et al., 2000; Chazarra et al., 2001; Nagai and Suzuki 2001). PPOs catalyze the hydroxylation of o-monophenols to o-diphenols (E.C. 1.14.18.1; monophenol mono-oxygenase, tyrosinase or cresolase activity) and the oxidation of o-dihydroxyphenols to o-quinones (E.C. 1.10.3.2; diphenol oxidase or catecholase activity; Steffens et al., 1994; van Gelder et al., 1997). Generated o-quinones further react with amines and thiol groups or undergo self-polymerization to produce the dark/brown products that cause problems during food processing (Feillet et al., 2000; Anderson and Morris 2001).

The central domain of all PPOs consists of two Cu-binding sites (CuA and CuB); at each of these sites, a copper ion is bound by three conserved histidine residues. This pair of copper ions is the site of interaction of PPOs with molecular oxygen and phenolic substrates (van Gelder et al., 1997; Steffens et al., 1994). There is a high degree of sequence similarity in the copper-binding regions of PPOs from different plant species, but considerably more divergence in other parts of the respective polypeptide chains (Demeke and Morris 2002). For example, there is 92% amino-acid sequence similarity at the copper A binding region, but overall only 42% sequence similarity between *Vicia* and tomato PPOs (Lax and Cary 1995). Even when comparing PPO sequences from a wide

variety of organisms, the Cu-binding regions are the most conserved domains of the polypeptide, with 42% sequence identity between the CuA and CuB regions of bacterial and human PPO (van Gelder et al., 1997).

Polyphenol Oxidase Substrates

Among other factors, PPO activity is affected by the chemical nature of the processed substrates. Different substrates may be oxidized at different rates (Lamkin et al., 1981; Interesse et al., 1982). Phenolic acids that are potential PPO substrates have been found in wheat flour extracts (Hatcher and Kruger 1997). Okot-Kotber et al. (2001) have shown that, in addition to the differences in the rates at which various phenolic substrates are oxidized by wheat bran PPO extract, some of the slowly oxidizable phenolics (chlorogenic acid, caffeic acid and ferulic acid) and those that are unoxidizable (3-phenyl-2-propenoic acid) may inhibit the oxidation of readily oxidized phenolics, such as L-DOPA. Anderson and Morris (2001) have reported that, among different substrates tested (phenol, L-tyrosine, catechol, methylcatechol, L-DOPA and caffeic acid), L-DOPA and catechol are the best substrates, leading to the highest enzyme activities in whole seed assays.

Jiménez and García-Carmona (1996) divided substrates of tyrosinases into three groups, depending on the development of o-quinonic products: (1) o-quinone products that are cyclizable and sustain intramolecular 1,4-addition to the benzene ring. (2) o-quinone products that are not cyclizable, but can undergo a water addition. (3) o-quinone products that are highly stable through the reaction (Martinez et al., 1993). Mushroom tyrosinase is able to use mono-, di-, and trihydroxyphenols as substrates, but has greatest

affinity for dihydroxyphenols. It was reported that, among a number of monohydroxyphenols (p-cresol and tyrosine), dihydroxyphenols (catechol, L-DOPA, D-DOPA, catechin and chlorogenic acid) and trihydroxyphenols (pyrogallol), catechol was the best substrate (Zhang et al., 1999). *Vicia faba* PPO showed higher activity with 4-methylcatechol than t-butylcatechol or chlorogenic acid (Jiménez and García-Carmona, 1996). 5-Caffeoylquinic acid (5CQA; chlorogenic acid), followed by 4-methylcatechol were shown to be the best substrates for both coffee leaf and endosperm PPO (Mazzafera and Robinson, 2000).

Optimum pH of Polyphenol Oxidases

pH is a determining factor for enzyme activity; it alters the ionization state of amino acid side chains or ionization of the substrate (Voet and Voet, 1990). The pH profile studies for plant PPOs have shown the optimum pH range to be 4.5-8.0 (Kavrayan and Aydemir 2001; Duangmal and Apenten 1999). Moore and Flurkey (1990) have reported that the low pH optimum ($\leq$ pH 4.0) obtained for broad bean latent enzyme was abolished in the presence of SDS. PPO activity from both coffee leaf and endosperm was reported to be optimal at pH 6-7 (Mazzafera and Robinson 2000). Gandia-Herrero et al. (2005) have reported that among the substrates tested (tyramine, tyrosine, L-DOPA, catechol, 4-methyl catechol and 4-tertiary butyl catechol) an optimum pH over 5.5 was observed for soluble PPO from beet root in absence of detergent SDS. They have also reported that under similar conditions (no detergent) as above, the membrane-bound PPO showed activity at acidic pH values (optimum pH = 4.5). However, in presence of detergent they observed a pH range of 6-7 in both the tissues tested. A shift in the optimum pH profile

was also observed in presence of detergent (SDS; pH=5.5) and in its absence (pH=3.5; no SDS) for partially purified persimmon (*Diospyros kaki*) fruit PPO using 4-tert-butylalcohol as a substrate (Núñez-Delicado et al., 2003).

Nagai and Suzuki (2001) have reported the optimum pH for Chinese cabbage (*Brassica rapa* L.) PPO to be 5.0 and they have also reported that the enzyme was unstable below pH 4.0 and above pH 7.0. Anderson and Morris (2001) indicated from their kinetic studies that L-DOPA and catechol at pH 6.5 produced the greatest enzyme activity with little auto-oxidation. Söderhäll (1995) has reported that a completely latent PPO purified from carrot cell extract was active only in the presence of Tris buffer at pH 8.0 and proteolytic processing or detergent treatment did not activate the PPO.

Sub-Cellular Localization

Plant PPOs are nuclear-encoded, but gene products are associated with the thylakoid lumen in photosynthetically active tissues (Hind et al., 1995; Jiménez and García-Carmona 1996; van Gelder et al., 1997). Consistent with this finding, N-terminal transit peptides are present in the corresponding genes. Typical transit peptides consist of 80-100 amino acids; peptide cleavage during chloroplast import reduced the molecular mass of several plant PPOs from 65-70 kD to ~60 kD or less for the mature protein (Cary et al., 1992; Dry and Robinson 1994; Anderson and Morris 2003). The peptide bond cleaved by the processing proteases was identified as alanine-alanine or alanine-serine in all plant pro-tyrosinases examined so far (Whitaker 1995). The transit peptide is characterized by the presence of three domains (Newman et al., 1993; Joy et al., 1995). The three domains of transit peptides are: (i) the N-terminal 25 amino acids mostly consists of hydroxyl

containing amino acids. (ii) The middle region (between 'N' and 'C' region of transit peptide) is more loosely conserved and called the 'n'-region. (iii) The C-terminal region is made of approximately 25 amino acids forming a hydrophobic thylakoid transfer domain (lumen targeting; van Gelder et al., 1997). It has been proposed that the presequence of the transit peptide is removed by stromal processing peptidase (SPP) upon import of the precursor (Robinson and Ellis 1984). The thylakoid-targeting domain is proposed to be removed by a thylakoid processing peptidase (TPP) while crossing the thylakoid or immediately thereafter (Cline and Henry 1996; Robinson and Mant 1997).

All pPPOs (precursors; 66-71 kDa) contain a two-domain transit peptide (as explained above) typical of lumen proteins and are also among the largest lumen proteins described (Steffens et al., 1994; Boss et al., 1995; Joy et al., 1995). Sommer et al. (1994) have shown that a tomato pPPO (67 kDa) is routed to the lumen in two steps by plastids from different plants. Import to the stroma was followed by the removal of the N-terminal domain of the transit peptide, and the resulting 62 kDa intermediate continued to the thylakoid lumen by a light-dependent reaction and accumulation as the 59 kDa mature protein (Sommer et al., 1994). Koussevitzky et al. (2004) have demonstrated that pretreatment with methyl jasmonate (MeJA) considerably increased the efficiency of isolated tomato plastids to import and process pPPO (precursor). The effect on PPO import and translocation was evident after 8-16 hrs exposure to MeJA. Nielsen et al. (1996) have not found an N-terminal transit peptide in fungal tyrosinases by the von Heijne method (von Heijne, 1983). This agrees with the absence of plastidial

compartments in fungi; accordingly, fungal tyrosinases have been localized in the cytosol (Wichers et al., 1995).

Polyphenol Oxidase Structure

The crystal structure of monomeric 39 kDa catechol oxidase from sweet potato (*Ipomoea batatas*) was analyzed and found to be ellipsoidal in shape with dimensions of 55x45x45 Å. The secondary structure is shown to be primarily made of α -helices with the core of the enzyme formed by a four-helix-bundle composed of α -helices $\alpha 2$, $\alpha 3$, $\alpha 6$ and $\alpha 7$ (Klabunde et al., 1998). This helical bundle was shown to accommodate the catalytic dinuclear copper center, and is surrounded by helices $\alpha 1$ and $\alpha 4$, and several short β -strands. The four helices of the α -bundle contribute three histidine residues, which are coordinated with the two active site coppers (Klabunde et al., 1998). In addition to the histidine ligands, the two active site copper ions are also coordinated by a bridging solvent molecule, most likely a hydroxide ion (Klabunde et al., 1998).

Activation of Polyphenol Oxidases

Though, plant PPOs have been located in thylakoid membranes (Golbeck and Cammarata 1981; Chazarra et al., 1996), they are not intrinsic membrane proteins. They can be released from the thylakoids by sonication or mild detergent treatment. PPOs have also been detected in soluble fractions in homogenates from different plant species (Mayer and Harel 1991). One unusual and intriguing characteristic of PPOs is their ability to exist in an inactive and latent state and they can be activated by various

methods, such as acid and base treatment or exposure to strong anionic detergents like sodium dodecyl sulfate (SDS) and N-lauroylsarcosine (van Gelder et al., 1997; Chazarra et al., 2001; Okot-Kotber et al., 2002). Several PPOs have also been activated or solubilized after partial proteolytic degradation (Nozue et al., 1999; Pérez-Gilabert et al., 2001; Gandía-Herrero et al., 2005). Chaotropes like urea and guanidine.HCl (GND) were also shown to activate PPOs in bran extracts from several wheat cultivars (Okot-Kotber et al., 2002).

The use of SDS is of particular interest since few enzymes are known to be activated by this detergent, while many are inactivated (Moore and Flurkey 1990). The activation process by SDS was found to involve a reorganization of the tertiary structure (Gandía-Herrero et al., 2005). A limited conformational change due to binding of small amounts of SDS may induce or initiate the activation of latent enzyme, and it was reported that the low optimum pH obtained for the latent enzyme was abolished in the presence of SDS. The possible relationship between these two factors (pH and SDS) has been studied in broad bean, grape and lettuce PPOs (Jiménez and García-Carmona 1996; Valero and García-Carmona 1992a; Chazarra et al., 2001a/b). Furthermore, the reversibility of the SDS-mediated activation of latent PPO from peach has been demonstrated by use of cyclodextrins as an agent to trap SDS molecules (Laveda et al., 2000). With respect to the physiologically relevant counterpart of the detergent, it has been suggested that lipids might fulfill this role (van Gelder et al., 1997). SDS activation of tyrosinase has been demonstrated *in vivo* with *Terfezia clavervyi* sections (Pérez-Gilabert et al., 2004).

Two types of behavior (hyperbolic and sigmoidal) have been reported for beet root PPO activation by SDS, depending on substrate nature (Gandía-Herrero et al., 2005). A hyperbolic behavior was exhibited by soluble PPO activity with different hydrophobic substrates tested (catechol, 4-methyl catechol and 4-tert-butyl alcohol) with increasing SDS concentration (Gandía-Herrero et al., 2005). A sigmoidal behavior was observed for PPO activation with increasing concentrations of SDS towards different hydrophilic substrates (L-tyrosine, L-DOPA, tyramine and dopamine). Maximum activation of the enzyme using hydrophobic substrates was observed at low SDS concentrations (near 0.01mM) at which the degree of activation was negligible for the hydrophilic substrates. Differential activation of PPO mediated by SDS may be relevant in the control of PPO activity since the enzyme is able to express activity toward a specific substrate while remaining latent to others (Gandía-Herrero et al., 2005).

Trypsin seems to be the most effective protease against PPOs from plants: grape (Sánchez-Ferrer et al., 1989), broad bean (King and Flurkey 1987), spinach (Golbeck and Cammarata 1981) and carrot (Söderhäll et al., 1985). Gandía-Herrero et al., (2005) have reported that trypsin treatment may modify the structure of the enzyme, probably improving the accessibility of the active site to the substrate, without affecting its integrity. Their study also showed that conformational changes provoked by SDS and by trypsin proteolysis lead to the same final effect, indicating that the same portion of the enzyme is involved in both processes. Moreover, their data clearly demonstrated for the first time, the existence of common regulation of the activation processes mediated by the protease trypsin and the detergent SDS.

In the same context, it has been suggested that a C-terminal extension of the polypeptide chain might mask access to the active site, and that removal of this extension may be the basis for enzyme activation (Gerdemann et al., 2002). This was indicated based on the comparative modeling of latent sweet potato (*Ipomoea batatas*) catechol oxidase (CO), using the hemocyanin (odgHC) structure from giant octopus (*Octopus dofleini*). Hemocyanins are dinuclear copper (type-3 copper center) containing proteins involved in oxygen transport in mollusks and arthropods (Himmelwright et al., 1980; Solomon et al. 1994). The superposition of 3D structural data of CO and odgHC show a close relationship for almost the entire backbone, resulting in a root mean square deviance of 1.27 Å for 704 backbone atoms (Eicken et al., 1999). Hemocyanins show no or only weak catecholase activity due to this C-domain, probably indicating an inhibitory function of this extension peptide.

Acid and basic shock has also been used to activate PPOs (Kenten 1957; Valero and García-Carmona 1992b). PPO from *Vicia faba* was found to be enzymatically inactive in aqueous buffers at neutral pH, but is active at acidic pH range of 3-4 (Jiménez and García-Carmona 1996). However, the presence of SDS in the reaction medium was shown to eliminate the activity at such acid pH and greatly increase the activity at neutral pH. The pH profile for trypsin-activated red beet PPO shows that the highest value in the activation process was obtained at pH 6.5 (Gandía-Herrero et al., 2005). The same study revealed that the dependence of the pH profile for PPO activity on the presence of SDS disappeared for the enzyme incubated with trypsin. Söderhäll (1995) has reported that a

completely latent PPO purified from carrot cell extract was active only in the presence of Tris buffer at pH 8.0.

Inhibition of Polyphenol Oxidases

Plant polyphenols are produced as secondary metabolites by most plants, in which they have numerous biological activities. Flavonoids (benzo- γ -pyrone derivatives consisting of phenolic and pyrane rings) are one of the best-studied groups of plant polyphenol (Kim and Uyama 2005). Some flavonoids, such as kaempferol, quercetin and morin inhibit tyrosinase activity (Kubo and Kinst-Hori 1999a; No et al., 1999; Kubo et al., 2000). Interestingly, quercetin and kaempferol can chelate copper in the met form of tyrosinase. This behavior differs from that of kojic acid, a fungal metabolite, which chelates via the oxy form (Cabanès et al., 1994). Captopril [(2S)-N-(3-mercapto-2-methyl propionyl)-L-proline] can inhibit both monophenolase and diphenolase activity of tyrosinase. Captopril inhibits tyrosinase activity by chelating the copper ions at the active site (Jay et al., 1991; Bartosz et al., 1996). Metallothionein from *Aspergillus niger* also inhibits PPOs by acting as a highly effective demetallizing agent (chelating copper; Hamer 1986).

Gallic acid inhibits the oxidation of L-DOPA catalyzed by tyrosinase (Shahidi and Naczki 1995). In addition, gallic acid itself seems to act as a substrate, being oxidized prior to L-DOPA which accelerates the oxidation of gallic acid. The resulting o-quinones of gallic acid may condense with one another through Michael-type addition, yielding a relatively stable quinol-quinone intermediate (Sayre and Nadkarni 1994). 4-Substituted

resorcinols, which are structurally related to phenolic substrates such as other slow-binding inhibitors, have recently been recognized as tyrosinase inhibitors (Jiménez and García-Carmona 1997). Presently, 4-hexylresorcinol is considered to be the most effective inhibitor for use in the food industry since it is safe (noncarcinogenic/nonmutagenic/nontoxic) and free of harmful side effects (Kim and Uyama 2005). It has also been recognized as safe for prevention of shrimp melanosis (McEvily et al., 1991; Iyengar et al., 1991) and for browning control of fresh and hot-air-dried apple slices as well as potatoes and avocados (Frankos et al., 1991; Iyengar et al., 1991). Tropolone is one the most potent tyrosinase inhibitors. It is structurally analogous to o-diphenolic substrates of tyrosinases, as well as an effective copper chelator (Kahn and Andrawis 1985). A study by Núñez-Delicado et al., (2003) has shown tropolone to be the most potent inhibitor of persimmon fruit PPO.

A large number of aldehydes and other compounds were also characterized as tyrosinase inhibitors, such as cinnaldehyde, (2E)-alkenals, 2-hydroxy-4-methoxybenzaldehyde, anisaldehyde, cuminaldehyde and cumic acid (Kubo and Kinst-Hori 1998 a/b; Kubo and Kinst-Hori 1999b/c; Lee et al., 2000). Usually, aldehyde groups react with nucleophiles like sulfhydryl, amino and hydroxyl ions (Kim and Uyama 2005). Tyrosinase inhibitory mechanism of aldehydes presumably comes from their ability to form a Schiff base with a primary amino group in the enzyme (Kubo and Kinst-Hori 1998b/1999b).

