JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Three Polyphenol Oxidases from Red Clover (*Trifolium pratense*) Differ in Enzymatic Activities and Activation Properties

GEORGE E. SCHMITZ, MICHAEL L. SULLIVAN, AND RONALD D. HATFIELD*

U.S. Dairy Forage Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1925 Linden Drive West, Madison, Wisconsin 53706

Polyphenol oxidases (PPOs) oxidize *o*-diphenols to *o*-quinones, which cause browning reactions in many wounded fruits, vegetables, and plants including the forage crop red clover (*Trifolium pratense* L.). Production of *o*-quinones in red clover inhibits postharvest proteolysis during the ensiling process. The cDNAs encoding three red clover PPOs were expressed individually in alfalfa (*Medicago sativa* L.), which lacks detectable endogenous foliar PPO activity and *o*-diphenols. Several physical and biochemical characteristics of the red clover PPOs in alfalfa extracts were determined. In transgenic alfalfa extracts, red clover PPOs exist in a latent state and are activated (10−40-fold increase in activity) by long incubations (>2 days) at ambient temperature or short incubations (<10 min) at ≥65 °C. PPO1 appears to be more stable at high temperatures than PPO2 or PPO3. During incubation at ambient temperature, the molecular masses of the PPO enzymes were reduced by approximately 20 kDa. The apparent pH optima of latent PPO1, PPO2, and PPO3 are 5.5, 6.9, and 5.1, respectively, and latent PPO1 is slightly activated (~5-fold) by low pH. Activation of the PPOs shifts the pH optima to ~7, and the activated PPOs retain substantial levels of activity as the pH increases above their optima. The latent and activated PPOs were surveyed for ability to oxidize various *o*-diphenols, and activation of the PPOs had little effect on substrate specificity. Activation increases the V_{max} but not the affinity of the PPO enzymes for caffeic acid. Results indicate red clover PPOs undergo structural and kinetic changes during activation and provide new insights to their effects in postharvest physiology.

KEYWORDS: Polyphenol oxidase; red clover; *Trifolium pratense*; enzymatic activities

INTRODUCTION

Polyphenol oxidases (PPOs) (EC 1.10.3.1 or EC 1.14.18.1), also known as catechol oxidases, are copper-containing proteins that oxidize *o*-diphenols to *o*-quinones. The secondary polymerization reactions of quinonic compounds and proteins generate the brown or black pigments commonly associated with wounded fruits, vegetables, and plants. The physiological functions of PPO are not fully known, but PPO is associated with a variety of processes including defenses against insect predation (*1, 2*) and bacterial pathogenesis (*3, 4*). PPOs are encoded by nuclear genes, and nearly all PPOs are transported to the thylakoid lumen of the chloroplast (*5, 6*), but a role in the chloroplast has not been established. Several PPOs are involved in the biosyntheses of pigments such as aurones in the flowers of snapdragon (*Antirrhinum majus*) (*7*), of betalains in moss rose (*Portulaca grandiflora*) and beet (*Beta vulgaris*) (*8*), of 8–8′-linked lignans in creosote bush (*Larrea tridentata*) (*9*), and of melanins in the bacterium *Marinomonas mediterranea* (*10*).

The biochemical and kinetic properties of many PPOs from fruits and vegetables have been investigated because their associated browning reactions affect food flavor and quality. Many PPOs exist in forms with low or undetectable activity that is enhanced by fatty acids (*11*), mild heat (*12*), acid or base shock (*13*), proteolysis with trypsin (*14, 15*), extended incubation (*16*), or detergents such as sodium dodecyl sulfate (SDS) (*14, 17–19*). Some latent and activated PPOs differ by approximately15 kDa at the C terminus (*20–22*), and Gerdemann et al. (*23*) have predicted that the C-terminal peptide extension may act as a shield to the active site, thereby hindering substrate binding. Activation of *B. vulgaris* PPO by trypsin digest or by treatment with SDS yields similar levels of activation, and because these methods of activation are not additive (*14*), they may have similar consequences. The ease of activation may support a hypothesis that the properties of in vitro activated PPOs are comparable to those of in vivo PPOs. However, it seems likely that some chemical methods of activation such as that by SDS may affect not only the PPO enzyme but also the binding properties of some substrates. Indeed, increasing concentrations of SDS in assays for *B. vulgaris* PPO cause either a sigmoidal or hyperbolic trend of activation depending on the

* Corresponding author [telephone (608) 890-0062; fax (608) 890-0076; e-mail Ronald.Hatfield@ars.usda.gov.

10.1021/jf072488u
Published on Web 12/11/2007

relative hydrophobicity of the substrate (*24*). Thus, the properties of activated PPOs could vary depending on the method of activation that is employed.

Many PPOs have been tested for activity using different *o*-diphenol substrates including catechol, 4-methylcatechol, 4-*tert*-butylcatechol, L-DOPA (L-3, 4-dihydroxyphenylalanine), (−)-epicatechin, (+)-catechin, chlorogenic acid, and dopamine, and each PPO seems to differ in its substrate specificity, enzyme characteristics, and possibly its physiological role. Unfortunately, the in vivo presence of several commonly tested *o*-diphenols has not been demonstrated, and their physiological relevance remains uncertain. Some PPOs such as those from red clover (*Trifolium pratense* L.) oxidize the physiological compound caffeic acid, an intermediate in the phenylpropanoid pathway and a central component of many *o*-diphenol ester or methoxy compounds.

Red clover and alfalfa (*Medicago sativa* L.) are forage crops similar in protein content. Harvesting of forage crops such as these disrupts plant cell membranes and releases proteases that reduce true protein content. During ensiling, a popular method of preserving forage for animal feed, bacterial fermentation eventually lowers the pH of silage, decreases proteolysis, and stabilizes the protein content, but protein losses prior to a reduction in pH can be large. In the early stages of ensiling, postharvest protein losses can be as high as 44% to 87% for alfalfa (*25, 26*). Ruminants poorly utilize the nonprotein nitrogen products of proteolysis (ammonia, amino acids, and small peptides), resulting in increased nitrogen excretion. Farmers must therefore supplement ruminant diets with protein, resulting in additional costs. In contrast to alfalfa, red clover loses only 7−40% of its original protein (*25*) during ensiling. The greater retention of true protein content in red clover during ensiling does not result from differences in endogenous protease activity (*27*), but from the presence of PPO activity and endogenous *o*-diphenols (*28*). In fact, providing alfalfa with PPO activity and *o*-diphenols, both of which are undetectable in alfalfa extracts (*29*), lowers the rate of proteolysis to levels comparable to those of red clover (*16, 29, 30*). Exploitation of this effect could improve stability of protein from alfalfa and other forages during ensiling, reduce costs to farmers, and decrease nitrogenous wastes that have negative environmental impacts. Unfortunately, the mechanism of proteolysis inhibition and the components involved are not yet well-defined.

Like the PPOs of some other organisms, red clover PPOs expressed in transgenic alfalfa exhibit low activity when extracted but can be activated by extended (≥2 days) incubation at ambient temperature (*16*). Although imprecise, the term latent is used frequently in the literature to describe PPOs that have low activity in addition to those that do not display activity. For consistency, the term latent is used herein to describe red clover PPOs that have low activity upon extraction prior to extended incubation at ambient temperature. The effects of activation on PPO enzyme activity, stability, and substrate specificity are poorly defined but important to optimize the ensiling process for forage crops such as alfalfa. In this study, three red clover PPOs expressed in transgenic alfalfa were analyzed for their abilities to oxidize various *o*-diphenols, and several biochemical and physical characteristics (pH optima, temperature optima, thermal stability, and relative molecular mass) were determined. Direct isolation of latent and activated PPOs from red clover was not reasonable because the PPOs were activated only 2-fold in red clover extracts by extended incubation following a purification step to remove endogenous *o*-quinones (*16*). This observation may mean the PPOs in red clover extracts are activated primarily before or during extraction, that red clover extracts lack an activation factor, or that activation is inhibited. Although total PPO activity in red clover extracts is comparable to those found in PPO-expressing transgenic alfalfa extracts that have been activated, activity levels of individual PPOs in red clover extracts cannot be determined to distinguish between these two possibilities.