The enzymatic browning can be prevented by trapping the o-dopaquinone intermediate with cysteine or ascorbic acid (Friedman and Bautista 1995; Friedman,

1996). 2-Mercaptoethanol was reported to be the most effective inhibitor of PPO from Chinese cabbage (Nagai and Suzuki 2001). These authors have also reported an inhibitory effect of ascorbic acid, glutathione and L-cysteine (in that order). Hexadecyl trimethyl-ammonium bromide (HCTAB), polyvinylpyrrolidone 40, cinnamic acid, and salicylhydroxamic acid were shown to inhibit PPO activity from leaf as well as fruit endosperm of coffee (Mazzafera and Robinson 2000). Dihydrobenzoic acids (DBA) such as 3,4-DBA, 3,5-DBA and 2,4-DBA inhibited L-DOPA oxidation by mushroom tyrosinase (Schved and Kahn 1992). Dimethyl sulfide (DMS) was also reported as a slow-binding inhibitor of mushroom tyrosinase (Pérez-Gilabert and García-Carmon 2001). The effect of ascorbic acid, sodium bisulfate and other reducing agents on tyrosinases has been controversial (Golan-Goldhirsch and Whitaker 1984). Sulfites are being used in a restricted manner in the food industry due to their potential health hazards (Taylor and Bush 1986).

The inhibitory action of phenylthiourea (PTU) on catechol oxidase from sweet potato has been studied in detail (Klabunde et al., 1998). The sulphur of PTU has been shown to replace the hydroxo-bridge, present in the enzyme, and is coordinated to both copper ions, thereby increasing the metal-metal separation (Klabunde et al., 1998). The same study also reported that the aromatic ring of Phe 261 is crucial and that it controls access to the active site of the enzyme. Binding of phenylthiourea ($IC_{50} = 43\mu M$, $K_M = 2.5mM$ for catechol substrate) brings about a conformational change in the phenyl ring of Phe 261 and the imidazole ring of His 244 to form hydrophobic interactions with the aromatic ring of the inhibitor (PTU). In addition to these interactions with the dicopper center, van

der Waals interaction between the residues lining the hydrophobic cavity (Phe 261, Ile 241, His 244) contribute to high affinity of PTU to the enzyme and leading to its inhibition (Klabunde et al., 1998).

Physiological Roles of Polyphenol Oxidases

While the biological functions of PPOs are not yet understood in detail, available information indicates a role of these enzymes in defense against pathogens and herbivores. This view is supported by the following facts (Dongfeng et al., 2004): (i) the binding of o-quinones to proteins can reduce the nutritive value of nucleophilic amino acids and seems to induce an antinutritive defense (ii) o-quinones can restrain bacterial propagation and (iii) an increase in PPO activity has been observed in incompatible host-pathogen interaction. Thipyapong et al. (2004) have shown that antisense PPO expression in tomato dramatically increased susceptibility to *P. syringae* pv. *tomato* expressing the avirulence gene *avrPto* in both *Pto* and *pto* backgrounds. They hypothesize that at physiological pH nucleophilic quinones are more likely to undergo reverse disproportionation with phenols to form two equivalents of semiquinone radicals (Guyot et., 1995, 1996). These semiquinones may interact with O_2 to generate O_2^- . The role of PPO in generation of such reactive oxygen species (ROS) is evident from the large contribution PPO-based phenolic oxidation makes to H_2O_2 production in plant extracts (Jiang and Miles 1993; Richard-Forget and Gaillard 1997).

H_2O_2 plays an important role in the vicinity of pathogen attack by providing direct anti-microbial toxicity, by serving as a substrate for oxidative cross-linking of cell walls, as a diffusible inducer of protective genes, and as a trigger of cell death resulting in

restricted lesions delimited from surrounding healthy tissue (Levine et al., 1996; Grant and Laoke 2000; Garcia-Olmedo et al., 2001). In addition, an ROS-mediated systemic signaling network may also mediate establishment of plant immunity (Grant and Laoke 2000). PPO-overexpressing transgenic tomato plants oxidized the endogenous phenolic substrate pool at a higher rate than control plants. The PPO-overexpressing tomato plants exhibited a great increase in resistance to *P. syringae* (Li and Steffens 2002). Compared with control plants, transgenic lines showed less severity of disease symptoms, with over 15-fold fewer lesions, and strong inhibition of bacterial growth, with over 100-fold reduction of bacterial population in the infected leaves (Li and Steffens 2002).

The involvement of PPOs in the biosynthesis of low molecular weight products has been proven in two different pathways of plant pigment biosynthesis. A tyrosinase involved in betalain biosynthesis has been found in common portulaca (*Portulaca grandiflora*) and red beet (*Beta vulgaris*) (Steiner et al., 1999). Betalains are nitrogen-containing water soluble pigments accumulating in flowers, fruits and occasionally in vegetative tissues of plants of most families of the Caryophyllales (Steglich and Strack 1990). A chalcone-specific PPO participates in aurone biosynthesis and its involvement has been demonstrated in snapdragon (*Antirrhinum majus*) flowers (Strack and Schliemann 2001). Besides chalcones, flavanones, and dihydroflavonols, aurones belong to the group of “minor flavonoids” (Bohm 1982). Aurones are gold-colored (latin aurum=gold) pigments occurring as glycosides in petals of some members of the Asteraceae (e.g. Cosmos), Scrophulariaceae (e.g. Snapdragon), Cyperaceae and Fabaceae (Strack and Schliemann 2001).

Many other hypotheses surround the possible physiological role of PPOs in plants. Roles in the phenylpropanoid pathway (i.e., coumarate 3-hydroxylase; Vaughn and Duke 1984; Kojima and Takeuchi 1989), the Mehler reaction, electron cycling, oxygen regulation (Vaughn et al., 1988; Trebst and Depka 1995) have been proposed. However, data published by Thipyapong et al. (2004) are not consistent with a role for PPOs in the Mehler reaction.

Mapping of PPO Genes

Color is one of the main factors influencing noodle/pasta quality. A better understanding of genetic control of PPO activity will help in developing wheat varieties with reasonably low-PPO activity. The development of molecular markers as aids for plant selection will accelerate wheat breeding, since the selection of plants can be carried out based on genotype rather than phenotype (Simeone et al., 2002). Evidence for the presence of several PPO genes or isoforms has been found in species for which a more detailed analysis was performed, such as wheat (*Triticum aestivum* L.; Kruger 1976; Demeke and Morris 2002), tomato (*Lycopersicon esculentum* L.; Thipyapong et al., 1997) and poplar (*Populus spp.*; Constabel et al., 2000). PPO genes have been cloned from several plant species, including sugar cane (*Saccharum officinarum* L.; Bucheli et al., 1996), potato (*Solanum tuberosum* L.; Thygesen et al., 1995), tomato (*Lycopersicon esculentum* L.; Shahr et al., 1992), *Vicia faba* (Cary et al., 1992), grape (*Vitis vinifera* L.; Dry and Robinson 1994) and apricot (*Prunus spp.*, Chevalier et al., 1999).

Generally, PPOs from monocotyledonous plants are less well investigated than those found in dicotyledons. It has been shown that PPO expression and activity are influenced

both by environmental conditions and genotype (Lamkin et al., 1981; Baik et al., 1994; Park et al., 1997). Tetraploid durum wheat (*Triticum turgidum* L.; genome organization AABB) has significantly lower kernel PPO activity than hexaploid (genome organization AABBDD) bread wheat cultivars (*Triticum aestivum* L.; Kruger 1976; Lanning et al., 2003; Demeke and Morris 2002). Based on mapping efforts by Jimenez and Dubcovsky (1999), gene(s) responsible for this difference may be present on chromosome 2D; however, Simeone et al. (2002) have also located a quantitative trait locus for PPO activity on chromosome 2A, which is present both in tetraploid and hexaploid wheats. Demeke et al. (2001) have located QTL markers in their RIL populations on chromosomes 2A, 2B, 3D and 6B.

In spite of their potential commercial importance, the biochemical and molecular properties of wheat PPOs were largely unknown prior to the work presented in this dissertation. My research addressed several issues relating to the biochemical and molecular characterization of wheat PPOs with special emphasis on kernel PPOs, as explained in the following three research chapters. During the course of this study, we developed an improved enzymatic assay for PPO screening, which is especially useful for tissues/materials with inherently low PPO activity (e.g., flour; Jukanti et al., 2003). We also worked on the activation/solubilization effects of various detergents on wheat PPO(s) from kernel and flour samples. The results from this study indicated that PPOs extracted in the absence of detergents from kernels of high PPO-activity wheat varieties were activated by SDS, but this was not observed with low-PPO germplasm (Jukanti et

al., 2003). SDS was further shown to have a combination of improved extraction and activation effect on meal samples from high PPO-activity wheat varieties.

Exhaustive EST database searches and full-length re-sequencing of identified clones have indicated the presence of a multi-gene family of at least six wheat PPOs. Phylogenetic analysis of these six sequences has revealed two distinct clusters of three similar sequences each (Jukanti et al., 2004). We used both molecular and biochemical approaches to investigate which PPO gene(s) are predominantly expressed in wheat kernels, and how they are regulated. We have identified a cluster of three genes which play a more important role in darkening reactions of wheat-based products. A major kernel PPO was purified and its identity was confirmed using MALDI-TOF analysis. We have shown experimentally for the first time the regulation of wheat kernel PPOs both at transcriptional and post-transcriptional level. Proteolytic processing of wheat PPOs was demonstrated using immunoblot and trypsin activation studies. Together, results presented in this dissertation have firmly established the molecular basis of wheat PPO function, and have allowed novel insights into PPO expression and activation.

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CHAPTER 2

EXTRACTION AND ACTIVATION OF WHEAT POLYPHENOL OXIDASE BY
DETERGENTS: BIOCHEMISTRY AND APPLICATIONSIntroduction

Polyphenol oxidases (PPOs) are copper-containing metalloproteins which have been found or characterized in numerous higher plant species (Söderhäll 1995; Kwon and Kim 1996; Ho 1999; Chazarra et al 2001; Gooding et al 2001; Kim et al 2001; Nagai and Suzuki 2001). Evidence for the presence of several genes or isoforms has been found in species for which a more detailed analysis was performed (e.g. wheat, Kruger 1974; tomato, Thipyapong et al 1997). Higher plant PPOs catalyzes the hydroxylation of o-monophenols to o-diphenols (E.C. 1.14.18.1; monophenol monooxygenase, tyrosinase or cresolase activity) and the oxidation of o-dihydroxyphenols to o-quinones (E.C. 1.10.3.2; diphenol oxidase or catecholase activity; Steffens et al 1994; van Gelder et al 1997). At present, the physiological role of PPOs is not entirely clear, but evidence points to a defense-related role of these enzymes in higher plant tissues (Constabel et al 1995; van Gelder et al 1997).

Some of the PPOs partially or completely purified and characterized so far show interesting biochemical features. In several species, these enzymes are associated with plastidial membranes, specifically with thylakoid membranes of chloroplasts (Jiménez and García-Carmona 1996; van Gelder et al 1997). PPOs appear to be present in a latent

form in some plant tissues and can be activated by various treatments, such as acid and base shock or exposure to the strong anionic detergent sodium dodecyl sulfate (SDS; van Gelder et al 1997; Chazarra et al 2001, Okot-Kotber et al 2002). Several PPOs have also been found to be activated / solubilized or at least remain active after partial proteolytic degradation (van Gelder et al 1997; Nozue et al 1999; Pérez-Gilabert et al 2001). These characteristics demonstrate the potential for a complex in vivo regulation of this group of enzymes, and potentially complicate in vitro analyses.

PPO has been implicated in enzymatic browning of vegetables, fruits and cereal products (Feillet et al 2000). Specifically, it has been demonstrated that PPOs play a major role in the time-dependent darkening of noodles and other wheat products (Bhattacharya et al 1999; Feillet et al 2000). Since a bright color is usually preferred for noodles, it is one of the goals of many wheat breeding programs to develop lines with reasonably low PPO levels in mature grains. In spite of their potential commercial importance, the biochemical properties of wheat grain PPOs are at present not well-known; recent evidence suggests that PPOs from wheat bran may be influenced by certain organic solvents and SDS (Okot-Kotber et al 2002). Therefore, the main goal of the work presented here was to investigate the influence of different types (anionic, nonionic and cationic) of detergents on the solubilization, activation and stability of PPOs present in meal and flour of both high- and low-PPO wheat lines. Results from this characterization were utilized to develop a robust PPO assay able to reliably discriminate low enzyme activities.

Materials and Methods

Plant Material

Thirty winter wheat (*Triticum aestivum* L.) genotypes including named cultivars and experimental lines (listed in Table 1.1) were grown at Bozeman, Havre, Huntley, and Conrad, MT in a randomized complete block design with three replications during the 2000-2001 season. Seed for the three replications for each entry was bulked upon harvest. Thus, the locations served as the replications for subsequent PPO assays and noodle sheet preparation (Tables I and II).

Assays were performed on intact kernels, whole-kernel meal from a Perten Laboratory Mill, model 3303, equipped with a type 2 (fine) grinding disc (Perten Instruments Inc., Springfield, IL), and flour obtained by the use of a Quadrumat Senior mill (SW Brabender, South Hackensack, NJ) after a two-stage temper to 15% moisture.

PPO Assays

For the evaluation of detergent effects on PPO extraction and activity, whole-kernel meal or flour (125 mg) from a high- (Rampart) and low- (Utah 100) PPO variety was extracted with 500 μ L extraction medium, consisting of 50 mM MOPS buffer pH 6.5 with 0-100 mM SDS (Fig. 2.1), or 0-50 mM Tween-20, Tween-80, Triton X-100, cholic acid, N-lauroylsarcosine, hexadecyltrimethyl-ammonium bromide (HCTAB), or SDS (Fig. 2.2). Extractions and assays were performed at least four times for each detergent concentration with both the high- and low-PPO lines, and means and standard deviations were calculated from the independent assays. Each sample was extracted in a 1.5 mL

Eppendorf tube, using a motor unit and pestle fitting the conical bottom of the tube for 30 sec. The tubes were then shaken for 30 min on a MS1 minishaker (IKA-Works Inc., Wilmington, NC) before centrifugation during 10 min at 20,000 g. Supernatants were used for PPO activity assays either directly, or after shock-freezing in liquid nitrogen and storage at -20 °C. Supernatants (20 - 100 µL, depending on PPO activity) were transferred to wells of 96-well microplates, and extraction buffer (50 mM MOPS pH 6.5 with or without detergent, depending on sample) was added to a total volume of 100 µL. Enzyme assays were started by adding 100 µL substrate solution (10 mM L-3,4-dihydroxyphenylalanine [L-DOPA] in 50 mM MOPS buffer pH 6.5), and optical density at 475 nm was assayed kinetically during 20 min using a SPECTRAMax PLUS384 spectrophotometer (Molecular Devices, Sunnyvale, CA). Activities were calculated from the increase in optical density during the linear phase of the reaction, and expressed as $\Delta OD_{475} \text{ hr}^{-1} \text{ g}^{-1}$ flour or meal. Blanks were performed by omitting L-DOPA from samples extracted in presence and absence of SDS, and rates were found to be negligible. Additionally, darkening of substrate solution (L-DOPA) was tested without enzyme, both in presence and absence of detergents, and also found to be negligible during the time of the assay.

To determine if SDS enhanced PPO activity by improved extraction or enzyme activation (Fig. 2.3), assays were performed as described above, using 50 mM SDS in the extraction buffer. Whole-kernel meal from five high- (Tiber, Windstar, Bigsky, NuHorizon, Harding) and five low-PPO lines (Ransom, Utah 100, GM10003, Gary, MT9513) was used for this experiment. Activities measured from samples extracted in

presence of 50 mM SDS were defined as 100% for each line (bars labeled "50 mM SDS" in Fig. 2.3). Additional samples were extracted in absence of SDS. Extracts (supernatants) prepared in absence of SDS were either assayed directly as described (bars labeled "0 mM SDS" in Fig. 2.3), or brought to 50 mM SDS and incubated for 5 min at room temperature in microplate wells prior to starting the enzyme assay (bars labeled "Enzyme activation" in Fig. 2.3). Additionally, pellets from extracts prepared in absence of SDS were re-extracted with 50 mM MOPS pH 6.5 containing 50 mM SDS and shaken for 30 min at room temperature prior to measuring PPO activity (bars labeled "pellet extraction" in Fig. 2.3). The complete experiment was performed at least twice for each line, and means and standard deviations were calculated separately from results obtained with the five high- and low-PPO lines, respectively.

Based on these optimization experiments, flour and meal samples analyzed for Tables I and II were extracted directly in presence of 50 mM SDS and analyzed as described. PPO activity in whole kernels was assayed using a modification of the methods described in Anderson and Morris (2001). For each sample, two kernels were incubated in 200 μ L substrate solution (10 mM L-DOPA in 50 mM MOPS buffer pH 6.5) in a well of a standard 96-well microtitration plate. The plates were shaken for 2 hr at room temperature on a MS1 minishaker. The assay was duplicated in two wells (with a total of 4 kernels) and the two values were averaged. Blanks were set up by incubating kernels in buffer without L-DOPA. After 2 hr, solutions were transferred to a new microtitration plate, and OD₄₇₅ was determined using a SPECTRAmax PLUS384 spectrophotometer.

For all assays (kernels, meal and flour), activities were calculated as increase in OD475 (Δ OD) during 1 hr, based on 1 g of meal/flour, or on 100 kernels.

Noodle Sheet Preparation, Incubation and Color Determination

A subset of 16 (BigSky, Culver, DW, Gary, Golden Spike, Morgan, MT9513, Neeley, Nuplains, NuSky, NuWest, Paul, Rampart, Rocky, Tiber, Utah 100) of the 30 genotypes mentioned above was used for noodle preparation, and to assay for color stability. Some lines were selected based on known characteristics (high- or low-PPO), and compared to experimental germplasm. Chinese raw noodles were prepared from 100 g flour and 29.2 mL salt water solution (4.29% w/v) added to the flour over a 30 sec time period. Mixing continued on a National pin mixer (National Mfg., Lincoln, NE) for 5 min. After mixing, dough was rested in a plastic bag for 30 min at room temperature, then folded and passed through an Ohtake Laboratory noodle machine (Ohtake Manufacturing Co., Ltd., Tokyo, Japan) three times at a gap size of 5 mm and an instrument temperature of 30 °C. After a second 30 min rest at room temperature, dough was successively passed through five progressively narrower roller settings to achieve a final noodle sheet thickness of 1.2 mm. Dough sheets were stored in plastic bags at room temperature and evaluated for color using the Minolta CR-310 Chroma Meter (Minolta, Ramsey, NJ) using the Commission Internationale de l'Eclairage (CIE) L* (brightness) a* (red-green) b* (yellow-blue) color system 0 and 24 hr after sheeting. L* value expressed sample brightness on a scale from 0 (black) to 100 (perfect white; Park et al 1997). Values of a* and b* indicated the red-green and yellow-blue axes, with positive a* and b* values indicating increased redness and yellowness (Park et al 1997).

Statistical Analysis of Data

Analysis of variance was computed for each PPO assay and for noodle color traits to verify differences among genotypes using locations as replications. Correlations were computed between whole kernel, whole-kernel meal and flour PPO activity and between PPO activity and noodle color characteristics using genotype means.

Results

Influence of Detergents on PPO Extraction and Activity

Since PPO activity is mainly found in the external layers of wheat kernels (bran), only low activities are present in milled flour (Demeke et al 2001; Okot-Kotber et al 2001; Rani et al 2001). Therefore, it can be difficult to assay these enzymes with visible range spectrophotometry using standard substrates such as L-DOPA, catechol, methyl catechol, tyrosine and phenol (Anderson and Morris 2001). Since an influence of the strong anionic detergent SDS on PPO activities from several other (non-cereal) species has been described in the past (van Gelder et al 1997), and a recent report also indicates that this detergent influences PPO activity extracted from wheat bran (Okot-Kotber et al 2002), flour and meal from one high- (Rampart) and one low-PPO (Utah 100) winter wheat line were extracted in presence of 0-100 mM SDS. Figure 2.1 (A, B) demonstrates a 13- to 15-fold increase in extractable PPO activity when flour from low- or high-PPO lines was extracted in presence of 50 mM SDS. A similar increase was observed in whole-kernel meal extracts from the low-PPO line (Figure 2.1 C), and a ~5-fold increase was seen in the line with the highest extractable PPO activities (Figure 2.1 D).

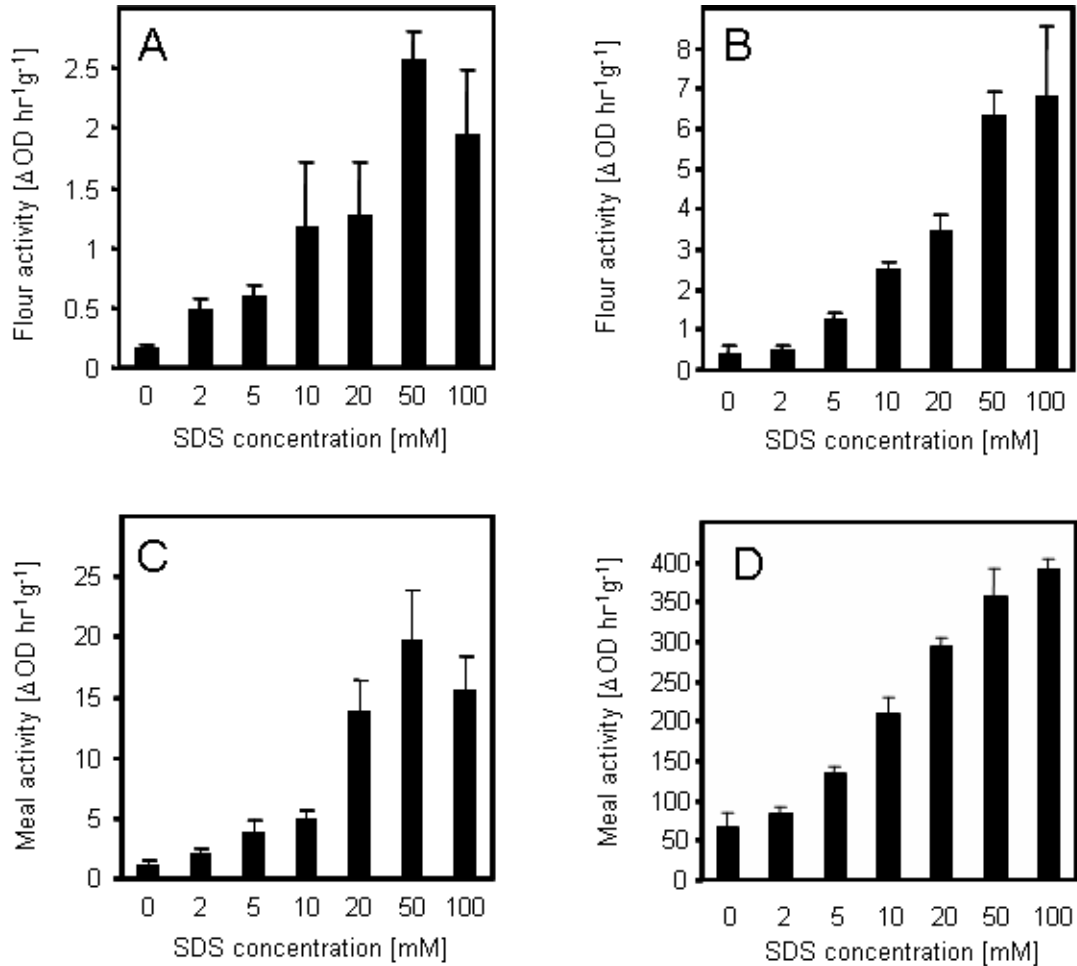


Fig. 2.1 Influence of SDS concentration on polyphenol oxidase (PPO) extraction and activity. Flour (A,B) and whole kernel meal (C,D) from a low-PPO (A,C; Utah-100) and a high-PPO (B,D; Rampart) winter wheat line were extracted with 0-100 mM SDS. Means and standard deviations of four independent extractions are shown for each SDS concentration.

An influence of time on extraction efficiency by SDS might be expected. Therefore, whole-kernel meal and flour from both Rampart and Utah 100 was incubated at room temperature on a rotary shaker during different time spans from 0 to 240 min after initial mechanical extraction. Interestingly, no major influence of extraction time was observed (data not shown); in order to standardize the method and accommodate for time needed to analyze series of samples, all extracts were incubated 30 min before centrifugation and

enzyme activity analysis. It is important to notice that PPOs appeared to be very stable in both flour and meal SDS extracts, and that even after 4 hr of incubation no major activity losses occurred.

To investigate if the increase in wheat kernel PPO activity after SDS extraction is specific, the effect of several additional anionic and nonionic as well as one cationic detergent (hexadecyl trimethyl-ammonium bromide, HCTAB) on enzyme activity was tested at concentrations of 10, 20 and 50 mM, again using one low- (Utah 100) and one high-PPO line (Rampart; Fig. 2.2).

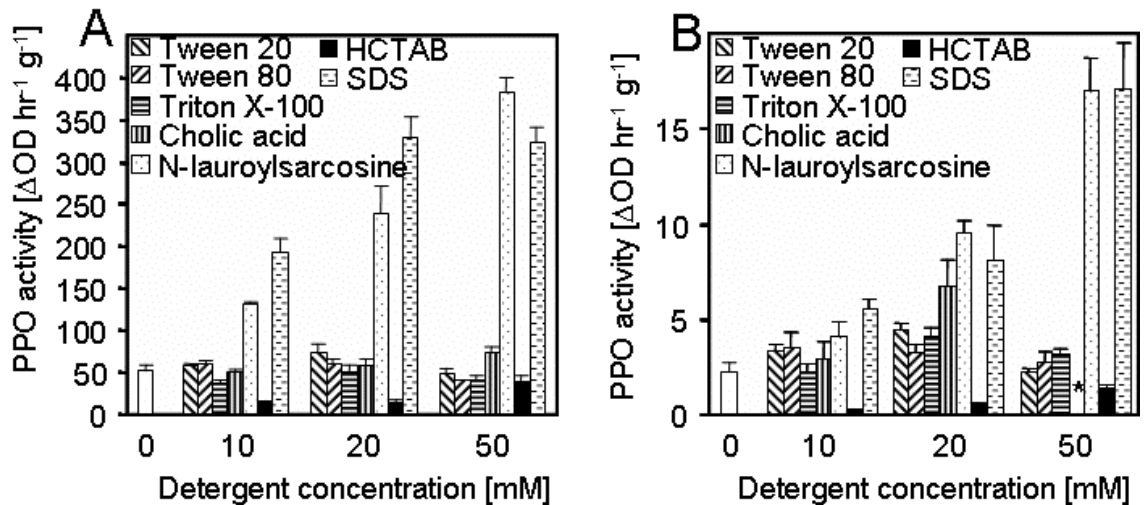


Fig. 2.2 Influence of different detergents on polyphenol oxidase (PPO) extraction and activity. Whole-kernel meal from a high-PPO (A, Rampart) and a low-PPO winter wheat (B, Utah-100) were extracted in presence of 0-50 mM of the detergents indicated. Means and standard deviations of four independent extractions are shown for each detergent at different concentrations. HCTAB, hexadecyltrimethyl-ammonium bromide. *Data point for cholic acid missing due to formation of precipitates.

In the high-PPO line, HCTAB inhibited PPO activity; among the other detergents, N-lauroylsarcosine increased extractable PPO activity comparably to SDS, while none of the other detergents tested were effective. Essentially the same result was obtained with

the low-PPO line, although some of the other detergents (Tween-20, Triton X-100 and cholic acid) caused a slight increase at moderate concentrations (20 mM).

To decide if the effect of SDS on extractable PPO activity was due to improved extraction/solubilization or to enzyme activation, activities obtained after direct extraction of whole-kernel meal from five high- (Tiber, Windstar, BigSky, NuHorizon, Harding) and five low-PPO lines (Ransom, Utah 100, GM10003, Gary, MT9513) were compared with activities obtained a) after enzyme extraction and assay without SDS; b) after enzyme extraction without SDS, but addition of 50 mM SDS prior to enzyme assay; and c) after re-extraction in presence of 50 mM SDS of meal previously extracted without detergent (Fig. 2.3). Results indicated clearly that SDS greatly enhanced extraction/solubilization of wheat kernel PPO(s); however, our data also indicated that PPOs extracted from high-activity lines were activated in presence of this detergent (Fig. 2.3A). Interestingly, no activation was found when the same experiment was performed with low-PPO lines.

Total proteins were assayed in some extracts using the detergent-compatible BCA protein assay (Pierce, Rockford, IL). These results indicated that SDS increased overall protein extraction between 30 and >100%, depending on the genotype; however, our results also demonstrated a higher increase in extractable PPO activity than in overall protein extraction.

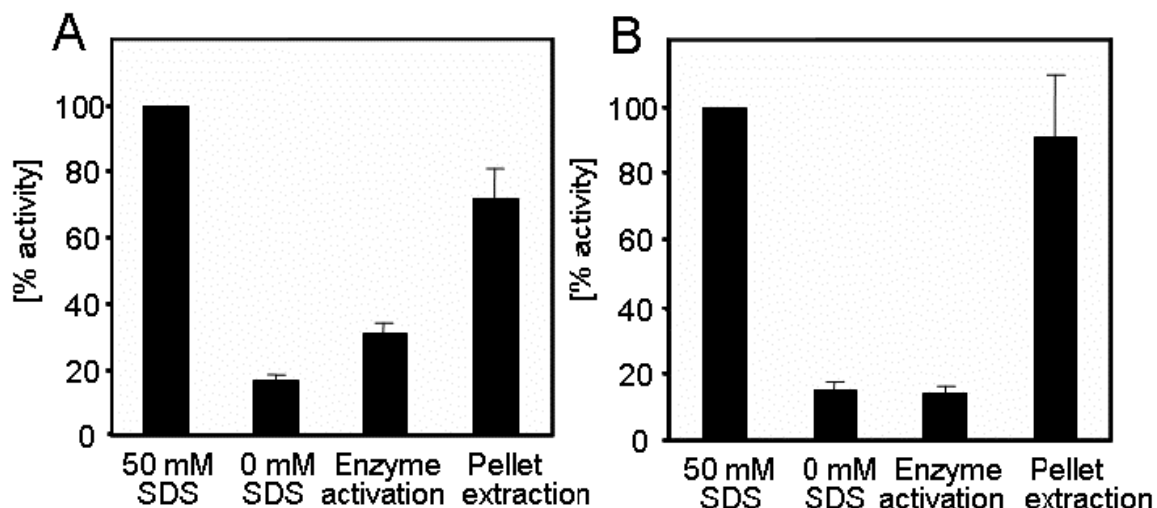


Fig. 2.3 Influence of SDS on PPO solubilization and activation in meal from five high- (A; Tiber, Windstar, BigSky, NuHorizon, Harding) and five low-PPO winter wheat lines (B; Ransom, Utah 100, GM10003, Gary, MT9513). Meal was extracted directly in presence of 50 mM SDS (defined as 100% activity) or in absence of SDS. Extracts (supernatants) prepared in absence of SDS were either assayed directly (0 mM SDS), or brought to 50 mM SDS prior to starting the assay (Enzyme activation). Pellets from extracts prepared in absence of SDS were re-extracted in presence of 50 mM SDS and incubated for 30 min prior to activity assays (Pellet extraction). Means and standard deviations calculated from the five lines are shown for both A and B. 100% activity corresponds to $155.2 \Delta\text{OD}_{475} \text{ hr}^{-1} \text{ g}^{-1}$ for A, and to $28.8 \Delta\text{OD}_{475} \text{ hr}^{-1} \text{ g}^{-1}$ for B.

Comparison of PPO Activity in Flour, Meal and Whole Kernels

To decide if PPO activities obtained after extraction of flour and meal with SDS reliably discriminate high- and low-PPO lines, activities obtained with three different methods (SDS extraction of flour and meal, and a whole-kernel assay in absence of detergents) were compared (Table 2.1). Analysis of variance showed that genotypes differed in PPO activity for all assay methods. The three PPO assay methods were also highly positively correlated with flour and meal being most highly correlated ($r=0.88$; $P<0.01$), while flour with whole kernel ($r=0.68$; $P<0.01$) and meal with whole kernel ($r=0.65$; $P<0.01$) were less highly correlated. The lower correlations involving whole kernels may reflect kernel-to-kernel variability inherent in this assay (McCaig et al 1999),

Table 2.1 Mean values for polyphenol oxidase (PPO) activity obtained from milled flour, whole-kernel meal, and intact kernels for 30 winter wheat genotype^a.

Genotype	Milled flour	Whole-kernel meal	Intact kernels
	[$\Delta\text{OD hr}^{-1} \text{ g}^{-1}$]	[$\Delta\text{OD hr}^{-1} \text{ g}^{-1}$]	[$\Delta\text{OD hr}^{-1}$ 100 kernels ⁻¹]
Utah 100	0.7	10.9	0.67
NuWest	1.4	51.3	0.86
NuSky	1.6	50.4	0.61
GM10003	1.8	27.2	0.73
Golden Spike	2.0	41.2	0.59
MT9513	2.0	28.5	0.71
BZ9W97-761	2.2	49.5	0.71
Gary	2.2	36.2	0.71
DW	2.5	86.7	0.84
McGuire	3.2	111.3	0.92
NuFrontier	3.8	144.3	1.00
Nuplains	4.2	100.7	0.88
Ransom	4.2	23.0	0.81
Pryor	4.3	105.8	1.45
Erhardt	4.6	148.2	1.24
Neeley	4.6	154.7	1.00
Windstar	4.9	169.3	1.27
BigSky	5.0	143.8	1.01
Judith	5.3	117.9	1.12
CDC Falcon	5.5	117.4	1.37
Morgan	5.5	168.5	1.00
Prower 99	5.5	143.1	0.92
NuHorizon	6.2	182.6	1.15
Culver	6.5	188.1	1.03
Rocky	6.7	171.0	1.13
Paul	6.8	150.9	1.28
BZ9W96-895	6.9	155.2	0.96
Tiber	6.9	173.5	0.95
Harding	8.0	175.9	1.19
Rampart	8.4	302.3	1.07
LSD(0.05)	2.4	55.5	0.35

^a Means of four replicates (material grown in four different locations) are shown for each genotype.

because the sample is not as homogenized as whole-kernel meal or flour. The correlations and the mean values (Table 2.1) show that high- and low-PPO genotypes can be separated by any of the assay methods.

Usefulness of PPO Assay Methods for Quality Trait Prediction

PPO has been implicated in the formation of an undesirable brown discoloration of wheat products such as Asian noodles (Feillet et al 2000). The genotypes differed for noodle brightness (L^*) at 0 and 24 hr and for decrease in noodle brightness with time (0 hr–24 hr; data not shown). All PPO assay methods showed statistically significant negative correlations with noodle brightness (L^*) at 24 hr and statistically significant positive correlations with decrease (0 hr–24 hr) in noodle brightness, while the correlations with L^* at 0 hr were significant only for the intact kernel and flour assays (Table 2.2). The three assay methods were about equal in their association with noodle brightness (L^*) at 24 hr and with time-dependent color change.

Table 2.2 Correlations between polyphenol oxidase (PPO) activities obtained from winter wheat intact kernels, whole kernel meal, and milled flour and noodle sheet color traits using 16 winter wheat genotypes^{a,b}.

Noodle sheet color trait	Intact kernel	Whole-kernel meal	Milled flour
L^* 0 hr	-0.60*	-0.51	-0.57*
L^* 24 hr	-0.67**	-0.68**	-0.71**
L^* 0–24 hr	0.70**	0.75**	0.78**
B^* 0 hr	0.13	0.03	0.14
B^* 24 hr	-0.03	-0.27	-0.15
B^* 24–0 hr	-0.26	-0.65**	-0.54*

^a Noodle sheets were evaluated for color after 0 and 24 hr. L^* values indicate sample brightness (higher values are equivalent to brighter sheets) and b^* values on the yellow-blue axis (higher values are equivalent to increased yellowness).