The red clover PPO-expressing transgenic alfalfa plants aided the analyses of these enzymes in several ways. First, the absence of endogenous PPO activity and *o*-diphenols in alfalfa allowed the immediate assay of the latent red clover PPOs without enzyme purification steps that could inadvertently activate the PPOs. Second, PPOs could be activated via factors inherent to alfalfa extracts (i.e. via long incubations at ambient temperature) rather than by external manipulations such as SDS treatment or trypsin digestion, and controlled comparisons could be made between latent and activated PPOs. Third, due to similarities in protein content between alfalfa and red clover, the activated red clover PPOs in alfalfa extracts might more closely resemble the active PPOs naturally found in red clover. Knowing the molecular and biochemical events during activation of red clover PPO in transgenic alfalfa may help to optimize a PPO-based method to preserve forage protein during ensiling. This work was undertaken to strengthen our knowledge of red clover PPO enzymes in order to develop strategies for their utilization to improve forage preservation during ensiling.

RESULTS AND DISCUSSION

Red Clover PPOs Are Processed Similarly in Red Clover and Transgenic Alfalfa Leaf Extracts. Consistent with previous results (*16*), red clover PPOs expressed in alfalfa were stable in leaf extracts, and after incubation of PPO-alfalfa extracts at room ambient temperature for 6−8 days, the activities of red clover PPO1, PPO2, and PPO3 increased by approximately 25-, 10-, and 40-fold, respectively, in standard assay conditions. The pH of the activated, buffered extracts did not change substantially during the incubation, and the levels of activation peak by approximately 4 days and plateau with similar levels of activity in extracts incubated for 6−8 days.

To assess molecular changes to the PPO enzymes during activation, desalted extracts from red clover and the transgenic alfalfa PPO plants were monitored by immunoblot analyses during 2 weeks of incubation at ambient temperature (**Figure 1**). Without desalting, activity in red clover extracts decreases rapidly to diminutive levels, presumably due to inhibition byproduct of the PPO reaction. Consistent with previous results (*16*), the dominant protein bands recognized by the anti-PPO1 antibodies at time = 0 reveal that the PPOs expressed in transgenic alfalfa and red clover were approximately 65 kDa, whereas doublets were observed in extracts of red clover and PPO1- and PPO3-expressing transgenic alfalfa, and several minor bands (approximately 50, 37, and 35 kDa) were observed in PPO2-expressing transgenic alfalfa. By 1−2 days of incubation, an additional protein band of approximately 45 kDa became visible in extracts of red clover and transgenic alfalfa expressing PPO1 or PPO2, and two protein bands (approximately 44–47 kDa) appeared in transgenic alfalfa expressing PPO3. No proteins in extracts of leaves of alfalfa transformed with the empty vector pILTAB357 reacted with the anti-PPO1 antiserum during the 2 week incubation (data not shown). Each PPO-expressing extract showed some degree of PPO activation with

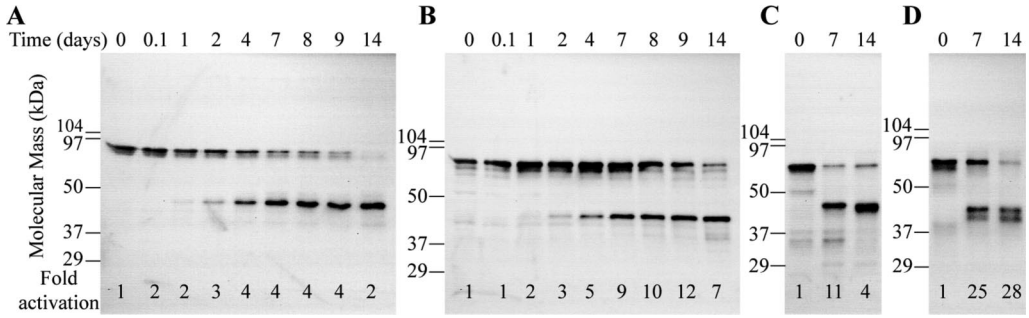

Figure 1. Relative molecular masses of clover PPOs decrease during incubation and activation. Desalted leaf extracts from red clover (**A**) or alfalfa transformed with PPO1 (**B**), PPO2 (**C**), or PPO3 (**D**) were incubated for 14 days at ambient temperature. At various times, aliquots were removed, submitted to immunoblot analyses (volume loaded was equivalent to 5 μg of protein at time = 0), and assayed for activity in standard conditions (2 mM caffeic acid). The extent of enzyme activation relative to initial activity and the length of incubation are indicated. Initial PPO activities of the extracts were 22 (red clover), 8.7 (PPO1-alfalfa), 0.15 (PPO2-alfalfa), and 3.2 (PPO3-alfalfa) nmol s^{-1} mg^{-1}.

red clover and PPO3-expressing transgenic alfalfa displaying the least (4 times) and the greatest (28 times) increases, respectively. Interestingly, the pattern of PPO processing was most similar between red clover and PPO1-expressing transgenic alfalfa, suggesting that this strain of red clover predominantly expresses PPO1 and that proteolytic events are similar in alfalfa and red clover extracts. However, the extent of activation in red clover was consistently lower (2−4-fold) than that of PPO1-expressing transgenic alfalfa (approximately 10-fold). Initial PPO activity was higher in red clover than in PPO1-expressing transgenic alfalfa (22.4 versus 8.7 nmol s^{-1} mg^{-1}), but it was unclear whether PPO concentration was also higher in the red clover extract. By 14 days, nearly all of the 65 kDa protein band of each extract had been degraded, but the maximum activation was generally obtained before all of the 65 kDa peptide was degraded. In some cases, the loss of the 65 kDa peptide appeared to be disproportionate to the level of activation and the concentration of the 45 kDa peptides and suggests activation may not depend entirely on the appearance of the 45 kDa peptide. The lack of a smaller band (20 kDa) appearing (**Figure 1**) may be due to simple elution of this fragment off the gel, additional degradation of the fragment to sizes not retained on the gel, or lack of specific recognition sites for the PPO antibody to bind. In preliminary tests to determine if proteolysis is a requirement for activation, PPO3 (displaying the greatest increase in activity upon activation) was incubated with a plant protease inhibitor cocktail (Sigma P9599) during the normal activation period. Protease inhibitors appeared to decrease the rate of activation and the extent (50% of maximum activation) for this PPO but did not eliminate activation. Western blot analysis of PPO3 activation in the presence of protease inhibitors indicates a reduction in total proteolysis (data not shown). These preliminary results suggest that proteolysis is involved in PPO activation but that it is not a sole requirement. Additional future work is needed to clearly define the roles of protease(s) in PPO activation. The alfalfa PPO system may be ideal for clearly defining the relationship between proteolysis and enzyme activation.

In the experiments below comparisons are made between crude extracts of transgenic alfalfa leaves expressing red clover latent PPOs (LtPPOs) and PPOs that have been activated by approximately 1 week of incubation at ambient temperature (AcPPOs).