^b*, ** significant at $P < 0.05$ and 0.01 levels, respectively.

Data from noodle sheet quality analysis indicated an increase in yellowness (rising b^* values) over a 24 hr time period (data not shown). Initial and 24 hr b^* values were not significantly correlated with PPO activities measured with any of the methods (Table 2.2). PPO activities based on meal and flour were negatively correlated with increase (24 hr–0 hr) in yellowness (Table 2.2). PPO activity assayed in intact kernels was a less reliable predictor of time-dependent change, as correlation with intact-kernel PPO was less negative than those with meal and flour and not statistically significant. None of the PPO assay methods showed any trend or correlation between PPO activity and a^* values (noodle redness; data not shown).

Discussion

The most interesting finding of this research, both from an applied and a basic perspective, is the enhanced PPO activity found in wheat flour and meal after extraction in presence of SDS and N-lauroylsarcosine. It is especially noteworthy that high concentrations (50 mM) are most effective, and that wheat flour and meal PPO activities are quite stable under these conditions. No significant changes were observed between 1 and 4 hr at room temperature in presence of SDS (data not shown). From an applied point of view, the relative stability of these proteins probably enhances their role in undesirable food darkening processes.

Many of the plant and fungal PPOs characterized so far seem to be latent in their mature form (van Gelder et al 1997). A number of treatments to activate PPOs have been described, including detergent (SDS) treatment, acidification, lipids, and partial

proteolysis (Jiménez and García-Carmona 1996; van Gelder et al 1997; Pérez-Gilabert et al 2001); one recent publication (Okot-Kotber et al 2002) also demonstrates an influence of SDS and other (non-detergent, denaturing) compounds (e.g., guanidine-HCl) on extractable PPO activities in wheat bran, with a higher activation at 2% (~69 mM SDS) than at 1 or 5% SDS. Interestingly, only a ~3-fold PPO activation was found by these authors; this may be explained by the fact that specific activities were shown, and that improved PPO extraction / activation was partially masked by an increase in overall protein extraction. Based on flour or meal weight, a 5- to 15-fold increase in PPO activities was obtained after extraction with 50 mM SDS in our experiments (Figs. 2.1 and 2.2).

PPOs in different species have been reported to be membrane-associated (Jiménez and García-Carmona 1996). As the molecular and biochemical properties of wheat PPOs are at present largely unknown, the question arises if the observed increase in extractable PPO from wheat flour and meal in presence of SDS is due to enzyme activation, improved extraction (solubilization of membrane-associated PPO forms), or a combination of both. Our results (Fig. 2.3) indicate that, while SDS considerably increases PPO extraction in all varieties tested, some enzyme form(s) present in high-PPO lines are also activated by this detergent (Fig. 2.3A). A considerable fraction (~70% in high-PPO lines, and ~90% in low-PPO lines) of wheat kernel PPO activity can only be detected in presence of detergent, suggesting that wheat kernel PPOs, as PPOs from other plant species and tissues, are at least partially membrane-associated. Interestingly, among a series of additional detergents tested, only the anionic detergent N-lauroylsarcosine was

efficient at increasing PPO extraction (Fig. 2.2); this detergent is often used in biochemistry for membrane protein solubilization (Angel et al 1990).

While the influence of detergents on PPO biochemistry is interesting from a basic point of view, and can be used to improve PPO activity assays, this finding also points to the possibility that compounds naturally present in wheat kernels, such as fatty acids or lipids, might activate or improve extraction of these enzymes during manufacturing processes, thus contributing to undesirable food darkening. More detailed biochemical studies will be necessary to understand the importance of such effects.

It has been mentioned that PPO in wheat kernels is mostly associated with the bran, which is removed during milling (Rani et al 2001). Our results confirm this observation; on a weight basis, PPO activity was 8- to 15-fold higher in whole-kernel meal than in flour. While the reduction of PPO during the milling process is favorable with regard to quality-diminishing browning processes, the remaining activity appears to be sufficient to cause problems. From a practical point of view, low activities are more difficult to quantify reliably, at least in simple high-throughput assays using visible-range spectrophotometry. The addition of SDS during enzyme extraction appears to be an easy and elegant solution to this problem, allowing the analysis of a considerable number of flour samples per day by one person, especially if a multichannel photometer is used. It is conceivable, though, that this method would even improve visual rating of PPO activities if access to a spectrophotometer is not available.

Comparison of PPO activities obtained after SDS extraction of whole-kernel meal and flour, and after whole-kernel assays without detergents (Table 1.1) indicate that data

obtained after detergent extraction are reliable at discriminating low- and high-PPO lines. This is confirmed by statistically highly significant correlation coefficients between activities obtained with the three methods. Data presented in Table 1.2 also indicate that all three methods are comparable in their correlation with changes in noodle brightness, and final L^* values after 24 hours, and whole-kernel meal and flour assays also correlated with changes in b^* values over time. Together, these data strongly indicate that all three assay methods are reliable at quantifying PPOs present during noodle preparation; therefore, in breeding programs, screening for low PPO activity can be performed with the material most easily accessible in any given situation. The only possible restriction refers to the number of kernels necessary to obtain reliable results from whole-kernel assays. It appears likely that the lower correlations between the whole-kernel assay and other assay methods are due to high kernel-to-kernel variability.

Conclusions

This paper describes novel, interesting biochemical features of wheat kernel PPOs. Based on our data, it appears likely that these enzymes are partially membrane-bound. Their extraction / solubilization can be considerably improved by using relatively high (50 mM, or ~1.4% w/v) of sodium dodecyl sulfate or N-lauroylsarcosine, while a series of other detergents was not effective. Additionally, PPO(s) extracted from high-activity wheat lines in absence of detergents can be activated by SDS. Our data also indicate that wheat kernel PPO(s) are very stable in detergent extracts, and this property was utilized to develop a more robust assay for low activities present in milled flour.

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CHAPTER 3

EVALUATION OF WHEAT POLYPHENOL OXIDASE GENES

Introduction

Polyphenol oxidases (PPOs) are ubiquitous, copper-containing metalloproteins that have been found or characterized in numerous higher plants (Flurkey 1989; Cary et al 1992; Dry and Robinson 1994; Hind et al 1995; van Gelder et al 1997; Constabel et al 2000; Demeke and Morris 2002). These enzymes catalyze the hydroxylation of o-monophenols to o-diphenols (E.C. 1.14.18.1; monophenol mono-oxygenase, tyrosinase or cresolase activity) and the oxidation of o-dihydroxyphenols to o-quinones (E.C. 1.10.3.2; diphenol oxidase or catecholase activity) (Steffens et al 1994; van Gelder et al 1997). In subsequent reactions, the quinones react with amines and thiol groups or undergo self-polymerization to produce dark / brown products, which are usually undesirable in fresh or processed food (Feillet et al 2000; Anderson and Morris 2001). The central domain of all PPOs consists of two Cu-binding sites (CuA and CuB); at each of these sites, a Cu ion is bound by conserved histidine residues. This pair of Cu ions is the site of interaction of PPOs with both molecular oxygen and phenolic substrates (van Gelder et al 1997). When comparing PPO sequences from a wide variety of organisms, the Cu-binding regions are the most conserved domains of the polypeptide, with 42% sequence identity between the CuA and CuB regions of bacterial and human PPOs (van Gelder et al 1997). Several plant PPOs analyzed so far are associated with plastidial thylakoid membranes and

contain N-terminal transit peptides needed for plastid import (Hind et al 1995; Jiménez and García-Carmona 1996; van Gelder et al 1997). Typical transit peptides consist of 80-100 amino acids; peptide cleavage during chloroplast import reduces the molecular mass of several plant PPOs from 65-70 kD to <60 kD for the mature protein (Cary et al 1992; Dry and Robinson 1994; van Gelder et al 1997).

Evidence for the presence of several PPO isoforms or genes has been found in species for which a more detailed analysis was performed (e.g. wheat [*Triticum aestivum* L.], Kruger 1976; Demeke and Morris 2002; tomato [*Lycopersicon esculentum* L.], Thipyapong et al 1997; poplar [*Populus spp.*], Constabel et al 2000). At present, the physiological role(s) of these enzymes are not entirely clear, but available data point to a defense-related role of PPOs in higher plants (Constabel et al 1995; 2000; van Gelder et al 1997; Constabel and Ryan 1998; Mohammadi and Kazemi 2002). In this context it is interesting that the highest PPO activities are usually associated with young tissues, which may be especially susceptible to challenges such as insect attack (Hunt et al 1993; Dry and Robinson 1994). From an applied point of view, PPOs have been implicated in enzymatic browning of vegetables, fruits and cereal products (Feillet et al 2000; Nagai and Suzuki 2001). Specifically, it has been suggested that these enzymes play a major role in the darkening of noodles and other wheat products (Bhattacharya et al 1999; Feillet et al 2000). Because a bright color is usually preferred by consumers, especially for Asian noodles, it is one of the goals of many US wheat breeding programs to develop and/or maintain varieties with reasonably low PPO activities (and, therefore, higher commercial value) in mature grains. Interestingly, within the mature cereal grain, PPOs

are mostly found in the aleurone layer, which is removed during the milling process; however, the remaining activity appears to be high enough to cause problems during food processing (Rani et al 2001). In spite of their commercial importance, the genetic, molecular and biochemical properties of wheat PPOs are not yet well understood. Data have been published on substrate preferences of (unpurified) wheat kernel PPOs (Anderson and Morris 2001). Additionally, it has been demonstrated that wheat kernel PPOs are activated to some extent by detergents and chaotropes (urea, guanidine.HCl) in crude extracts (Okot-Kotber et al 2002; Jukanti et al 2003).

Different groups (Jiménez and Dubcovsky 1999; Demeke et al 2001) have also published data on the chromosome location of PPO genes or genes influencing PPO activity; these results may be useful for breeding and variety development. Biochemical (Kruger 1976, Anderson and Morris 2003) as well as molecular data (Demeke and Morris 2002) indicate the presence of several (3-12) PPO isoforms or genes in wheat, but only one (partial-length) wheat PPO gene has been cloned so far (Demeke and Morris 2002), and one wheat kernel PPO was purified from bran of a high-PPO variety (Anderson and Morris 2003).

To accelerate the systematic functional characterization of wheat PPOs, especially of those involved in food biochemistry, the main goal of this research was the identification of all wheat PPO genes, utilizing bioinformatic tools and publicly accessible expressed sequence tag (EST) databases.

Materials and Methods

To mine public databases for potential wheat PPO sequences, the sequence published by Demeke and Morris (2002; GenBank accession number AF507945) was utilized for a basic local alignment search tool (BLAST; Altschul et al 1990; <http://www.ncbi.nlm.nih.gov/BLAST/>) search against the National Center for Biotechnology Information (NCBI) non-human, non-mouse (i.e., including all plants) EST databases. All *Triticum* sequence “hits” (see results) were exported to the San Diego SuperComputer Center biology workbench (<http://workbench.sdsc.edu/>) for further analysis. Sequences were aligned using the CLUSTALW tool (Thompson et al 1994), either directly (not shown), or after translation to protein sequences (Fig. 3.1). To make sequence identities/similarities more easily visible, aligned sequences were shaded using the BOXSHADE tool (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic relationships between the sequences were calculated using PHYLIP (Felsenstein 1989; Fig. 3.2).

All EST clones which could be obtained from the originating laboratories (GenBank accession numbers BQ839339, CA594200, CA716843, BQ246043, BQ239083 and BM136370) were re-sequenced using standard fluorescent chain-terminating dideoxynucleotides (full length of vector insert, Prober et al 1987); deposited sequences were used for the previously published wheat PPO (Demeke and Morris 2002; GenBank accession AF507945) and for an EST clone with GenBank accession number CD935448.

Results

To the best of our knowledge, only one wheat PPO DNA sequence has been published so far (Demeke and Morris 2002), and a few wheat PPO peptide sequences were obtained after purification and tryptic digestion of a kernel PPO (Anderson and Morris 2003). However, in the last two years, a large number of wheat EST sequences have been deposited by different laboratories. A detailed search of these resources (as outlined under Materials and Methods) suggests the presence of a PPO multigene family with six members, falling into two phylogenetic clusters, in wheat (Figs. 3.1 and 3.2).

Utilizing the published sequence (Demeke and Morris 2002; GenBank accession number AF507945) as a search argument for a NCBI BLAST search, six “hits” with sequence identities of >60% in overlaps of at least 200 base pairs (bp) were obtained. Interestingly, among these, based on 100.0% sequence identity in a 260-bp overlap, one EST (GenBank accession number CD935448) was found to be identical to the gene used as a search argument, but extended its sequence by 303 bp at the 5'-end. Based on sequence comparisons (not shown) of the combined sequence (from GenBank accession numbers AF507945 and CD935448) with PPOs from potato (*Solanum tuberosum* L., Q06355), tomato (*Lycopersicon esculentum* L., Q08307), tobacco (*Nicotiana tabacum* L., CAA73103), fava bean (*Vicia faba* L., Q06215), poplar (*Populus* sp., AF263611), grapevine (*Vitis vinifera* L., P43311) and spinach (*Spinacia oleracea* L., P43310), this sequence represents the first wheat PPO cDNA which is almost (no N-terminal methionine residue) full-length, including a transit peptide (needed for plastid import). Consequently, this combined sequence (designated as AF507945* in Fig. 3.1) was used

as a reference for comparisons with all other wheat PPOs, both at the cDNA and, after translation, at the protein level (Fig. 3.1).

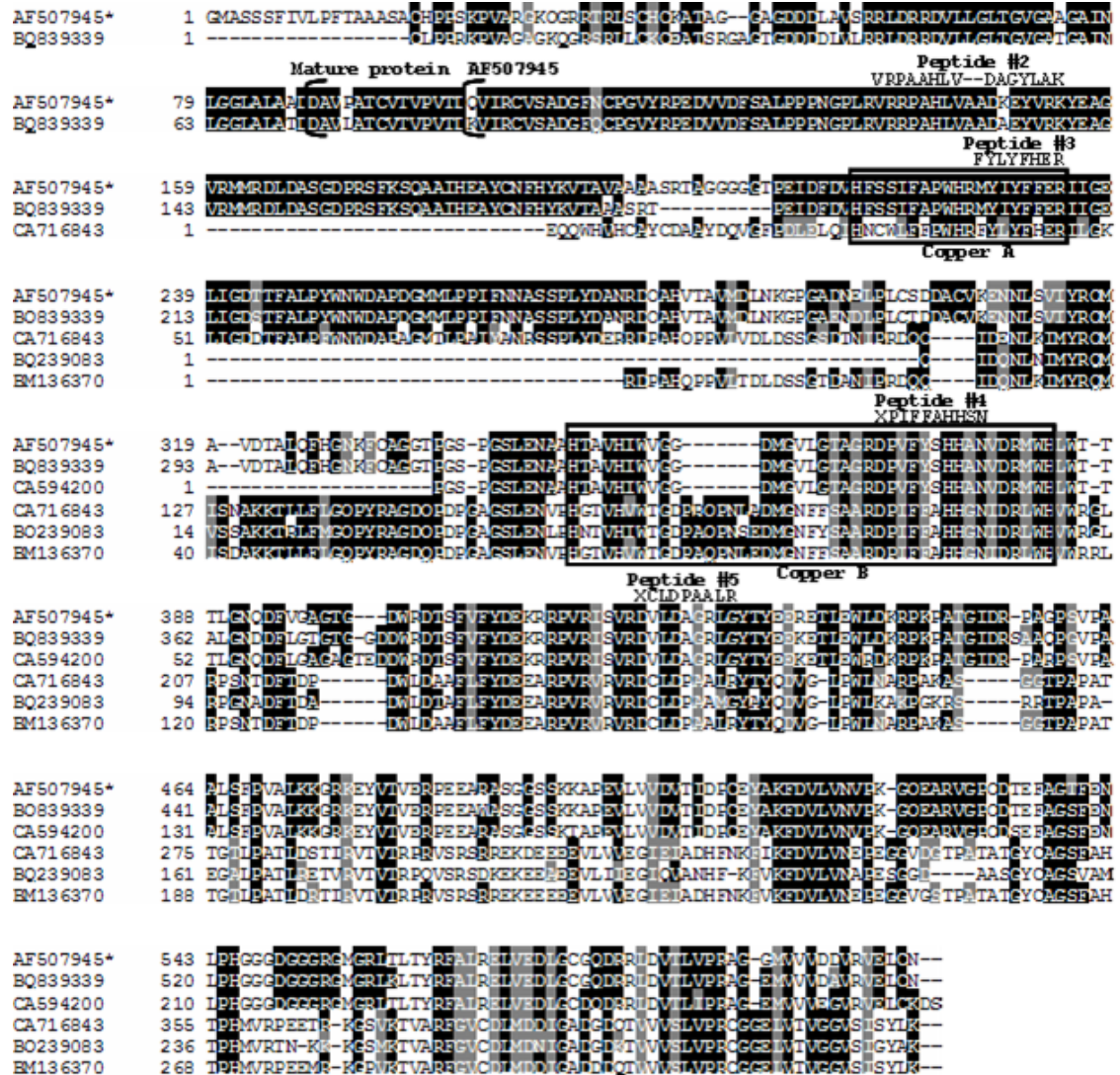


Fig. 3.1 Sequence alignment of wheat PPO ESTs. EST sequences were identified through a NCBI BLAST search, using GenBank accession #AF507945 (Demeke and Morris 2002) as search argument. Tentative PPO sequences were translated and aligned. Peptide sequences obtained after purification of a seed kernel PPO (Anderson and Morris 2003) are shown above the aligned sequences. Conserved copper binding regions (A and B; according to Demeke and Morris 2002) are framed. Sequence #CD935448 was found to be identical to #AF507945, but extended its 5'-end by 303 bp / 101 N-terminal amino acids. The combined sequence is used as a reference sequence for this figure and labeled AF507945*. The start of the original sequence (AF507945) is indicated; additionally, the tentative N-terminus of mature polypeptides (obtained from alignment with other plant PPOs) is also shown.

Based on sequence alignments with the other higher plant PPOs mentioned, the tentative start of the mature polypeptide chain (after cleavage of the transit peptide) is also shown in Fig. 3.1.

An EST with GenBank accession number CA594200 appears to represent a second, novel PPO gene. This clone was re-sequenced in our laboratory; it is 5'-truncated but contains a complete 3'-end, including a poly-A tail. This clone demonstrates ~86% sequence identity with the reference sequence over its full length, including the 3'-untranslated region (UTR). A clone with accession number CA594195 may represent the same gene, but sequence quality is poor; additional controls by the authors of this sequence indicated potential problems with this clone (S. Tingey, DuPont, personal communication).

A third interesting clone has accession number BQ839339, with 84.6% sequence identity to the reference sequence in a 610-bp overlap. This clone was also re-sequenced in our lab; while it is, most likely, still 5'-truncated (missing part of the sequence information corresponding to a tentative transit peptide), it is the longest of all currently available wheat PPO ESTs/cDNAs (Fig. 3.1). This clone may represent a third wheat PPO gene, closely related to both the reference sequence and to clone CA594200.

A clone with accession number CA716843 clearly represents an additional wheat PPO gene. This clone was re-sequenced in our lab; it demonstrated ~56% sequence identity with the reference sequence over its full length (it is 5'-truncated, but contains a complete 3'-UTR). While this gene was more different from the reference sequence than

those mentioned above, it still appears to be a PPO, based on sequence comparison at and around the Cu-binding regions (Fig. 3.1). Furthermore, Anderson and Morris (2003) reported several peptide sequences obtained after purification, tryptic digestion and microsequencing of one kernel PPO, and the sequences of their peptides #3 and 5 are exact matches with CA716843; one amino acid was different in peptide #4 (Fig. 3.1). Based on this finding, and on the fact that this EST was isolated from a cDNA library prepared from developing kernels, at 14 days past anthesis, clone CA716843 most likely encodes a PPO actively expressed in developing kernels.

A last, tentative wheat PPO EST from this first BLAST search has GenBank accession number BQ239083. Again, this clone was re-sequenced in our lab; comparably to CA716843, it demonstrates ~55% sequence identity with the reference sequence, but is closely related to CA716843. As this clone was also isolated from a kernel cDNA library (mRNA isolated at 5 days post anthesis), it may represent a second wheat kernel PPO.

Research in our lab strives to understand the functional role of kernel PPOs in the discoloration of wheat products. Since results from C. Morris' laboratory (Demeke and Morris 2002; Anderson and Morris 2003) indicate that several PPOs might be present in developing kernels, two more BLAST searches were performed, using the tentative kernel PPOs (clones CA716843 and BQ239083) as search arguments. Four additional (not discovered in the first search, with >80% sequence identity in >100-bp overlaps) sequences were found by this approach. Two of these (GenBank accession numbers BQ246043 and BM136370) also showed significant sequence identity with the original reference sequence; the others, while clearly related to BQ239083, are more distant from

the original reference sequence, and may not represent functional PPO genes. Re-sequencing of BQ246043 in our lab (including, again, the 3'-UTR) demonstrated that this clone is identical to CA716843 (cDNAs derived from the same gene). On the other hand, re-sequencing clone BM136370 suggested that this EST is derived from an additional wheat PPO gene, which is different from all others mentioned above, but closely related to CA716843 and BQ239083 (Fig. 3.1). This EST was isolated from a cDNA library prepared from developing spikes.

All PPO sequences have been visualized after translation and alignment (Fig. 3.1). As this figure suggests the presence of two distinct sequence groups, phylogenetic relationships between the six wheat PPOs were determined using PHYLIP (Felsenstein 1989).

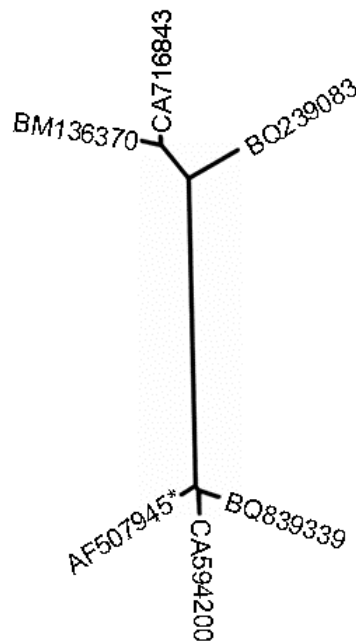


Fig. 3.2 Phylogenetic relationships of wheat PPOs shown in Fig. 3.1.

This analysis (Fig. 3.2) confirms that known wheat PPOs fall into two distinct clusters of three sequences each, and that members of each cluster are closely related. Novel sequence information obtained from this research (full-length sequencing of EST clones) has been submitted to GenBank. New GenBank accession numbers AY596266 – AY596270 correspond to EST clones BQ839339, CA594200, CA716843, BQ239083 and BM136370 (in this order). Additionally, a sequence with accession number AY515506 has been submitted by J. Anderson's laboratory (USDA, Fargo, ND) to GenBank while this publication was under review; it appears to be derived from the same gene as EST clone BM136370 (new accession number AY596270) utilized for this manuscript, but contains additional 5'-sequence information.

Discussion

Higher plant PPOs have been intensely studied during the last few years, both because of their potential roles in defense against pathogens and insects (Constabel et al 1995; Mohammadi and Kazemi 2002), and because of their involvement in post-harvest food biochemistry (Feillet et al 2000; Nagai and Suzuki 2001). Specifically, wheat PPOs have been investigated by a number of research groups due to their role in the undesirable darkening of Asian noodles produced from high-PPO germplasm (Feillet et al 2000; Anderson and Morris 2001). Investigating the biological basis of the high- vs. low-PPO activity trait in mature kernels of established or new wheat lines (as needed for variety improvement) has proven difficult, mostly due to a lack of information on the number of PPO genes actively expressed during grain development.

Based on the analysis of publicly accessible EST data, our research has identified six different wheat PPO DNA sequences, falling into two clusters with three similar sequences each (Figs. 3.1 and 3.2). As these ESTs were, in part, isolated from different wheat cultivars, some of these differences might be due to allelic variation. However, the following points strongly argue against this interpretation: (1) Southern blots performed by Demeke and Morris (2002) on DNA extracted from the wheat cultivar 'Penawawa', using a probe derived from GenBank accession number AF507945, detected 2-4 bands (depending on the restriction enzymes used for DNA digestion). Three bands each were detected in DNA from a number of additional wheat cultivars digested with HindIII, but some differences were detected in the banding pattern between low- and high-PPO varieties. Considering the sequence differences shown in Fig. 3.1, it is reasonable to assume that the probe used in this study detected all members of the "lower", but not of the "upper" cluster presented in Fig. 3.2, suggesting that this cluster contains three (or possibly more) different sequences (i.e., genes) in each cultivar. (2) Two ESTs situated in the "upper" cluster in Fig. 3.2 (BQ246043 and BQ239083) were isolated from cDNA libraries prepared from the same cultivar, 'Glenlea'. BQ246043 is not shown in Fig. 3.1, as it is essentially identical to clone CA716843. This suggests the presence of at least two different PPO sequences (i.e., genes) within this cultivar. (3) Careful comparative analysis of all sequences presented in this manuscript at the DNA level, both in the coding region and 3'-UTR (not shown, but accessible through GenBank, see results) indicates too important differences between sequences in each of the two clusters to be explained by allelic variation. Additionally, it has to be kept in mind that PPOs are

ubiquitous enzymes; it is reasonable to assume that such genes are present on each of the three (A, B and D) genomes of hexaploid wheat. In this context, Demeke et al. (2001) found quantitative trait loci (QTL) influencing PPO activity on chromosomes 2A, 2B, 3D and 6B. Therefore, our data are best explained by postulating a PPO multigene family with at least six members for hexaploid wheat.

Considering the tissues from which RNA for cDNA library preparation was extracted, it appears that three genes (members of the "upper" cluster in Fig. 3.2; GenBank accession numbers CA716843, BQ239083 and BM136370) are expressed at some point during kernel development, and may therefore be important for food biochemistry. Members of the "lower" cluster (AF507945, BQ839339 and CA594200) were isolated from other tissues and were not present in kernel cDNA libraries. Therefore, these genes are probably not expressed at high levels during kernel development, and are not likely to contribute to undesirable food darkening processes. Interestingly, an affinity-purified anti-peptide antibody developed in our laboratory against the sequence GTAGRDPVFYSHHAN (derived from the CuB-binding region of sequence AF507945, but identical in BQ839339 and CA594200; compare Fig. 3.1) reacted only weakly with proteins extracted from bran of the high-PPO winter wheat variety 'Rampart' (data not shown), again suggesting that these PPOs are not, or only at low levels, present in kernels. Additionally, the PPO isoform purified by Anderson and Morris (2003) clearly belongs to the first cluster, as demonstrated by the alignment of obtained peptide sequences with translated ESTs in Fig. 3.1. The single amino acid difference between the peptide #4 sequence obtained by these authors and clones CA716843, BQ239083 and

BM136370 (serine instead of glycine in the penultimate position of the peptide) appears intriguing; considering the fact that the sequences of peptides #3 and 5 are in complete agreement with the sequence of clone CA716843, this might be explained by allelic variation in this gene. However, at this point, the presence of an additional wheat PPO, which would be very closely related to clone CA716843, cannot be excluded. Among other higher plant PPOs, serine residues (instead of alanine or glycine) are present in this position in genes from potato (*Solanum tuberosum* L., U22921), fava bean (*Vicia fava* L., Q06215), poplar (*Populus spp.*, AF263611) and spinach (*Spinacia oleracea* L., P43310).

Data presented in this manuscript represent a necessary and essential first step towards the systematic functional characterization of wheat PPOs. They will allow the identification of those PPO gene(s), which are principally involved in food biochemical processes such as darkening of Asian noodle preparations. At the same time, this data set will also initiate research leading to an improved understanding of the (potential) role of wheat PPOs in defense against pests or pathogens. The development of new germplasm, improving end product quality without making new varieties more susceptible to known insects or diseases, will be the final goal of these efforts. To work towards this goal, isolation of full-length cDNA and genomic clones, studies of expression patterns of the different genes in developing kernels of low- and high PPO germplasm, and subcloning into appropriate vector systems for protein expression (allowing antibody production and biochemical analysis) have been initiated. Additionally, using nulli-tetrasomic lines (Sears 1954), it will be possible to determine on which chromosome(s) of the A, B and D genomes the different genes are present; comparison of these data with previous mapping

efforts (Jiménez and Dubcovsky 1999; Demeke et al 2001) will yield additional data for the identification of those gene(s) associated with the high-PPO activity trait.

Conclusions

Data presented in this manuscript suggest, for the first time, that wheat polyphenol oxidases are organized in a multigene family with at least six members, falling into two clusters with three similar sequences each. Members of one cluster are expressed in developing kernels and may therefore be involved in undesirable food darkening processes.

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CHAPTER 4

MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF POLYPHENOL
OXIDASES FROM DEVELOPING WHEAT (*Triticum aestivum* L.) KERNELS AND
SENESCING LEAVES.Introduction

Polyphenol oxidases (PPOs) are ubiquitous, copper-containing metalloproteins that have been found or characterized in several higher plant species (Flurkey, 1989; Cary *et al.*, 1992; Kwon and Kim, 1996; Constabel *et al.*, 2000; Chazarra *et al.*, 2001; Nagai and Suzuki, 2001). PPOs catalyze the hydroxylation of o-monophenols to o-diphenols (E.C. 1.14.18.1; monophenol mono-oxygenase, tyrosinase or cresolase activity) and the oxidation of o-dihydroxyphenols to o-quinones (E.C. 1.10.3.2; diphenol oxidase or catecholase activity; Steffens *et al.*, 1994; van Gelder *et al.*, 1997). Generated o-quinones further react with amines and thiol groups or undergo self-polymerization to produce dark/brown products that are usually undesirable in fresh or processed food (Feillet *et al.*, 2000; Anderson and Morris, 2001). While the biological function(s) of PPOs are not yet entirely clear, available evidence indicates a role for these enzymes in defense against insect and pathogen attack (Li and Steffens, 2002; Constabel *et al.*, 1998). The central domain of all PPOs consists of two Cu-binding sites (CuA and CuB); at each of these sites, a copper ion is bound by conserved histidine residues. This pair of copper ions is the site of interaction of PPOs with molecular oxygen and phenolic substrates (Steffens *et*

al., 1994; van Gelder *et al.*, 1997). There is a high degree of sequence similarity in the copper-binding regions of PPOs from different plant species, but considerably more divergence in other parts of the respective polypeptide chains (Demeke and Morris, 2002). For example, there is 92% amino acid sequence similarity at the copper A binding region, but overall only 42% sequence similarity between Vicia and tomato PPOs (Lax and Cary, 1995). Plant PPOs are nuclear-encoded, but gene products are associated with plastidial thylakoid membranes in photosynthetically active tissues (Hind *et al.*, 1995; Jiménez and García-Carmona, 1996; van Gelder *et al.*, 1997). In consistence with this finding, N-terminal transit peptides are present in the corresponding sequences. Typical transit peptides consist of 80-100 amino acids; peptide cleavage during chloroplast import reduced the molecular mass of several plant PPOs from 65-70 kD to ~60kD or less for the mature protein (Cary *et al.*, 1992; Dry and Robinson, 1994; Anderson and Morris, 2003).

PPOs are present in a latent form in some plant tissues and can be activated by various methods, such as acid and base treatment or exposure to strong anionic detergents like sodium dodecyl sulfate (SDS) and N-lauroylsarcosine (van Gelder *et al.*, 1997; Chazarra *et al.*, 2001; Okot-Kotber *et al.*, 2002; Jukanti *et al.*, 2003). PPOs extracted in absence of detergents from kernels of high-activity wheat varieties were activated by SDS, but this was not observed with low-PPO germplasm (Jukanti *et al.*, 2003). Several PPOs have also been activated or solubilized after partial proteolytic degradation (Nozue *et al.*, 1999; Pérez-Gilabert *et al.*, 2001; Gandia-Herrero *et al.*, 2005).

Evidence for the presence of several genes or isoforms has been found in species for which a more detailed analysis was performed, such as wheat (*Triticum aestivum* L.; Kruger, 1976; Demeke and Morris, 2002; Jukanti *et al.*, 2004), tomato (*Lycopersicon esculentum* L.; Thipyapong *et al.*, 1997) and poplar (*Populus* sp.; Constabel *et al.*, 2000). PPO genes have been cloned from several plant species (Shahar *et al.*, 1992; Thygesen *et al.*, 1995; Bucheli *et al.*, 1996), but the genes from monocotyledonous plants are less well investigated than those found in dicotyledons. It has been shown that PPO expression and activity are influenced both by environmental conditions and genotype (Lamkin *et al.*, 1981; Baik *et al.*, 1994; Park *et al.*, 1997). Tetraploid durum wheat (*Triticum turgidum* L. var. *durum*; genome organization: AABB) has significantly lower kernel PPO activity than hexaploid (genome organization: AABBDD) bread wheat cultivars (*Triticum aestivum* L.; Kruger, 1976; Demeke and Morris, 2002; Lanning *et al.*, 2003). Based on mapping efforts by Jiménez and Dubcovsky (1999), gene(s) responsible for this difference may be present on chromosome 2D; however, Simeone *et al.* (2002) have also located a quantitative trait locus for PPO activity on chromosome 2A, which is present in both tetraploid and hexaploid wheats. Demeke *et al.* (2001) have found quantitative trait loci on chromosomes 2A, 2B, 3D and 6B.

Previous work in our laboratory (Jukanti *et al.*, 2004) has indicated the presence of at least six different PPO genes in hexaploid wheat varieties. Based on sequence similarity, these genes can be grouped into two distinct clusters of three highly similar sequences each. The two groups, with their original EST and GenBank accession numbers (obtained after full-length sequencing of each clone) are shown in Table 4.1. Interestingly, all

sequences in cluster 2 (Table 4.1) were isolated from cDNA libraries prepared from developing wheat grains, while genes grouped in cluster 1 (Table 4.1) were isolated from other tissues. Because of their potential ecological (defense) function and also because of their impact on food quality, a comprehensive understanding of the high-PPO activity trait in major crops is imperative. Identification of most, if not all, wheat PPO genes (Jukanti *et al.*, 2004; Table 4.1) has allowed us to initiate, for the first time, a thorough molecular and biochemical characterization of PPO function in wheat.

Table 4.1 Wheat PPO genes with their EST and GenBank (obtained from full-length sequencing of cDNA clones) accession numbers. Genes are grouped by their phylogenetic relationships (Jukanti *et al.*, 2004).