Activation Increases the V_{max} of Red Clover PPOs. We hypothesized that extended incubation could activate red clover PPOs by increasing their limiting rates (V_{max}), their affinities for an *o*-diphenol substrate, or both. LtPPOs and AcPPOs in alfalfa extracts were assayed in the presence of various

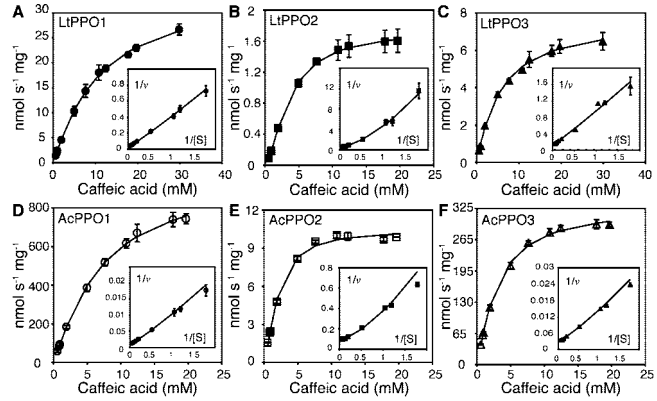

Figure 2. PPO activity as a function of caffeic acid concentration. Initial rates of caffeic acid oxidation were determined with fixed volumes of transgenic red clover PPO-expressing alfalfa extracts of latent (solid symbols) or activated (open symbols) PPO1 (circles), PPO2 (squares), or PPO3 (triangles), and the results were plotted and fitted to the shown curve by a least-squares method. The inset graphs are double-reciprocal (Lineweaver–Burk) plots of the same data and fitted curves. The characters *v* and [S] represent initial velocity and caffeic acid concentration, respectively. Error bars indicate the standard deviation of an average of values from at least three samples.

Table 1. Kinetic Constants of Latent and Activated PPOs[a]

		H	$K_{0.5}$ (mM)	V_{max} (nmol s^{-1} mg^{-1})
PPO1	latent	1.1	11	35
	activated	1.1	7.0	990
PPO2	latent	1.6	3.6	1.7
	activated	1.5	2.0	10
PPO3	latent	1.1	5.5	7.6
	activated	1.2	3.0	330

[a] The Hill coefficient (*H*), $K_{0.5}$, and V_{max} constants of latent and activated PPO1, PPO2, and PPO3 were determined by least-squares fitting of initial velocity data versus caffeic acid concentration. Parameters are defined under Materials and Methods.

concentrations of caffeic acid (**Figure 2**). When the data for LtPPO2 and AcPPO2 are plotted in double-reciprocal (Lineweaver–Burk) graphs (inset graphs of **Figure 2B**,**E**), the curves are nonlinear with slightly upward concavity that is characteristic of positive cooperativity. To adjust for the deviation from Michaelis–Menten kinetics, the V_{max} and $K_{0.5}$ of each PPO for caffeic acid were calculated (**Table 1**) by least-squares fitting of the data to eq 1 (under Materials and Methods), which contains the Hill coefficient, *H*. PPO1 and PPO3 deviate only

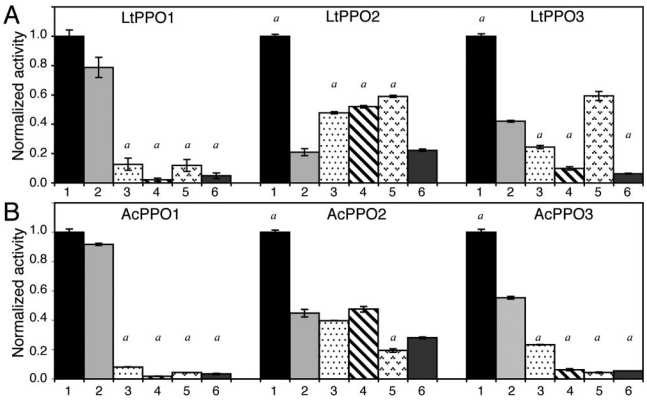

Figure 3. Substrate specificity of latent and activated PPOs. Latent (**A**) and activated (**B**) PPOs were assayed for activity in the presence of the indicated *o*-diphenol (2 mM): 1, caffeic acid; 2, chlorogenic acid; 3, (−)-epicatechin; 4, (+)-catechin; 5, catechol; 6, dopamine. Values shown are normalized to the specific activity (in nmol s^{-1} mg^{-1}) of each PPO for caffeic acid (LtPPO1, 3.6; AcPPO1, 99; LtPPO2, 0.34; AcPPO2, 5.8; LtPPO3, 1.5; AcPPO3, 68). The letter designation "*a*" indicates the difference from chlorogenic acid is significant ($P < 0.01$) by analysis of variance in two independent experiments. Error bars indicate the standard deviation of an average of values from two samples.

slightly ($H \leq 1.2$) from the Michaelis–Menten equation (where $H = 1$), whereas the Hill coefficients for LtPPO2 and AcPPO2 are approximately 1.5. The $K_{0.5}$ values of AcPPOs are more than half of those of the LtPPOs, but the V_{max} values for AcPPO1, AcPPO2, and AcPPO3 are increased by approximately 25-, 6-, and 40-fold compared to their latent counterparts. Increases in activity are consistent with levels of activation observed when using standard assay conditions with a nonsaturating caffeic acid concentration (2 mM). Extended incubation at ambient temperature activates red clover PPOs primarily through increasing the V_{max} of the enzymes.

One hypothesis to explain the mechanism of PPO activation could be that proteolytic cleavage in vivo "matures" the PPO enzyme and that in vitro methods of activation simulate the natural maturation mechanism. In seeming contradiction to this hypothesis, fresh, desalted red clover extracts exhibit high initial PPO activity, lack the 45 kDa peptide, and have a low activation potential despite similar trends of change in PPO molecular mass. From this hypothesis one might predict that an activated PPO would have a higher affinity for *o*-diphenol substrates, but our data show that activation exerts only minor effects on the $K_{0.5}$ and would therefore not agree with this prediction. Others have discovered that SDS-activated or acid-activated field bean PPO had a larger Stokes radius than the native (latent) PPO, and SDS activation increased the intrinsic fluorescence of tryptophan residues, indicating the enzyme's tertiary structure had changed to expose more tryptophan residues (*31*). The hypothesis that activation occurs through alteration of the enzyme's size or shape by proteolysis or other treatment would be consistent with our data that show large increases in the V_{max} of activated PPOs.

Substrate Specificity Differs between Red Clover PPOs, and Activation Yields Only Minor Changes in Substrate Specificity. The rates of oxidation of diverse *o*-diphenols at a final concentration of 2 mM by LtPPOs and AcPPOs were measured (**Figure 3**). Among the *o*-diphenols tested in this assay, all LtPPOs and AcPPOs oxidized caffeic acid at the fastest rates. The preference of red clover PPOs for caffeic acid is consistent with the high levels of caffeic acid derivatives such as clovamide (caffeoyl DOPA) and phasalic acid (caffeoyl malate) in the leaves of red clover (*32*) that are presumably major in vivo substrates. Unfortunately, it was not possible to measure the activities of red clover PPOs for clovamide or phasalic acid because these *o*-diphenols are not commercially available.