EST number	GenBank accession number
<i>Cluster 1</i>	
CD935448	AF507945 ¹
BQ839339	AY596266 ²
CA594200	AY596267 ²
<i>Cluster 2</i>	
CA716843; BQ246043	AY596268 ²
BQ239083	AY596269 ²
BM136370	AY515506; AY596270 ²

¹Demeke and Morris, 2002

²Jukanti *et al.*, 2004

Materials and Methods

Southern Blot Analysis

To obtain DNA, leaves were collected from three bread wheat (*Triticum aestivum* L.) varieties ('Rampart', a high-PPO winter wheat; 'Utah-100', a low-PPO winter wheat; 'Reeder', a low-PPO spring wheat) and one durum wheat (*Triticum turgidum* L.) variety

('Vic', with very low measurable PPO activity) at 14 days after germination, frozen in liquid N₂, and stored at -80 °C. DNA was extracted from leaves using the cetyl-trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) and digested with *EcoRV*, *BamHI* and *HindIII*. Southern analysis was performed on the four different varieties to determine the number of hybridizing fragments, and to test for differences between high- and low-PPO genotypes. Southern blot analysis was carried out according to Sambrook *et al.* (1989). The PPO gene with GenBank accession number AY596268 (cluster 2, Table 4.1) was used as a probe. The 1.5 kb cDNA was randomly labeled with α -[³²P]-dCTP according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The pre-hybridization and hybridization solutions contained 5x SSC (Sambrook *et al.*, 1989), 5x Denhardt's solution, 1% (w/v) SDS and 100 µg/ml of salmon sperm DNA. Hybridization solution additionally contained 5% (w/v) dextran sulfate. Hybridization was performed at 65 °C overnight. The hybridized membrane was washed twice for 20 min at 65 °C in 2x SSC/ 0.1% SDS, and twice with 0.2x SSC / 0.1% SDS. Signal was detected after 5 days of exposure to X-ray film (at -80 °C, using an intensifying screen).

Protein Purification

Wheat kernel PPOs were extracted from bran of the high-PPO variety Rampart. Total soluble proteins were extracted in 20 mM Tris buffer pH 8.0. Proteins were first fractionated by anion exchange chromatography (Macro-Prep[®] High Q, 1.5 x 20 cm; Biorad, Hercules, CA), using a three-step gradient from 0 - 0.3 M NaCl. The main peak was collected, fractions were pooled and brought to 1 M (NH₄)₂SO₄, loaded on a Macro-Prep[®] Methyl HIC column (1.5 x 20 cm; Biorad) equilibrated with 20 mM Tris pH 8.0 / 1

M (NH₄)₂SO₄ and fractionated with a linear gradient from 1 - 0 M (NH₄)₂SO₄. The first (main) activity peak was collected, pooled and concentrated using Centriplus YM-10 filter devices (Millipore, Bedford, MA) and further purified using gel chromatography on a Sephacryl S-200 HR (1.5 x 50 cm; Amersham, Uppsala, Sweden) column equilibrated with 20 mM Tris pH 8.0 / 50 mM NaCl. For all purification steps, activities were assayed as outlined under "enzyme activity assays", using 10 mM 3,4-dihydroxy-L-phenylalanine (L-DOPA) as a substrate.

Mass Spectrometry

Proteins eluted from gel chromatography were concentrated and separated on 13% SDS-PAGE gels, using the Laemmli (1970) buffer system. Two bands of ~56 and 58 kD were separately excised, subjected to in-gel tryptic digestion using a commercially available kit (Pierce, Rockford, IL), and resulting peptides were analyzed by Matrix-assisted laser desorption/ionization (MALDI) – time of flight (TOF) mass spectrometry in a Bruker Biflex III (Karlsruhe, Germany) instrument, using sinapinic acid as a matrix. Mass peaks were identified with the help of the MS-Digest program at the ProteinProspector (<http://prospector.ucsf.edu>) website, using the (translated) sequences shown in Table 4.1.

Northern Blot Analysis

RNA was extracted from kernel and flag leaf samples harvested at different developmental stages (kernels at 7 – 42 days post anthesis (dpa) and leaves at 7 - 28 dpa, in 7-day intervals) from the same varieties as used for Southern blotting, using a modified

Trizol protocol (Hogg *et al.*, 2004). Northern blot analysis was performed using the method described by Giroux and Morris (1997). PPO genes with GenBank accession numbers AY596268 and AY596266 (Table 4.1) were used as probes. RNA (10 µg per sample) was fractionated on agarose formaldehyde gels and blotted onto a nylon membrane (Millipore, Bedford, MA) by overnight capillary transfer in 20x SSC. The blots were hybridized, washed, and exposed to film in the same way as Southern blots.

PPO Antibody Production

As only relatively small quantities of (partially) purified PPO were obtained from the protocol outlined above, a partial-length PPO gene (GenBank accession AY596268, 2nd cluster in Table 4.1) was expressed as maltose binding protein fusion in *E. coli*. A 1251 bp-fragment was cloned into the pMalc2 (New England Biolabs Inc.,) vector, using forward primer 5'-AATATGAATTCGAGCAGCAGTGGCACGTGCACTGCGCCTAC-3' (including an *EcoRI* restriction site) and reverse primer 5'-TATACAAGCTTTCACTTGAGGTAGCTGATGCTGACGCCGCC-3' (including a *HindIII* restriction site). Correct insertion was verified by sequencing, and the PPO-maltose binding protein (MBP) fusion was expressed in *E. coli* BL21(DE3)-RIL (Stratagene, La Jolla, CA) cells. Total soluble proteins were harvested by sonication and purified by affinity chromatography on amylose resin, following the manufacturer's instructions (New England Biolabs Inc.). Polyclonal (rabbit) antibodies against the PPO-MBP fusion protein were raised commercially (Alpha Diagnostic International, San Antonio, TX), and obtained sera were highly reactive against protein expressed in *E. coli* (data not shown).

SDS-PAGE and Immunoblotting

Proteins were analyzed in the same plant material as used for Northern blotting, again at 7-42 dpa. Proteins were extracted in 50 mM Na phosphate buffer pH 7.5 with a complete set of protease inhibitors (10 μ M E-64, 100 μ M leupeptin, 10 mM 1, 10, phenanthroline, 1 mM PMSF and 1 μ M pepstatin). Plant material (100 mg) was ground with 400 μ l extraction medium, using a motor unit and small pestles fitting the bottom of standard 1.5 ml-tubes for ~30 s, followed by centrifugation (10,000 g for 10 min, at 4 °C). Soluble proteins from supernatants were quantified using a modified Bradford reagent (Coomassie Plus Protein Assay, Pierce, Rockford, IL), denatured and separated by SDS-PAGE in a minigel apparatus (Hoefer Inc., San Francisco, CA) using the Laemmli (1970) buffer system. Proteins (22.5 μ g per lane) were separated on 11% polyacrylamide gels and visualized either with Coomassie Blue or blotted to nitrocellulose membrane (Osmonics, Westborough, MA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA) at 100V / hr. Immunoblots were developed essentially as outlined by Fischer *et al.* (1999), except that only 0.05% (v/v) Tween-20 was used in the primary and secondary antibody solutions. The primary (anti-PPO) antibody was used at 1:1000, and the secondary antibody (goat anti-rabbit IgG, horseradish peroxidase conjugate; Sigma, St. Louis, MO) at 1:2000. Signal was developed using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Enzyme Activity Assays

PPO activities were measured in the same samples as used for immunoblotting, following the protocol of Jukanti *et al.* (2003), except that MOPS buffer was replaced

with 50 mM Na phosphate buffer pH 6.5. Briefly, 50 μ l extract (supernatant) was mixed with 50 μ l 50 mM Na phosphate buffer pH 6.5 in 96-well microtitration plates, and enzyme activities were quantified after adding 100 μ l 10 mM 3,4-dihydroxy-L-phenylalanine (L-DOPA) in the same buffer. Absorbance at 475 nm was assayed kinetically for 15 min, using a multichannel spectrophotometer (SPECTRAMax Plus³⁸⁴, Molecular Devices, Sunnyvale, CA). Activities were calculated from the increase in absorbance during the linear phase of the reaction and expressed as $\Delta OD_{475}/hr/g$ of protein. Means and standard deviations of at least three independent assays are shown.

PPO Activation with Trypsin

Proteins were extracted from kernels of the high-PPO variety Rampart, collected at 14 dpa. Extractions were performed as outlined above, in 20 mM Tris pH 7.5 (without protease inhibitors). The extract (400 μ l) was mixed with 44 μ l trypsin solution and incubated at room temperature for 16 hours. Aliquots (50 μ l each) were sampled at 0, 2, 4, 6, 8 and 16 hours, shock-frozen in liquid nitrogen and stored at -80 °C until analysis of enzymatic activity (as outlined above).

Trypsin solution (trypsin from bovine pancreas, 13,600 units / mg solid, Sigma, St. Louis, MO) was prepared at 1.0 mg solid in 5 ml 500 mM Tris pH 8.0, containing 200 mM $CaCl_2$. Trypsin was omitted for control incubations. The experiment was repeated three times, and means and standard deviations are shown.

Results

Wheat Polyphenol Oxidase Genes

Previous work from our and other groups (Demeke and Morris, 2002; Jukanti *et al.*, 2004) has indicated the presence of a PPO multigene family in allohexaploid (bread) wheat. To corroborate these data, we performed Southern blots on three different hexaploid wheat varieties ('Rampart', a high-PPO winter wheat; 'Utah-100', a low-PPO winter wheat; 'Reeder', a low-PPO spring wheat) and a tetraploid durum wheat with low PPO activity ('Vic'), using the gene with GenBank accession number AY596268 as a probe. This probe detected 2-4 bands in all varieties tested (Figure 4.1), depending on the restriction enzyme used. However, differences in the number of detected bands between high- and low-PPO varieties were also visible, e.g. for DNA cut with *EcoRV* and *HindIII* (Figure 4.1).

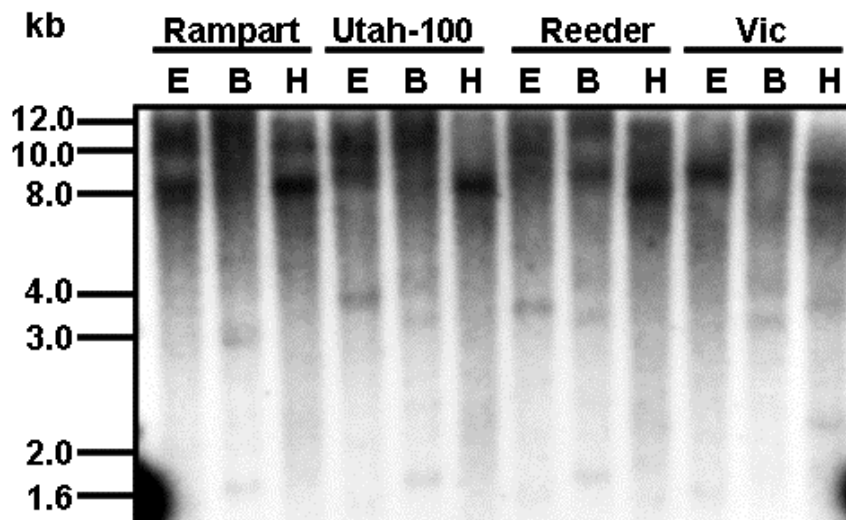


Fig. 4.1 Southern Blot analysis of wheat genomic DNA extracted from different varieties and digested with *EcoRV* (E), *BamHI* (B) and *HindIII* (H). A PPO gene with GenBank accession number AY596268 was used as a probe.

Based on known sequence identities (Jukanti *et al.*, 2004), it is reasonable to assume that AY596268 hybridizes with all members of cluster 2 (but not cluster 1; Table 4.1), confirming the presence of a small gene family with three (or possibly more) members. Demeke and Morris (2002), using a probe from cluster 1 (AF507945; Table 4.1) also detected 3-4 bands, confirming the presence of 3 (or more) genes in this cluster as well. Together, these data strongly indicate that wheat PPOs are organized in a family with at least six different genes.

Purification and Characterization of Kernel Polyphenol Oxidases

To identify the major PPO(s) present in mature wheat grains, they were partially purified from bran of the high-PPO variety Rampart, using a combination of anion exchange, hydrophobic interaction and size exclusion chromatography (Figure 4.2A). This approach led to a ~110-fold purification of PPOs; the specific activity was 0.10 $\Delta\text{OD}_{475} \text{ mg}^{-1} \text{ protein min}^{-1}$ in crude extracts from wheat bran, and the specific activity of the fraction obtained from the size exclusion column was 11.3 $\Delta\text{OD}_{475} \text{ mg}^{-1} \text{ protein min}^{-1}$.

The partially purified PPO fraction was analyzed by SDS-PAGE, resulting in two major proteins with apparent molecular weights of ~56 and 58 kD (Figure 4.2B), and analysis of the same preparation with a polyclonal wheat PPO antibody detected a strong signal at 58 kD (Figure 4.2C), confirming that at least one of the two proteins is a PPO.

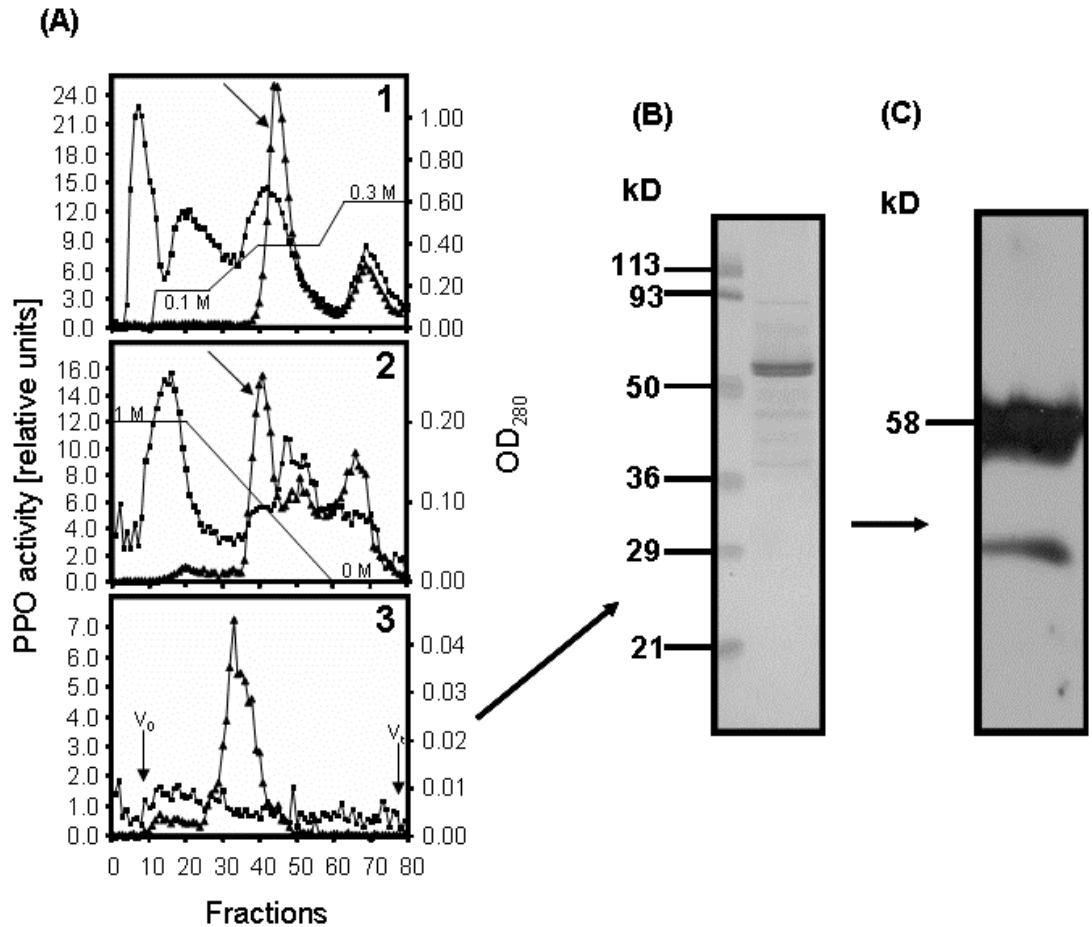


Fig. 4.2 Purification of wheat kernel PPO(s). (A) Enzyme was partially purified by anion exchange (1), hydrophobic interaction (2) and size exclusion chromatography (3). Squares represent protein concentration (OD_{280}), and triangles represent enzyme activity. Arrows in (1) and (2) designate fractions selected for the next chromatographic step. (B) Purified protein from size exclusion chromatography was analyzed by SDS-PAGE. (C) Purified protein was analyzed by immunoblotting, using a polyclonal antibody raised against the product of the gene with GenBank accession number AY596268.

To further analyze the isolated proteins, both bands were excised from the gel separately and subjected to tryptic digestion followed by MALDI-TOF mass spectrometry (Figure 4.3A). This approach confirmed the identity of the 58 kD band as a wheat PPO; careful analysis of the obtained peptide mass peaks indicated that this protein is derived from a gene in cluster 2 (GenBank accession number AY596268; Figure 4.3B).

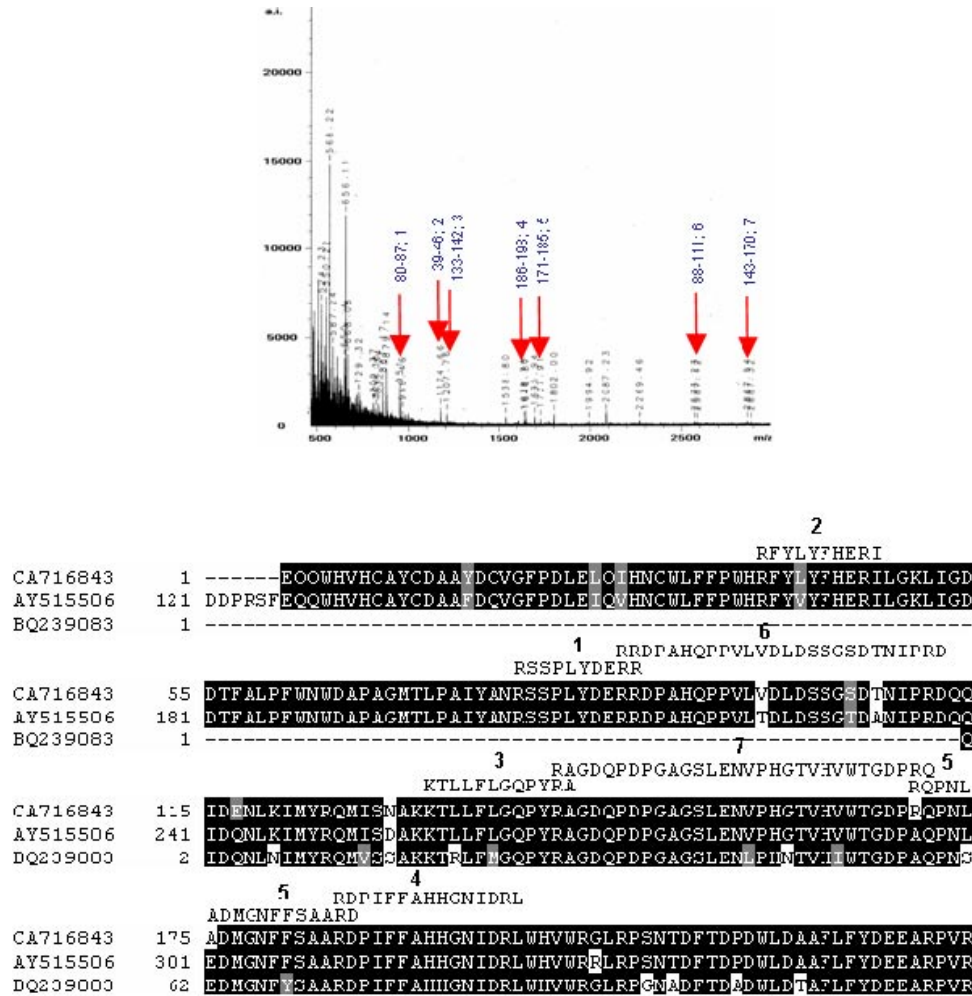


Fig. 4.3 Mass spectrometric analysis. (A) The 58 kD band from Figure 4.2B was subjected to in-gel tryptic digestion, and analyzed by MALDI-TOF mass spectrometry. Arrows designate mass peaks corresponding to the peptides shown in (B), with amino acid numbers derived from sequence AY596268. (B) Multiple sequence alignment of sequences in PPO gene cluster 2 (Table 4.1) in the area covered by mass spectrometry. Peptide numbers are derived from their masses, as shown in (A).

The presence of additional mass peaks may be due to the fact that no full-length clone of this gene is available yet. The partially purified PPO fraction was analyzed by SDS-PAGE, resulting in two major proteins with apparent molecular weights of ~56 and 58 kD (Figure 4.2B), and analysis of the same preparation with a polyclonal wheat PPO antibody detected a strong signal at 58 kD (Figure 4.2C), confirming that at least one of

the two proteins is a PPO. To further analyze the isolated proteins, both bands were excised from the gel separately and subjected to tryptic digestion followed by MALDI-TOF mass spectrometry (Figure 4.3A). This approach confirmed the identity of the 58 kD band as a wheat PPO; careful analysis of the obtained peptide mass peaks indicated that this protein is derived from a gene in cluster 2 (GenBank accession number AY596268; Figure 4.3B). The presence of additional mass peaks may be due to the fact that no full-length clone of this gene is available yet.

Regulation of Polyphenol Oxidase Gene Expression and Activity in Developing Wheat Kernels and Flag Leaves

To investigate PPO regulation in developing wheat kernels and flag leaves, we performed a series of Northern blots, using the sequences with GenBank accession numbers AY596266 (cluster 1; Table 4.1) and AY596268 (cluster 2; Table 4.1) as probes. Interestingly, no signal was detected with the probe derived from AY596266, even when blots were exposed for considerably longer time than those developed with the probe derived from AY596268, confirming that the first cluster of genes is not, or only at marginal levels, expressed in developing kernels (data not shown). In contrast, a transcript of ~2 kb was detected with the probe derived from AY596268, with maximal expression levels at 14 ('Rampart', 'Utah-100') to 28 dpa ('Reeder'; Figure 4.4A). Expression levels were higher in the high-PPO variety 'Rampart', as compared to both 'Utah-100' and 'Reeder'. A very faint signal (clearly visible on original X-ray films, but poorly reproducible) was detected for the durum wheat variety, 'Vic' (Figure 4.4A).

Similar to the situation in developing kernels, a probe derived from the gene with GenBank accession number AY596266 did not detect any signal in senescing flag leaves between 7 and 28 dpa (data not shown), but a transcript of 2 kb was again detected with the probe derived from AY596268 (Figure 4.4B). Gene expression in leaves reached maximal levels at 21-28 dpa (Figure 4.4B); again, transcript levels were higher in the variety 'Rampart' than in 'Utah-100' and 'Reeder'. PPO expression was undetectable in leaves of the durum wheat variety, 'Vic'.

To compare PPO gene expression with protein levels, soluble proteins were extracted from developing kernels and analyzed by immunoblotting, using a polyclonal antibody produced against the product of gene AY596268. In hexaploid wheat varieties, the antibody detected two principal protein bands with molecular weights of ~85 and 67 kD, with a minor band around 75 kD visible at 14-35 dpa (Figure 4.5A). The heavier (85 kD) band was detected at higher levels at 14-28 dpa (when gene expression was maximal); during later developmental stages (35-42 dpa), this band weakened in favor of the lighter (67 kD) form of the enzyme (Figure 4.5A). The situation appears different in durum wheat, where the 75 kD band was more persistent and bands <75 kD became detectable very late during kernel development (Figure 4.5A). Interestingly, PPO activity only became measurable starting at 28 dpa in kernels, when the 67 kD band first appeared in the varieties Rampart and Utah-100 (Figure 4.5B).

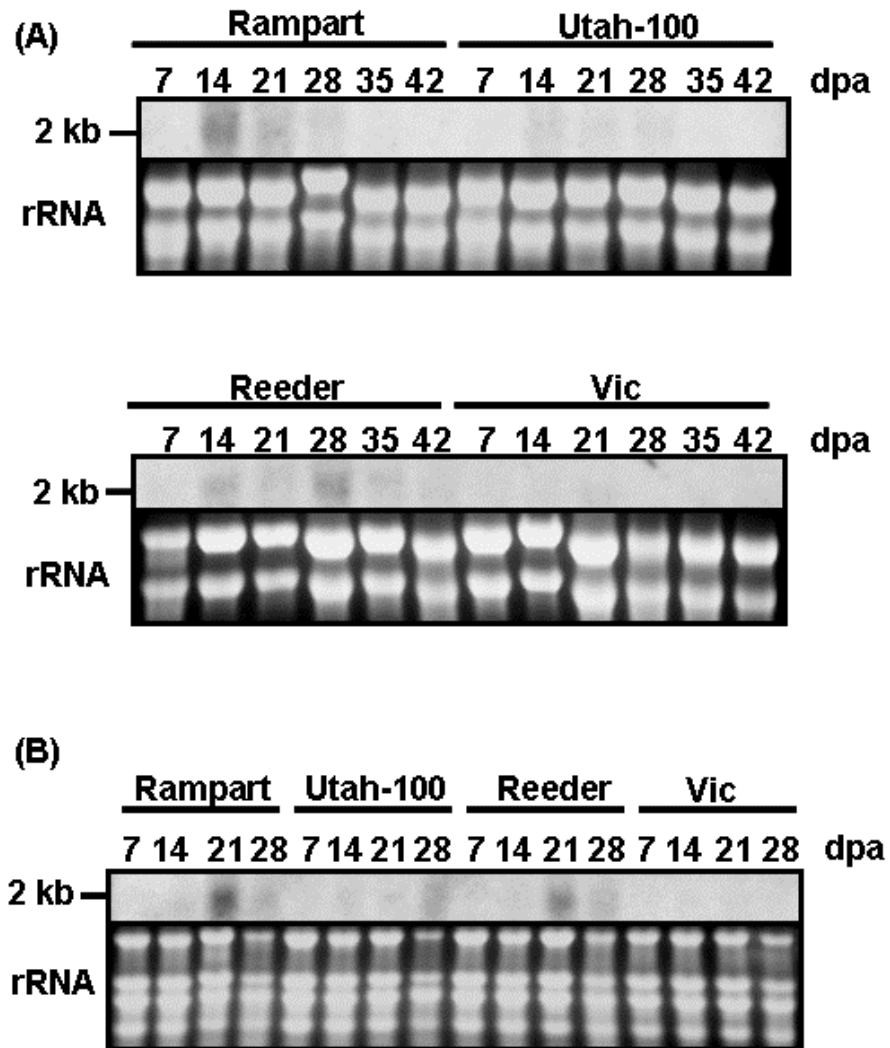
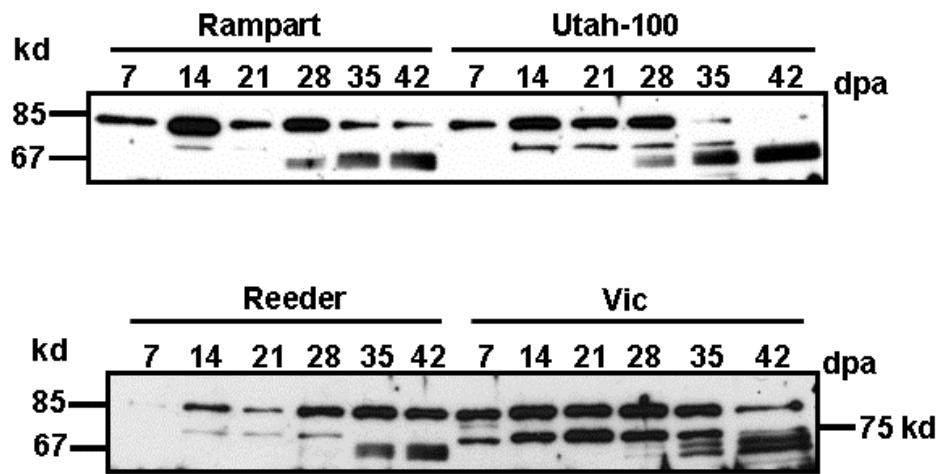


Fig. 4.4 PPO gene expression in developing kernels and senescing leaves. (A) Total RNA (10 μ g per lane) from kernels of 4 wheat varieties was analyzed by Northern blotting, using a probe derived from the gene with GenBank accession number AY596268. (B) Identical to (A), but RNA was extracted from flag leaves. dpa, days past anthesis.

Our data indicate that PPOs are synthesized as inactive precursors early during wheat kernel development, and that they are proteolytically processed and activated (potentially involving more than one step) during later phases of kernel development (28-42 dpa). In order to corroborate this finding, we extracted soluble proteins at 14 dpa from the high-

PPO variety, 'Rampart' (no measurable PPO activity; Figure 4.5B) and treated this preparation with purified trypsin.

(A)



(B)

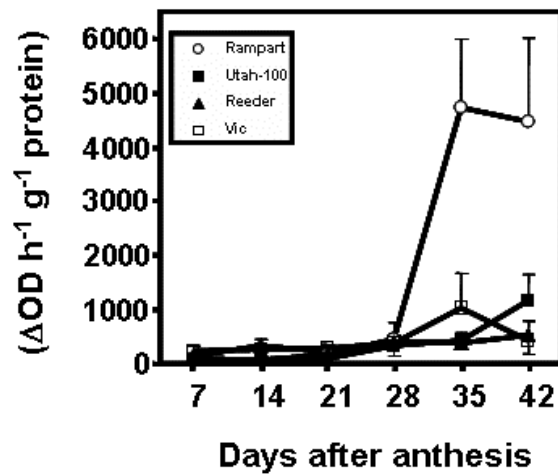


Fig. 4.5 PPO protein levels and activities in developing kernels. (A) Kernel proteins (22.5 μg per lane) from 4 wheat varieties were analyzed by immunoblotting, using a polyclonal antibody raised against the product of the gene with GenBank accession number AY596268. (B) PPO (diphenolase) activities were measured in the same samples as shown in (A), using 3,4-dihydroxy-L-phenylalanine (L-DOPA) as a substrate. Means and standard deviations of three replicates are shown. dpa, days past anthesis.

As suspected, this treatment was able to activate precursor PPO protein(s) present at this developmental stage, with activities increasing during the first 4 hours and remaining stable thereafter (up to 16 hours; Figure 4.6). No activation was detectable in controls (without trypsin), suggesting that the proteolytic system naturally activating wheat PPOs at later developmental stages is not yet present at 14 dpa. Immunoblotting of trypsin-treated fractions did not detect any bands with a clearly defined, lower molecular weight (data not shown), indicating that modification of PPO precursor proteins by trypsin is different from modifications which are naturally occurring during kernel development.

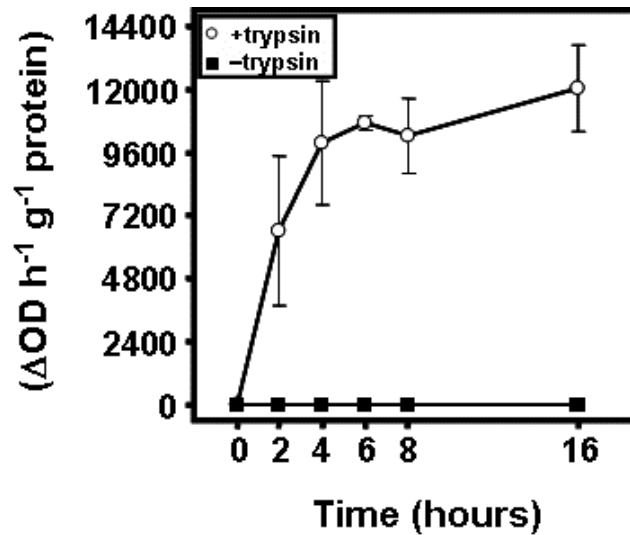


Fig. 4.6 PPO activation by trypsin. Total proteins were extracted from kernels of the variety 'Rampart' at 14 days past anthesis and incubated with purified trypsin (circles) or in absence of trypsin (squares, control) at room temperature. PPO (diphenolase) activities were measured using 3,4-dihydroxy-L-phenylalanine (L-DOPA) as a substrate. Means and standard deviations of three experiments are shown.

To compare the protein levels of kernels and leaves, soluble proteins were extracted from senescing flag leaves and analyzed by immunoblotting, using the same polyclonal

antiserum as above. The antibody detected a major protein band of ~75 kD in all the four varieties with maximal levels at 21 ('Utah-100', 'Reeder') to 28 dpa ('Rampart'; Figure 4.7A).

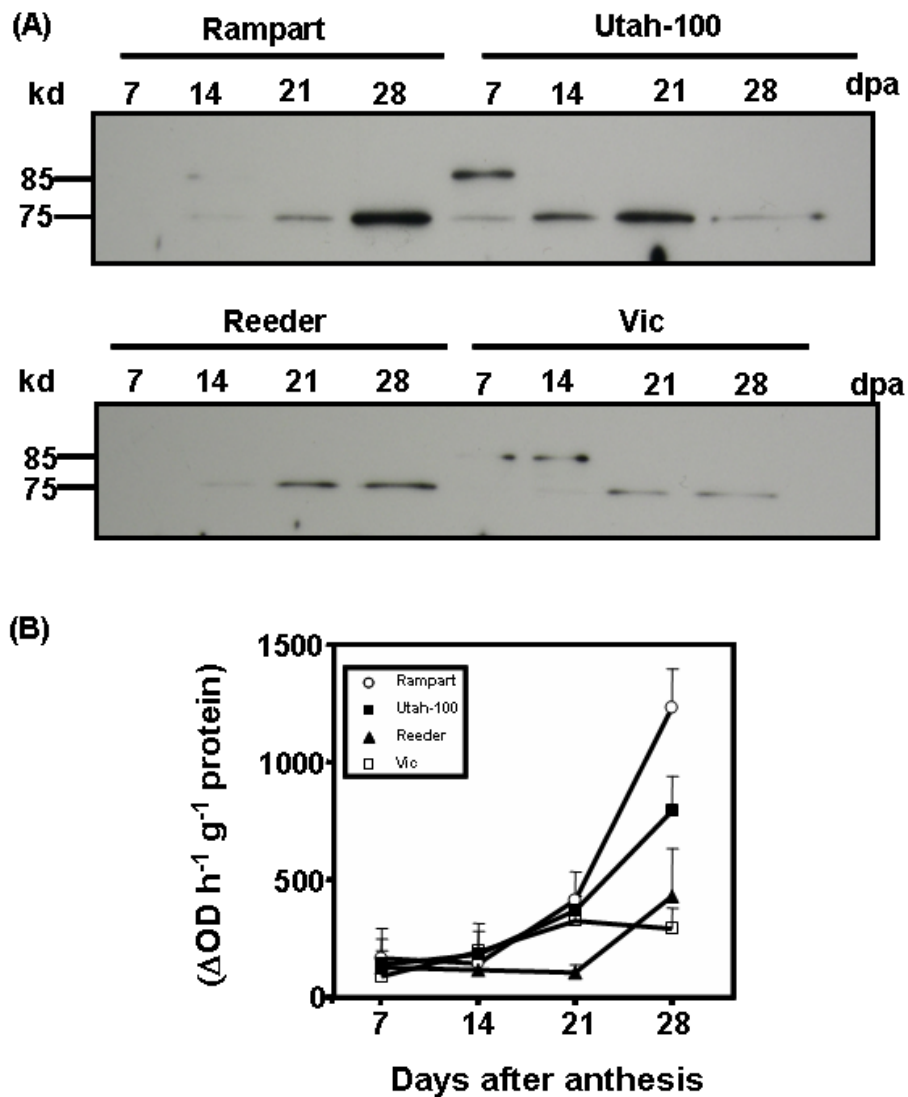


Fig. 4.7 PPO protein levels and activities in senescing leaves. (A) Leaf proteins (22.5 μ g per lane) from 4 wheat varieties were analyzed by immunoblotting, using a polyclonal antibody raised against the product of the gene with GenBank accession number AY596268. (B) PPO (diphenolase) activities were measured in the same samples as shown in (A), using 3,4-dihydroxy-L-phenylalanine (L-DOPA) as a substrate. Means and standard deviations of three replicates are shown. dpa, days past anthesis.