Generally, appreciable oxidation rates for chlorogenic acid, an ester of quinic and caffeic acids [3-(3, 4-dihydroxycinnamoyl)quinic acid], were also observed. To correct for the differences in PPO expression and to display the relative specificity for the *o*-diphenol substrates, the activities of each LtPPO or AcPPO were normalized to the rate of caffeic acid oxidation by that enzyme. For a given enzyme, activities for (−)-epicatechin, (+)-catechin, catechol, and dopamine were significantly different ($P < 0.01$ using analysis of variance) from the activity for caffeic acid. Statistical significance was calculated also by comparing the oxidation rate for each *o*-diphenol to the oxidation rate for chlorogenic acid. From this analysis, it is clear that PPO1, PPO2, and PPO3 differ in their substrate specificities. Both LtPPO1 and AcPPO1 rapidly oxidize only caffeic and chlorogenic acids, and the difference in oxidation rates between these *o*-diphenols by LtPPO1 or AcPPO1 was not statistically significant. The normalized rates of chlorogenic acid oxidation by LtPPO2, AcPPO2, LtPPO3, and AcPPO3 are lower than those seen for LtPPO1 or AcPPO1. However, LtPPO2 and AcPPO2 oxidize a broader range of substrates than the PPO1 enzymes, catalyzing reactions also for (−)-epicatechin, (+)-catechin, catechol, and dopamine. It should be noted that the actual (not normalized) activities of LtPPO2 and AcPPO2 are much lower than those of the respective latent or activated PPO1 or PPO3 enzymes despite having comparable levels of expression in leaves of transgenic alfalfa (*16*). PPO2 is most different from the other PPOs in terms of sequence identity and expression level and pattern in red clover (*16*), so the differences in its substrate specificity and activity may not be surprising and may indicate that PPO2 has an in vivo function different from those of PPO1 or PPO3. The normalized activities of LtPPO3 and AcPPO3 for (−)-epicatechin, (+)-catechin, and dopamine are generally not as high as those of LtPPO2 or AcPPO2 and bear greater similarities to the PPO1 enzymes. The monophenol L-tyrosine, the *o*-diphenol protocatechuic acid, and the triphenol gallic acid were not oxidized by any latent or activated red clover PPO in this assay (data not shown).

When considering the effects of activation on substrate specificity, we expected that PPO activation would either alter substrate specificity or amplify all activities proportionately. The substrate preferences of any LtPPO and its respective AcPPO (compare panels **A** and **B** of **Figure 3**) are approximately the same with two exceptions. First, the relative activities of PPO2 and PPO3 for catechol, the simplest *o*-diphenol, are reduced by 3−10-fold in the activated forms, indicating the activities for catechol are not increased proportionately to those for caffeic acid. The meaning of this observation is unclear but may indicate catechol is not an important physiological substrate of these enzymes. Second, AcPPO2 had a 2-fold greater activity for chlorogenic acid than LtPPO2. Even when these exceptions are taken into consideration, activation seems to have only minor effects on the relative substrate affinities.

Activation Changes the pH-Dependent Activity Profiles of Red Clover PPOs. Figure 4 displays the rates of caffeic acid oxidation by the LtPPOs and AcPPOs at various pH values. The pH curves are strikingly different between the LtPPOs and the AcPPOs. LtPPO1 exhibits an activity optimum at pH 5.5, resulting in an approximately 14-fold higher activity than that at pH 7 (**Figure 4A**). AcPPO1 has a pH activity

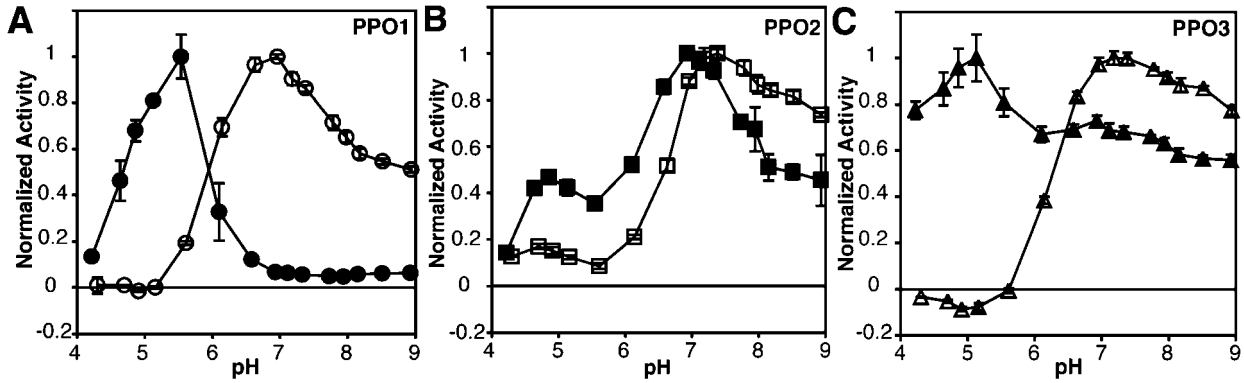

Figure 4. Optimal pH for latent and activated PPOs. Extracts containing latent (solid symbols) or activated (open symbols) PPOs were assayed in citrate−phosphate buffer at pH values from 4 to 9 using 2 mM caffeic acid as substrate. Values shown are normalized to the peak specific activity in nmol s^{-1} mg^{-1} for each PPO (**A**, LtPPO1, 61; AcPPO1, 98; **B**, LtPPO2, 0.23; AcPPO2, 4.1; **C**, LtPPO3, 2.0; AcPPO3, 55). Error bars indicate the standard deviation of an average of values from two to four samples.

peak at pH 7.0 that is broader than that of LtPPO1. The pH profile of LtPPO2 is bimodal with a small peak at pH 4.9 and a large peak at pH 6.9, and LtPPO3 shows no large changes in activity in the pH range from 4.2 to 8.9 but has a slight activity peak at pH 5.1. Optima pH of AcPPO2 and AcPPO3 are 7.4 and 7.2, respectively. In addition, each AcPPO retains at least 50% of its peak activity as the pH is increased above its optimum up to 8.9.

To test if LtPPO1 is activated by acidic pH, 2.5 μL of 1 N acetic acid or extraction buffer (see Materials and Methods) was added to 40 μL of LtPPO transgenic alfalfa extracts to lower the pH to 4.9 or to leave the extract pH unaffected. Precisely 1 min after mixing, the resulting extracts were assayed in standard conditions (pH 7.0). The activity of the acidified LtPPO1 was moderately higher than the activity of the LtPPO1 that was maintained at pH 7.5 (15.2 ± 5.7 vs 2.9 ± 0.2 nmol s^{-1} mg^{-1}, respectively). Acid activation of LtPPO1 (~5-fold) seems to account for much, but possibly not all, of the enhancement of activity (~10-fold) at pH 4.9 in the pH curve. The inability to generate the higher activities by acid shock could be explained if acid activation is reversible. Activities of LtPPO2 or LtPPO3 were not substantially affected by acidification in parallel experiments (data not shown).

Low pH optima (≤5.5) among PPOs are not unusual (*12, 33, 34*), and plateaus of activity above pH 5.5 have been seen also in *B. vulgaris* and *E. japonica* PPOs (*15, 24, 35*), but not in all PPOs. The elimination of a low pH optimum by activation occurs also for some PPOs of other organisms when activated by SDS (*17, 35*). However, the pH optima of PPOs vary, and even the same PPO enzyme can have different pH optima depending on the *o*-diphenol substrate. For example, the pH optimum of purified garland chrysanthemum PPO is 4.0 for chlorogenic acid but 8.0 for epicatechin (*36*). Thus, responses in PPO activity to changes in pH vary between species, and comprehensive themes are difficult to discern, but red clover PPOs, especially when activated, display similar trends across a pH spectrum. The AcPPOs have approximately neutral pH optima and activity plateaus at higher pH. This result may represent the presence of key histidine residues in the active site. The side-chain pK_a of histidine is 6.0, and histidine side chains would be predominantly reduced at pH >7. Six histidine residues coordinate the two copper atoms in catechol oxidase (*37*) and could conceivably be involved in the plateau effect with red clover PPOs.

Role of Temperature in PPO Activation and Optimum Acitivity. Retention of red clover PPO activity when stored at 30 °C for 2 weeks (*16*) indicates the PPO enzymes are especially stable. The activities of red clover LtPPOs and AcPPOs were

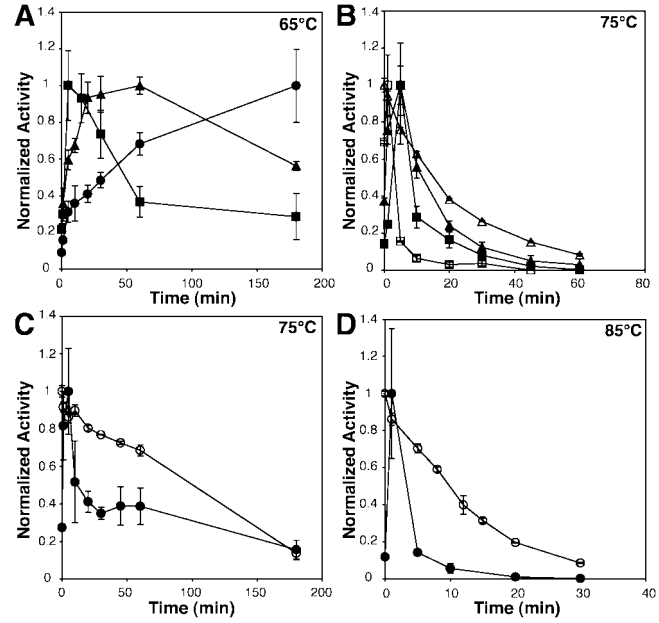

Figure 5. Thermal stability of latent and activated PPOs. Extracts containing latent (solid symbols) or activated (open symbols) PPO1 (circles), PPO2 (squares), and PPO3 (triangles) were incubated at 65 °C (**A**), 75 °C (**B**, **C**), and 85 °C (**D**). Aliquots were removed at the indicated times and assayed for activity at 25 °C using 2 mM caffeic acid as substrate. Specific activities are normalized to the peak activity achieved by the individual extract (**A**, LtPPO1, 32; LtPPO2 1.5; LtPPO3, 5.7;**B**, LtPPO2, 2.1; AcPPO2, 8.0; LtPPO3, 4.0; AcPPO3, 71; **C**, LtPPO1, 17; AcPPO1, 117; **D**, LtPPO1, 58; AcPPO1, 112). The activity at 0 min was determined immediately before the extract was shifted to the incubation temperature. Error bars indicate the standard deviation of an average of values from two samples.

determined when the extracts were preincubated in a water bath at high temperatures (**Figure 5**). Incubation of extracts at 65 °C (**Figure 5A**) results in activation of the LtPPOs, with LtPPO1, LtPPO2, and LtPPO3 attaining peak activities at ≥180, ~5, and ≥60 min (11-, 4.5-, and 4-fold), respectively, suggesting that short incubations (<3 h) at high temperature do not activate red clover PPOs as much as long incubations (6–8 days) at ambient temperature. PPO activation by heating suggests that alteration of enzyme conformation by refolding or slightly unfolding the PPO enzymes activates them. LtPPO1 retains an elevated level of activity for >3 h at 65 °C, whereas the activity levels of LtPPO2 and LtPPO3 decline after long periods at 65 °C, although they retain elevated activity levels even at 3 h.

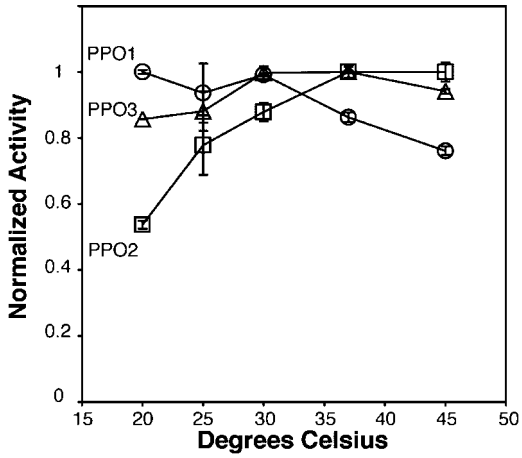

Figure 6. Temperature optima of activated PPOs. Extracts containing activated PPOs were assayed for activity at various temperatures using 2 mM caffeic acid as substrate. Activities are normalized to the peak activity (in nmol s^{-1} mg^{-1}) observed for each extract (PPO1, circles, 39; PPO2, squares, 5; PPO3, triangles, 77). Error bars indicate the standard deviation of an average of values from two samples.

The data indicate the red clover LtPPOs rank in thermostability in the order LtPPO1 > LtPPO3 > LtPPO2. When incubated at 75 °C, gradual activity losses were observed for both LtPPOs and AcPPOs after attaining their highest activities, but LtPPO1 (**Figure 5C**) and LtPPO3 (**Figure 5B**) lose activity more quickly than AcPPO1 and AcPPO3, respectively, indicating that activation of PPO1 and PPO3 by extended incubation at ambient temperature increases the thermal stability of these enzymes. LtPPO1 and AcPPO1 activities are fully eliminated by 30 min of incubation at 85 °C (**Figure 5D**), and consistent with the results at 75 °C, the rate of activity loss for LtPPO1 at 85 °C is substantially faster than that for AcPPO1. AcPPO2 is slightly activated (1.4-fold) by heating at 75 °C (**Figure 5B**), indicating that incubating PPO2 for approximately 1 week at ambient temperature does not fully activate it. Unlike AcPPO1 and AcPPO3 after attaining peak activity when heated at 75 °C, AcPPO2 loses activity at approximately the same or a slightly faster rate than its latent counterpart, LtPPO2.

Red clover PPOs appear to be more heat resistant than other PPOs (*35, 38*). PPOs from garland chrysanthemum (*36*) and broad bean (*17*) retain ~50% or more of their activity with incubations at 50 °C between 10 and 30 min, but not at higher temperatures like the red clover PPOs. In addition, AcPPO1 and AcPPO3 are denatured more slowly than their latent counterparts at higher temperatures (**Figure 5B−D**), indicating that activation by extended incubation at ambient temperature helps stabilize PPO1 and PPO3.

The activities of the red clover AcPPOs at different assay temperatures are shown in **Figure 6**. Because high temperatures activate LtPPOs, only AcPPOs were assayed at temperatures between 20 and 45 °C. AcPPO1 and AcPPO3 activities remain relatively constant in this range and indicate that these enzymes do not have distinct temperature optima between 20 and 45 °C. However, the activity of AcPPO2 nearly doubles as the assay temperature is increased to 45 °C. Because heating at 75 °C activated AcPPO2, it was possible that this modest increase in activity at 45 °C reflects further activation of the AcPPO2 enzyme rather than a true temperature optimum. However, an AcPPO2 transgenic alfalfa extract was incubated at 45 °C for 3 min and assayed at 30 °C but showed no increase in activity, indicating that AcPPO2 is not activated at 45 °C.

Conclusions. Activation of red clover PPOs occurs by extended incubation at ambient temperature and is accelerated at higher temperatures and, for PPO1, by acidification. The red clover PPOs exhibit a high degree of thermal stability, especially PPO1, which is stable at temperatures up to 65 °C for >3 h. This paper demonstrates that red clover PPOs have enzymatic and biochemical properties different from those of many other reported PPOs. These unique features include utilization of a wide range of substrates, shifts in pH optimum from latent to activated forms, and high degrees of thermal stability. A comparison between latent and naturally activated PPOs indicates differences in the V_{max}, but not the $K_{0.5}$, for each enzyme. The changes in relative molecular mass during extended incubation for PPO in red clover and PPO1 expressed in transgenic alfalfa are comparable, suggesting PPO1-expressing transgenic alfalfa will be a useful tool to further investigate the inhibition of proteolysis by PPO and *o*-diphenols and in optimizing this natural system of protein protection for the ensiling process.