Unsurprisingly, the highest protein levels were found in the high-PPO winter wheat variety ('Rampart'; Figure 4.7A). Additionally, a minor protein band of ~85 kD was detected in the low-PPO winter wheat variety ('Utah-100') at 7 dpa, and in the durum wheat ('Vic'; Figure 4.7A) at 14 dpa. In contrast to kernels, low(er) molecular weight forms of the protein, and enzyme activities became detectable almost simultaneously with high transcript levels (21-28dpa; Figure 4.7B), indicating differences in protein synthesis and activation between the two tissue types.

Discussion

Previous data from our group indicated that wheat PPOs are organized in a multigene family with at least six members, falling into two distinct phylogenetic clusters of three highly similar sequences each (Jukanti *et al.*, 2004). Using Southern analysis, with a gene from cluster 1 (GenBank accession AF507945; Table 4.1) as a probe, Demeke and Morris (2002) detected between 2 and 4 distinct bands in DNA from the high-PPO variety 'Penawawa', depending on the restriction enzyme used. Judged by sequence comparisons of the genes shown in Table 4.1 (Jukanti *et al.*, 2004), it is reasonable to assume that the probe used in this study detected all members of cluster 1, but most likely not of cluster 2. Therefore, to estimate the number of genes present in cluster 2, we performed Southern analysis using the gene with accession number AY596268 as a probe, and also found between 2 and 4 hybridized DNA bands in each lane, depending on the combination of wheat variety and restriction enzyme (Figure 4.1). As data from Northern blots confirm poor cross-hybridization of cluster 1 genes/probes with members

of cluster 2, the combined results of these experiments (Demeke and Morris, 2002; and those presented in this study) corroborate the presence of several (6 or possibly 1 - 2 more) PPO genes in hexaploid (bread) wheat. If the number of six PPO genes is correct, and bearing in mind that PPOs are ubiquitous, it is tempting to speculate that each of the three (A, B and D) genomes contributed 2 PPO genes. Supporting the idea of PPO genes located on all three genomes, activity-based mapping efforts have located quantitative trait loci for PPOs on chromosomes 2A, 2B and 2D (Jiménez and Dubcovsky, 1999; Simeone *et al.*, 2002; Watanabe *et al.*, 2004). Additionally, Demeke *et al.* (2001) have found QTLs on chromosomes 3D and 6B.

Based on the tissues, from which the different wheat PPO ESTs were originally isolated, we have previously suggested that genes present in cluster 2 (Table 4.1) are expressed in developing kernels, while genes grouped in cluster 1 might be active in other tissues (Jukanti *et al.*, 2004). Here, we have utilized a two-pronged approach, including both biochemical and molecular methods, to experimentally confirm this interpretation and initiate the functional characterization of wheat PPOs. Data presented in Figures 2 and 3, using a combination of PPO activity-based enzyme purification, immunoblot and mass spectrometric analysis, confirm that the product of one gene from cluster 2 (again with GenBank accession AY596268) is present in mature wheat kernels. Based on the fact that additional (minor) activity peaks were also present in the different fractions (Figure 4.2A), it is possible that detailed analysis of the corresponding samples will provide additional data on the presence of the other (two) genes. However, as these experiments only yielded small quantities of partially purified protein, it may be more

profitable to use an immunoaffinity-based purification strategy to identify all PPO proteins present in mature wheat kernels. Data presented in Figure 4.3 should be interpreted in context with results published by Anderson and Morris (2003). Peptide sequences obtained by these authors after purification of a kernel PPO from a different wheat variety also assigned that enzyme to cluster 2; based on available sequence information, only one amino acid was different from the corresponding sequences in AY596268, but the sequences were more divergent from the other 2 genes in cluster 2 (as shown in Table 4.1). It is at present unclear if this difference is based on an allelic variation, or if the protein isolated by these authors represents the product of a fourth gene in the "kernel" cluster of wheat PPOs.

While EST data (Jukanti *et al.*, 2004) suggested that cluster 2 genes are expressed in wheat kernels, the biochemical approach used in this study confirmed the presence of at least one gene product from this cluster in mature grains. However, prior to the experiments outlined in this study, no data on developmental regulation of gene expression in wheat were available. Similarly, it was unclear if the high-PPO trait is based on transcriptional or post-transcriptional events. Finally, no studies directly assessing relative expression of cluster 1 and cluster 2 genes in kernels and other tissues had been performed. Data presented in Figures 4.4-4.7 address these problems, and allow the following pertinent conclusions:

- (1) Based on the use of one gene as a probe (AY596266), Northern blotting confirmed that cluster 1 genes are not, or only at marginal levels, expressed in either developing kernels or senescing (past-anthesis) flag

leaves. As the relevant literature suggests that PPOs are involved in the defense of mainly young plant tissues against pests/pathogens (Constabel *et al.*, 2000; Li and Steffens, 2002;), it might be interesting to assess such a role for cluster 1 wheat PPOs by systematically investigating their expression in (young) vegetative tissues.

- (2) While high(est) enzyme activities are present in kernels at 35-42 dpa (Figure 4.5B), maximal expression of gene AY596268 (and, possibly, other cluster 2 genes) is found between 14 and 28 dpa, with low to undetectable transcript levels during the later developmental stages (Figure 4.4A). Similarly, maximal gene expression in senescing flag leaves occurred ~7 days before high enzyme activities were measurable.
- (3) Gene expression levels were higher in both developing kernels and senescing flag leaves of the high-PPO variety 'Rampart' than in low(er)-PPO hexaploid varieties ('Utah-100' and 'Reeder'). Signals were very low (faintly visible on original X-ray films, but poorly reproducible) in the durum wheat, 'Vic'. These data suggest that the high-activity trait is, at least partially, due to transcriptional regulation.
- (4) Evaluation of protein relative to transcript levels and enzyme activities yielded the most interesting results of this study. From data presented in Figures 4-7, it is apparent that kernel PPO(s) are synthesized as inactive, high-weight (~85 kD) precursors, which reach highest levels around the time of maximal gene expression (Figures 4.4A and 4.5A). Subsequently,

these precursors are proteolytically processed and activated, possibly involving a two-step process. PPO (diphenol oxidase) activity became measurable at 28 dpa, when the fully processed (67 kD) form first became detectable in the varieties 'Rampart' and 'Utah-100' (Figure 4.5A/B). Proteolytic activation of inactive PPO precursor proteins was directly confirmed for the variety 'Rampart', using purified trypsin (Figure 4.6). Indications of protein processing were also found in leaves, but the (apparently) mature form of the polypeptide, appearing at ~21 dpa, was heavier than in kernels (~75 kD; Figure 4.7A).

- (5) Protein processing in kernels of durum wheat differed from the other varieties, with a heavier (intermediary?) form being more prominent, before final (and slow) processing to <70 kD polypeptides (Figure 4.5A). This difference in processing, besides very low gene expression, may also explain the low PPO activity generally associated with mature durum wheat kernels.

While the conclusions made in the last few paragraphs allow important insights into PPO regulation, they also point to open questions and clearly indicate future lines of research:

- (6) PPO protein levels (including the mature 67 kD form) appear surprisingly high in the low-PPO activity varieties or, in other words, while activity in mature kernels is positively correlated with transcript levels found earlier

during development, this is not the case for protein levels. This finding indicates that additional mechanism(s) besides the regulation of gene expression and protein processing may be involved in the regulation of PPO activity.

- (7) The molecular weight of the mature PPO(s) shown in Figure 4.5A is different from the weight of the protein purified from mature kernels (67 vs. 58 kD, Figure 4.2). Since Anderson and Morris (2003) have also estimated the weight of a major, purified kernel PPO at ~67 kD, it appears likely that the molecular weight shown in Figure 4.2B/C is the result of further processing, either *in vivo* or, more likely, during the purification process. It would be interesting to determine how far plant PPOs can be degraded (either N- or C-terminally) without losing their enzymatic function.
- (8) It is, at present, unclear why the mature polypeptide chain in leaves appears heavier than in kernels. It is possible that the major leaf PPO(s), while also members of cluster 2, are different from the principal gene expressed in kernels, and that this is associated with altered protein processing.
- (9) The presence of latent PPOs, which can be activated by proteolytic or chemical (detergents, extreme pH, etc.) has been postulated for other organisms. Specifically, it was suggested that a C-terminal extension of the polypeptide chain might mask access to the active site, and that

removal of this extension may be the basis for enzyme activation (Gerdemann *et al.*, 2002). Considering the data discussed in the last few paragraphs, including trypsin activation, developing wheat kernels clearly represent an ideal system for the detailed analysis of such a mechanism. Additionally, it appears likely that the protease(s) responsible for *in vivo* processing of wheat PPO only become active during later phases of kernel development. Obviously, the identification and characterization of the responsible enzyme or enzymes will be important for the functional characterization of wheat PPOs.

In summary, wheat PPOs emerge as a fascinating example of a multigene family with complex transcriptional and post-transcriptional regulation. While our work answers a number of questions regarding the molecular biology and biochemistry of these enzymes, it also opens several new lines of research. Specifically, using gene-specific analyses at the transcriptional and post-transcriptional level, the contribution of all genes in both clusters to PPO function in the different plant tissues has to be assessed. Of equal importance, mechanism(s) involved in the transcriptional (e.g., promoter analyses) and post-transcriptional regulation (activation of precursor proteins) need to be investigated. These studies will establish the necessary basis to understand both the applied (influence on product quality) and basic (functional importance for plant development) aspects of wheat PPOs, and may well have additional value for our overall understanding of plant PPO function.

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CHAPTER 5

COMPREHENSIVE DISCUSSION

In spite of their commercial and biological importance, little information about the molecular biology of wheat polyphenol oxidases (PPOs) was available prior to the research presented in this dissertation. This research has firmly established wheat PPOs as a fascinating example of a plant multigene family with complex transcriptional and post-transcriptional regulation. Application of bioinformatic tools, and thorough use of publicly available EST databases has identified most, if not all, wheat PPOs. These data indicated the presence of six PPO genes, falling into two different phylogenetic clusters of three highly similar sequences each, in hexaploid (bread) wheat (chapter 3; Jukanti et al., 2004). While it could be argued that (some of) the sequence differences within the two clusters in Table 4.1 are due to allelic differences, the following points make this interpretation highly unlikely:

(1) Two ESTs (BQ246043 and BQ239083) of cluster 2 in Table 4.1 were isolated from cDNA libraries prepared from the same cultivar, 'Glenlea'. This suggests the presence of at least two different cluster 2 sequences (genes) within this cultivar. (2) The 3'- UTR sequence of BQ239083 is completely different from the other two sequences (CA716843 and BM136370) of cluster 2 (Table 4.1), and there are also differences between CA716843 and BM136370 in this area. Similarly, 3'-UTRs of genes in cluster 1 (Table 4.1) are quite different. Additionally, we have corroborated our interpretation of

EST data with Southern blot analysis of three hexaploid ('Rampart', 'Utah-100' and 'Reeder') and one tetraploid durum wheat variety ('Vic'). Southern blot analysis using AY596268 (cluster 2 of Table 4.1) as a probe detected 2-4 bands in all the varieties tested, depending on the restriction enzyme used (Fig. 4.1). Demeke and Morris (2002), using AF507945 as a probe (cluster 1 of Table 4.1) also detected 3-4 bands, suggesting the presence of at least three genes in this cluster as well. Based on sequence differences between cluster 1 and 2 genes, it is reasonable to assume that probes made from cluster 1 genes do not cross-hybridize with cluster 2 sequences, and vice versa. Furthermore, mapping efforts by Jiménez and Dubcovsky (1999), Demeke *et al.* (2001) and Simeone *et al.* (2002) have located quantitative trait loci for PPO activity on chromosomes in all three (A, B and D) wheat genomes. Considering all these data, it becomes clear that wheat PPOs are organized in a multigene family with at least six genes.

Gene expression studies using three different hexaploid and one tetraploid durum wheat variety (same as above) have revealed early expression of PPO genes [14-28 days post anthesis (dpa) in developing kernels, and 21-28 dpa in flag leaves]. Two probes (AY596266 and AY596268) from the two different clusters of Table 4.1 were used for Northern analysis. Intriguingly, the first probe did not detect any signal in either developing kernels or senescing (post-anthesis) flag leaves, suggesting that cluster 1 genes are not, or only at marginal levels, expressed in these tissues. In contrast, a probe derived from EST AY596268 detected a ~2 kb signal in both these tissues. These findings strongly indicate that cluster 2, but not cluster 1 genes, are responsible for the undesirable darkening of wheat products. Based on literature data indicating a defense

role for plant PPOs in young tissues (Li and Steffens 2002; Constabel and Ryan 1998), we suspect such a role for wheat cluster 1 PPOs. Expression analysis of cluster 1 genes in young leaves and other (vegetative) tissues will constitute a logical, next step to verify this interpretation.

Comparison of gene expression and enzyme activity with protein levels yielded the most interesting results of this study (besides the establishment of the multigene family). It is apparent from our protein work that wheat kernel PPOs are synthesized as heavier precursor protein(s) (~85 kD), which reach maximal levels at the same time as PPO transcripts, but well before enzyme (diphenolase) activity becomes measurable. While 85 kD appears heavy for a plant PPO precursor protein, Anderson and Morris (2003) have recently reported immunological detection of both ~83 and 67 kD PPO forms in wheat. Our data strongly indicate that wheat kernel PPO(s) are synthesized as inactive precursors, and that they are proteolytically processed to a ~67 kD active form during kernel development. It is noteworthy that protein processing was different in the tetraploid variety, 'Vic', indicating that the low PPO activities generally associated with durum wheats could be due to (inefficient?) processing in addition to low gene expression (Figs. 4.4 and 4.5).

Activation of PPO precursors (extracted from kernels at 14 dpa) by partial proteolysis was directly confirmed using the purified serine peptidase trypsin (Fig. 4.6). Activities increased during the first four hours and remained stable thereafter. This indicates that trypsin, similarly to the *in vivo* system activating wheat PPOs, only leads to partial protein degradation. However, as immunoblotting of trypsin-treated fractions did not

detect any bands with clearly defined, lower molecular weight (data not shown), its action on PPO precursors is different from that of peptidase(s) active in maturing kernels. In this context, it is also of interest that the molecular weight of the (active) PPO purified from the variety "Rampart" (Fig. 4.2) was only 58 kD, indicating further processing beyond the 67 kD form detected by immunoblotting. It would be of considerable interest for our understanding of plant PPO function to determine exact cleavage sites after both *in vivo* enzyme activation, and after (further) *in vitro* degradation. Additionally, it is now clear that one or several peptidases are involved in regulating PPO activity levels found in mature wheat kernels, and their characterization will be important for both practical (wheat product chemistry) and basic reasons.

Early work (chapter 2) performed for this dissertation has demonstrated that wheat PPO activity, similarly to PPOs from other plants, is also influenced by strong anionic detergents such as SDS and N-lauroylsarcosine. Our work confirmed that SDS acts both on enzyme solubilization and activation, at least when high-PPO wheat varieties are used as a PPO source (Fig. 2.3; Jukanti et al., 2003). In this context, Gerdemann et al. (2002), applying comparative modeling studies to sweet potato PPO, have hypothesized that the C-terminal part of the polypeptide chain could have an inhibitory function, shielding the active site. These authors also stated that SDS might change the conformation of this shield, allowing substrate access to the active site. It is tempting to speculate that endogenous peptidases or trypsin might also act on this domain of the PPO polypeptide. Wheat, allowing the separate isolation of PPO precursor(s) (from 14 dpa kernels) and

mature protein (from bran, using an immunoaffinity strategy) prior to their analysis by mass spectrometry, constitutes an ideal system to directly test this hypothesis.

Besides their importance for our understanding of plant PPO function, our analysis of SDS influence on wheat PPO activities also has practical aspects. It has clearly made PPO assays (as used for germplasm screening) more robust, furnishing wheat breeders with an important analytical tool (chapter 2).

In conclusion, this work has unraveled several important aspects of wheat PPO function, both at the molecular (gene family and its expression) and biochemical level (activation of precursor proteins by limited proteolysis, and enzyme activation by detergents). Based on these data, the following approaches will lead to a more complete understanding of wheat PPO genetics, molecular biology and biochemistry: (1) The development of gene-specific primers/probes (e.g. for quantitative real-time RT-PCR) will allow to determine the contribution of all cluster 2 genes to PPO transcript levels in developing kernels. These data, possibly complemented with protein-level studies (necessitating the development of isoform-specific monoclonal or peptide antibodies) will indicate if one or several specific cluster 2 genes contribute to high PPO levels in mature kernels. Similar strategies will be applicable to investigate cluster 1 gene expression and protein synthesis in other tissues. (2) Utilization of gene-specific primers, in combination with the use of nullitetrasonic and ditelosomic “Chinese spring” wheat lines (Sears 1954) will allow to identify the chromosomal location of wheat PPOs. This approach will also allow the development of molecular markers for germplasm screening, facilitating the development of low-PPO varieties by breeders. (3) A combination of

immunoaffinity purification and mass spectrometry of both precursor and mature PPOs will allow to understand the activation mechanism of wheat PPOs. (4) Starting with peptidase inhibitor analysis, and possibly using recent techniques such as activity profiling (van der Hoorn *et al.*, 2004), it should be feasible to identify and characterize the peptidase(s) responsible for *in vivo* activation of PPO precursors during kernel development. Together, these experiments will lead to a more complete understanding of plant PPO function, and will also indicate which gene(s) need to be targeted for germplasm improvement, either through classical breeding, or through transgenic approaches such as antisense or RNAi (Baulcombe, 2004) technology.

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APPENDIX

AUTHORIZATION CERTIFICATE

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08/09/05

Dear Karen Cummings:

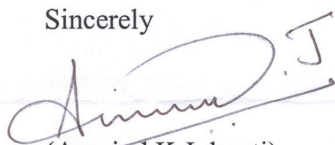
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