MATERIALS AND METHODS

Plant Materials and Propagation. Regent-SY alfalfa plants (*39*) expressing red clover PPO1, PPO2, or PPO3 or that were transformed with the binary vector pILTAB357 (*40*) were used in all experiments and have been described previously (*16*). A single red clover genotype was used in all experiments involving red clover and was selected from a population of WI-2 (lot C136) germplasm (*41*). All experiments were conducted with alfalfa primary transformants propagated from stem cuttings in vermiculite in a growth chamber at approximately 25 °C with 15−16 h day^{-1} of approximately 100 μmol m^{-2} s^{-1} illumination from fluorescent lamps. Red clover and transformed alfalfa were maintained in a greenhouse year-round and fertilized weekly (Peter's soluble 20–20–20; Scotts, Marysville, OH). Supplemental lighting (13 h/day) was used during all but summer months. Leaf tissue from plants maintained in a greenhouse was used in all experiments.

Extract Preparation. Fully expanded young alfalfa or red clover leaves were powdered in liquid nitrogen with a mortar and pestle and extracted with 20 mM Tris−100 mM ammonium acetate (pH 7.5 with 1 N acetic acid) using 3 mL of buffer per gram of fresh weight as described by Sullivan et al. (*16*). For red clover tissue, 50 mM ascorbic acid was included in the extraction buffer to prevent extensive browning by endogenous PPO and *o*-diphenol PPO substrates. Extracts were maintained on ice or at 4 °C throughout the procedure. Extracts were centrifuged twice at 16000*g* for 10 min, each time transferring the supernatants to new tubes. Aliquots of 100 μL were placed in 1.5 mL microcentrifuge tubes, frozen with liquid nitrogen, and stored at −80 °C. Protein concentrations were estimated using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. Aliquots were thawed just prior to assay to determine activities of latent extracts.

PPO Assays. PPO activity was determined using a modification of the assay of Esterbauer et al. (*42*). A standard assay contained 20 μL of 100 mM caffeic acid in ethanol (2 mM final concentration) and 20 μL of a 2-nitro-5-thiobenzoic acid (TNB) solution [prepared by suspending 19 mg of 5,5′-dithiobis(2-nitrobenzoic acid) (D-8130, Sigma Aldrich) in 10 mL of water and adding 30 mg of $NaBH_4$] and was brought to a final volume of 1 mL with a 10-fold dilution of McIlvaine's citrate−phosphate buffer (15 mM $Na_2HPO_4 \cdot 7H_2O$, 2.3 mM citric acid), pH 7.0 at 30 °C. Adjustments of the buffer pH with strong acids or bases such as HCl or NaOH were avoided as these seemed to enhance non-PPO oxidation of caffeic acid. Due to differences of expression and activity levels, 10, 80, and 20 μL (~70, 700, and 150 μg of protein) of transgenic alfalfa plant extracts expressing latent PPO1, PPO2, and PPO3, respectively, were added to initiate the assays. The oxidation of TNB was measured by monitoring the $A_{412\ nm}$ of the assay with a Beckman DU800 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) and the data capture software. Reaction rates were determined by plotting $A_{412\ nm}$ versus time, determining the slope of the linear portion

of the reaction by linear regression and converting to nanomoles of substrate using the conversion 91.0 nmol/unit of $A_{412\ nm}$ (*42*). PPO-independent oxidation rates were determined by monitoring reactions containing equivalent volumes of extracts of alfalfa that was transformed with the empty vector pILTAB357, and PPO-independent oxidation rates were subtracted from the experimental values to give a net oxidation rate. Specific activity of a given extract was calculated by dividing the net reaction rate by the amount of extract protein present in the reaction and expressed as nanomoles per second per milligram. All assays were performed at 30 °C except for the temperature optima and temperature stability assays (described below). In all experiments, the assay buffers were pre-equilibrated to the assay temperature in a water bath, and the assay temperature was maintained by a Peltier Temperature Controller (Beckman Coulter, Inc.) during the reaction. Unless stated otherwise, reported values are the average of measurements made from at least two extract samples.

PPO Activation. PPO-alfalfa extracts (stored at −80 °C) were activated by rapidly thawing and incubating at ambient (∼23 °C) temperature for 6−8 days in 1.5 mL microcentrifuge tubes. The activated extracts were centrifuged for 5 s to pellet the precipitates that had formed, and the supernatants were transferred to fresh 1.5 mL microcentrifuge tubes. To adjust for higher activities, the clarified, activated extracts were diluted in extraction buffer (see Extract Preparation) or assayed using smaller volumes as appropriate. Typically, 10 μL aliquots of 10-fold dilutions (∼7 μg of protein) of the activated PPO1 and PPO3 extracts and a 10 μL aliquot of the undiluted (∼70 μg protein) activated PPO2 extract were assayed. Activated PPO-alfalfa extracts were incubated for the same number of days in a given experiment. The protein concentration was determined prior to the approximately 1 week of incubation, and this value was used to calculate the specific activity of an activated extract.

Substrate Specificity. Other *o*-diphenols were substituted for caffeic acid at the same final concentration (2 mM) in the assay described above to determine the relative activities toward each substrate for the latent and activated PPOs. To minimize nonenzymatic oxidation, caffeic acid, protocatechuic acid, (+)-catechin, and catechol were dissolved at 100 mM in 95% (v/v) ethanol, but due to the different solubilities of the substrates, the other *o*-diphenols were dissolved at 100 mM in various solvents: chlorogenic acid, 80% (v/v) ethanol; (−)-epicatechin, 70% (v/v) ethanol; and dopamine, methanol. In a control experiment, caffeic acid was dissolved in different solvents (95% ethanol, 70% ethanol, and methanol) and used as the substrate in PPO assays, but the solvent did not affect PPO activity.

Temperature Stability. The activities of latent and activated PPO-alfalfa extracts were monitored following incubations at 65, 75, and 85 °C in circulating water baths. Volumes of 0.2, 0.3, or 0.9 mL of the PPO-alfalfa extracts (or their dilutions) in 1.5 mL polypropylene microcentrifuge tubes were incubated at the indicated temperature for up to 3 h. Assays were performed as above except that the assay temperature was maintained at 25 °C. The first time point (0 min) was determined immediately before the incubation was initiated. Aliquots were removed by micropipet, promptly added to a prepared assay cuvette, and mixed by inversion, and reactions were monitored in a spectrophotometer. The specific activity of an enzyme was normalized to the highest value obtained for that PPO during incubation at the specified temperature.

Determining the pH Optima of Latent and Activated PPOs. The activities of the PPO-alfalfa extracts were measured from pH 4 to 8 essentially as described above using a 10-fold dilution of McIlvaine's citrate−phosphate buffer (*43*) at the defined mixtures of 0.2 M $Na_2HPO_4 \cdot 7H_2O$ and 0.1 M citric acid at 30 °C. Because this buffer was not defined for pH >8 and a pH as high as 9.0 was desired, mixtures of $Na_2HPO_4 \cdot 7H_2O$ and citric acid that would yield a pH of 8.5 (18 mL of H_2O, 1.975 mL of 0.2 M $Na_2HPO_4 \cdot 7H_2O$, and 25 μL of 0.1 M citric acid) and a pH of 9.0 (18 mL of H_2O, 1.995 mL of 0.2 M $Na_2HPO_4 \cdot 7H_2O$, and 5 μL of 0.1 M citric acid) at 30 °C were determined empirically. Reported values are the averages of three measurements of activity at each pH except for latent PPO2 and latent PPO3, which are the averages of two and four measurements, respectively.

Enzyme Kinetics. Caffeic acid was dissolved in dimethyl sulfoxide (DMSO) at 11 concentrations between approximately 25 and 1500 mM. Latent and activated PPO-alfalfa extracts were assayed for activity as above but in triplicate and in undiluted McIlvaine citrate phosphate buffer (pH 7.0; 165 mM $Na_2HPO_4 \cdot 7H_2O$, 18 mM citric acid) containing 20 μL of the caffeic acid/DMSO stock solutions. Thus, DMSO was maintained at a constant concentration, whereas the final concentration of caffeic acid was varied between approximately 0.5 and 30 mM. At the highest caffeic acid concentration (30 mM), the pH of the assay buffer had decreased to 6.78, and higher concentrations of caffeic acid were not used. Initial velocity data obtained at 30 mM caffeic acid were used in subsequent data analyses for only LtPPO1 and LtPPO3, the activities of which, according to pH optima data, were not largely affected by this alteration in pH. The pH of the assay buffer was >6.85 at the other caffeic acid concentrations used (≤20 mM), and the effects of pH on activity at these concentrations were assumed to be negligible. The initial velocity data were used to determine the kinetic parameters V_{max} and $K_{0.5}$ and the Hill coefficient by fitting the initial velocity data to the equation

$$v = [V_{max} \times S^H]/[K_{0.5} + S^H] \quad (1)$$

where v is the initial velocity, S is the concentration of caffeic acid, V_{max} is the velocity at saturating caffeic acid concentration, $K_{0.5}$ is the concentration of caffeic acid, where $v = 0.5V_{max}$, and H is the Hill coefficient (*44*). The Hill coefficient is an index of cooperativity and helps compensate for deviation from Michaelis–Menten enzyme kinetics. Data were fitted (before averaging three experimental replicates) by least-squares analyses using the Microsoft Excel Solver tool.

SDS-PAGE and Immunoblotting. SDS-PAGE [10% acrylamide; 37.5:1 acrylamide:bis(acrylamide)] was performed using standard methods (*45*). Leaf extracts for SDS-PAGE were prepared as detailed elsewhere in Materials and Methods. Low molecular weight compounds were removed from extracts by gel filtration. Briefly, a spin column was prepared by plugging the bottom of a 10 mL disposable syringe barrel with Miracloth (Cal-biochem, San Diego, CA) and filling the syringe with Sephadex G-25, Superfine (Amersham Biosciences, purchased from Sigma, St. Louis, MO), equilibrated with extraction buffer lacking ascorbic acid. The column was placed in a 50 mL tube and centrifuged at 160*g* for 2 min at 4 °C (final volume of packed matrix was approximately 5 mL). Leaf extract (1.5 mL) was applied to the packed column, the centrifugation repeated, and the eluate collected (desalted extract). Desalted extract was immediately assayed in standard assay conditions for PPO activity (time = 0) and at designated times during 2 weeks of incubation at ambient temperature. Protein concentration was determined at time = 0 as described above. At the time of assay, an aliquot of the desalted extract was denatured for SDS-PAGE by boiling with an equal volume of SDS-PAGE sample buffer for 10 min. Denatured samples were stored at 4 °C until all samples during the incubation had been obtained and were reheated at 65 °C for 10 min just prior to SDS-PAGE electrophoresis. For immunoblotting, 5 μg of total cellular protein was separated by SDS-PAGE and transferred to polyvinylidene fluoride (Bio-Rad Laboratories), and blots were processed and developed according to the manufacturer's instructions. Detection was accomplished using anti-PPO1 antiserum (*16*) diluted 1:2500, conjugated goat-anti-rabbit alkaline phosphatase (Bio-Rad Laboratories) diluted 1:3000 as the secondary antibody, and Immun-Star Chemiluminescent Substrate (Bio-Rad Laboratories). Blots were imaged using a Chemidoc System (Bio-Rad Laboratories).

Statistical Analysis. Batches of extracts of PPO-expressing alfalfa were generated and frozen in aliquots sufficient for one to four enzymatic assays of PPO activity. The same batch of extract was assayed for PPO activity before and after long incubations in a single experiment, and experiments were reproducible with different batches of extracts. Reported values are the average of two to four assays of extract aliquots as indicated in the text. An ANOVA single-factor test was used to evaluate significant differences of the data in the substrate specificity experiment at the criterion of $P < 0.01$. Averages, standard

deviations, and statistical significance were calculated using Excel (Microsoft Corp., Redmond, WA).

ACKNOWLEDGMENT

We thank Sara Rierson for assistance in plant maintenance and technical support and Debby Samac and Mindy Dornbush for production of original T_0 transgenic alfalfa.

LITERATURE CITED

(1) Felton, G. W. Indigestion is a plant's best defense. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (52), 18771–18772.
(2) Constabel, C. P.; Bergey, D. R.; Ryan, C. A. Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octdecanoid defense signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 407–411.
(3) Li, L.; Steffens, J. C. Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. *Planta* **2002**, *215* (2), 239–247.
(4) Thipyapong, P.; Hunt, M. D.; Steffens, J. C. Antisense down-regulation of polyphenol oxidase results in enhanced disease susceptibility. *Planta* **2004**, *220* (1), 105–117.
(5) Sommer, A.; Ne'eman, E.; Steffens, J. C.; Mayer, A. M.; Harel, E. Import, targeting, and processing of a plant polyphenol oxidase. *Plant Physiol.* **1994**, *105* (4), 1301–1311.
(6) Sherman, T. D.; Gardeur, T. L.; Lax, A. R., Implications of the phylogenetic distribution of polyphenol oxidase in plants. In *Enzymatic Browning and Its Prevention*; Lee, C. Y., Whitaker, J. R., Eds.; American Chemical Society: Washington, DC, 1995; pp 103–119.
(7) Nakayama, T.; Sato, T.; Fukui, Y.; Yonekura-Sakakibara, K.; Hayashi, H.; Tanaka, Y.; Kusumi, T.; Nishino, T. Specificity analysis and mechanism of aurone synthesis catalyzed by aureusidin synthase, a polyphenol oxidase homolog responsible for flower coloration. *FEBS Lett.* **2001**, *499* (1–2), 107–111.
(8) Steiner, U.; Schliemann, W.; Böhm, H.; Strack, D. Tyrosinase involved in betalain biosynthesis of higher plants. *Planta* **1999**, *208* (1), 114–124.
(9) Cho, M. H.; Moinuddin, S. G. A.; Helms, G. L.; Hishiyama, S.; Eichinger, D.; Davin, L. B.; Lewis, N. G. (+)-Larreatricin hydroxylase, an enantio-specific polyphenol oxidase from the creosote bush (*Larrea tridentata*). *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (19), 10641–10646.
(10) Lopez-Serrano, D.; Solano, F.; Sanchez-Amat, A. Identification of an operon involved in tyrosinase activity and melanin synthesis in *Marinomonas mediterranea*. *Gene* **2004**, *342* (1), 179–187.
(11) Golbeck, J. H.; Cammarata, K. V. Spinach thylakoid polyphenol oxidase: isolation, activation, and properties of the native chloroplast enzyme. *Plant Physiol.* **1981**, *67* (5), 977–984.
(12) Sheptovitsky, Y. G.; Brudvig, G. W. Isolation and characterization of spinach photosystem II membrane-associated catalase and polyphenol oxidase. *Biochemistry* **1996**, *35* (50), 16255–16263.
(13) Kenten, R. H. Latent phenolase in extracts of broad-bean (*Vicia faba* L.) leaves. I. Activation by acid and alkali. *Biochem. J.* **1957**, *67* (2), 300–307.
(14) Gandia-Herrero, F.; Jimenez-Atienzar, M.; Cabanes, J.; Garcia-Carmona, F.; Escribano, J. Evidence for a common regulation in the activation of a polyphenol oxidase by trypsin and sodium dodecyl sulfate. *Biol. Chem.* **2005**, *386* (6), 601–607.
(15) Tolbert, N. E. Activation of polyphenol oxidase of chloroplasts. *Plant Physiol.* **1973**, *51*, 234–244.
(16) Sullivan, M. L.; Hatfield, R. D.; Thoma, S. L.; Samac, D. A. Cloning and characterization of red clover polyphenol oxidase cDNAs and expression of active protein in *Escherichia coli* and transgenic alfalfa. *Plant Physiol.* **2004**, *136*, 3234–3244.
(17) Moore, B. M.; Flurkey, W. H. Sodium dodecyl sulfate activation of a plant polyphenoloxidase. Effect of sodium dodecyl sulfate on enzymatic and physical characteristics of purified broad bean polyphenoloxidase. *J. Biol. Chem.* **1990**, *265* (9), 4982–4988.
(18) Laveda, F.; Nunez-Delicado, E.; Garcia-Carmona, F.; Sanchez-Ferrer, A. Reversible sodium dodecyl sulfate activation of latent peach polyphenol oxidase by cyclodextrins. *Arch. Biochem. Biophys.* **2000**, *379* (1), 1–6.
(19) Sanchez-Ferrer, A.; Bru, R.; Garcia-Carmona, F. Novel procedure for extraction of a latent grape polyphenoloxidase using temperature-induced phase separation in Triton X-114. *Plant Physiol.* **1989**, *91* (4), 1481–1487.
(20) Robinson, S. P.; Dry, I. B. Broad bean leaf polyphenol oxidase is a 60-kilodalton protein susceptible to proteolytic cleavage. *Plant Physiol.* **1992**, *99* (1), 317–323.
(21) Rathjen, A. H.; Robinson, S. P. Aberrant processing of polyphenol oxidase in a variegated grapevine mutant. *Plant Physiol.* **1992**, *99* (4), 1619–1625.
(22) Mayer, A. M.; Harel, E. Polyphenol oxidase in plants. *Phytochemistry* **1979**, *18*, 193–215.
(23) Gerdemann, C.; Eicken, C.; Galla, H.-J.; Krebs, B. Comparative modeling of the latent form of a plant catechol oxidase using a molluskan hemocyanin structure. *J. Inorg. Biochem.* **2002**, *89*, 155–158.
(24) Gandia-Herrero, F.; Jimenez-Atienzar, M.; Cabanes, J.; Garcia-Carmona, F.; Escribano, J. Differential activation of a latent polyphenol oxidase mediated by sodium dodecyl sulfate. *J. Agric. Food Chem.* **2005**, *53*, 6825–6830.
(25) Papadopoulos, Y. A.; McKersie, B. D. A comparison of protein degradation during wilting and ensiling of six forage species. *Can. J. Plant Sci.* **1983**, *63*, 903–912.
(26) Muck, R. E. Dry matter level effects on alfalfa silage quality. I. Nitrogen transformation. *Trans. ASAE* **1987**, *30*, 7–14.
(27) Jones, B. A.; Hatfield, R. D.; Muck, R. E. Characterization of proteolysis in alfalfa and red clover. *Crop Sci.* **1995**, *35* (2), 537–541.
(28) Sullivan, M. L.; Hatfield, R. D. Polyphenol oxidase and *o*-diphenols inhibit postharvest proteolysis in red clover and alfalfa. *Crop Sci.* **2006**, *46*, 662–670.
(29) Jones, B. A.; Hatfield, R. D.; Muck, R. E. Screening legume forages for soluble phenols, polyphenol oxidase and extract browning. *J. Sci. Food Agric.* **1995**, *67*, 109–112.
(30) Jones, B. A.; Muck, R. E.; Hatfield, R. D. Red clover extracts inhibit legume proteolysis. *J. Sci. Food Agric.* **1995**, *67*, 329–333.
(31) Kanade, S. R.; Paul, B.; Rao, A. G.; Gowda, L. R. The conformational state of polyphenol oxidase from field bean (*Dolichos lablab*) upon SDS and acid-pH activation. *Biochem. J.* **2006**, *395* (3), 551–562.
(32) Yoshihara, T.; Yousikawa, H.; Sakamura, S.; Sakuma, T. Clovamides; L-dopa conjugated with *trans*- and *cis*-caffeic acids in red clover (*Trifolium pratense*). *Agric. Biol. Chem.* **1974**, *38*, 1107.
(33) Parr, A. J.; Bolwell, P. G. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols concentration or profile. *J. Sci. Food Agric.* **2000**, *80*, 985–1012.
(34) Valero, E.; Garcia-Carmona, F. pH-induced kinetic co-operativity of a thylakoid-bound polyphenol oxidase. *Biochem. J.* **1992**, *286* (Part 2), 623–626.
(35) Selles-Marchart, S.; Casado-Vela, J.; Bru-Martinez, R. Isolation of a latent polyphenol oxidase from loquat fruit (*Eriobotrya japonica* Lindl.): kinetic characterization and comparison with the active form. *Arch. Biochem. Biophys.* **2006**, *446* (2), 175–185.
(36) Nkya, E.; Kouno, C.; Li, Y. J.; Yang, C. P.; Hayashi, N.; Fujita, S. Purification and characterization of polyphenol oxidase from garland chrysanthemum (*Chrysanthemum coronarium* L.). *J. Agric. Food Chem.* **2003**, *51*, 5467–5471.
(37) Eicken, C.; Krebs, B.; Sacchettini, J. C. Catechol oxidase − structure and activity. *Curr. Opin. Struct. Biol.* **1999**, *9* (6), 677–683.
(38) Rapeanu, G.; Van Loey, A.; Smout, C.; Hendrickx, M. Thermal and high-pressure inactivation kinetics of polyphenol oxidase in Victoria grape must. *J. Agric. Food Chem.* **2005**, *53*, 2988–2994.
(39) Bingham, E. T. Registration of alfalfa hybrid Regen-SY germplasm for tissue culture and transformation research. *Crop Sci.* **1991**, *31*, 1098.

(40) Verdaguer, B.; de Kochko, A.; Beachy, R. N.; Fauquet, C. Isolation and expression in transgenic tobacco and rice plants, of the cassava vein mosaic virus (CVMV) promoter. *Plant Mol. Biol.* **1996**, *31*, 1129–1139.
(41) Smith, R. R.; Maxwell, D. P. Registration of WI-1 and WI-2 red clover. *Crop Sci.* **1980**, *20*, 831.
(42) Esterbauer, H.; Schwarzl, E.; Hayn, M. A rapid assay for catechol oxidase and laccase using 2-nitro-5-thiobenzoic acid. *Anal. Biochem.* **1977**, *77*, 486–494.
(43) McIlvaine, T. C. A buffer solution for colorimetric comparison. *J. Biol. Chem.* **1921**, *49*, 183–186.
(44) Cornish-Bowden, A. *Fundamentals of Enzyme Kinetics*, rev. ed.; Portland Press: London, U.K., 1995; p xiii, 343 pp.
(45) Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Springs Harbor Laboratory: Cold Springs Harbor, NY, 1988; p 508.

Received for review August 19, 2007. Revised manuscript received October 22, 2007. Accepted October 26, 2007. This work was supported in part by a CSREES NRI GRANT, 2006-35318-16587. Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

JF072488U