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In Vitro Proteolytic Inhibition, Polyphenol Oxidase Activity, and Soluble *o*-Diphenols in Grasses and Cereals

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Producing high quality forage remains a challenge due to potential protein degradation during ensiling. Ten grass species and four cereals were screened for proteolytic inhibition, polyphenol oxidase (PPO) activity, and the presence of soluble PPO substrates. In orchardgrass, ryegrass, smooth bromegrass, and meadow fescue extracts, the addition of caffeic acid resulted in 70−98% reduction in proteolysis for both greenhouse and field grown environments. These same grass extracts exhibited 10- to 1000-fold higher PPO activity compared to all other grasses and cereals, depending on the substrate supplied and environment grown. Chlorogenic acid and caffeic acid were the two abundant soluble *o*-diphenols. Tall fescue and timothy exhibiting low PPO activity contained the highest levels of chlorogenic acid (3−11 μmol g^{-1} FW): 10- to 100-fold more than any *o*-diphenol in all other grasses. These results suggest that several grass species contain PPO activity, but may lack appropriate *o*-diphenol substrates to effectively inhibit proteolysis during ensiling while other grasses lack PPO activity but contain potential soluble substrate. Environmental factors appear to influence these parameters, creating an opportunity to exploit and enhance proteolytic inhibition during ensiling by genetic manipulation.

KEYWORDS: Proteolysis; polyphenol oxidase; *o*-diphenols; caffeic acid; chlorogenic acid; ensiling; silage

INTRODUCTION

Forages play an important nutritional role in ruminant animal production and are grown by farmers in order to meet the fiber requirements of the animals and to help offset the high cost of grain in total mixed feed rations. Grassland agriculture is a reliable source of forage and a primary feed base for ruminant livestock. The proportion of the diet that is provided by forage can be up to 80% for beef cattle and up to 60% for dairy cows (*1*). For a high-producing dairy cow, about 50% of the crude protein and net energy for lactation requirements is supplied by forage, along with 80−90% of the fiber (neutral detergent fiber) requirements (*2*). In the U.S., a large percent of land area is dedicated to crop production, with a significant percent dedicated to grasslands. These grasslands are divided into cool-season, warm-season, and subtropical zones, with transitional areas between. The dominant grass forages grown in the Midwestern region include smooth bromegrass (*Bromus inermis* Leyss), timothy (*Phleum pratense*), meadow fescue (*Festuca pratensis*), orchardgrass (*Dactylis glomerata* L.), reed canarygrass (*Phalaris arundinacea* L.), ryegrass (*Lolium perenne* L.), and tall fescue (*Festuca arundinacea*) (*3*).

Obtaining and maintaining high quality forage for optimal animal performance is particularly challenging in the humid regions of agricultural production. Ensiling forage is a common practice in these regions, helping to ensure preservation of readily digestible carbohydrates and producing a high quality nutritional product at feeding. Frequently, protein degradation during the ensiling process can be extensive even under good ensiling conditions. Protein degradation occurs as the result of native proteases being released once the plant begins to senesce, a process that begins almost immediately after the forage is harvested (cut). Proteolytic activity continues even after ensiling until the pH has decreased to a level that will inhibit most plant proteases, typically pH 4 (*4*). In the case of ryegrass and orchardgrass, the pH must decrease to less than pH 5 (*5*) and less than pH 4 (*6*), respectively, to sufficiently suppress proteolytic activity. The longer it takes to reach a sufficiently low pH or if low pH is not reached, the more extensive the protein degradation. In general, protein breakdown is highest during the first day after sealing and decreases rapidly as the pH decreases, with little protein breakdown occurring after one week of proper ensiling (*7*).

All ensiled forages (for that matter any plant material that is ensiled) require careful handling to prevent excessive protein degradation. Papadopoulos and McKersie examined proteolysis in ensiled alfalfa, red clover, orchardgrass, and smooth bromegrass. The results revealed twice the protein degradation in alfalfa than in red clover. The ensiled grasses performed comparably to the ensiled alfalfa (*8*). Muck et al. had similar results from proteolysis determinations of ensiled orchardgrass, alfalfa, and red clover (*9*). In both studies, the samples with the least protein degradation were from ensiled red clover, with the other forages performing equally.

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Red clover is a forage legume with similar nutritional characteristics to those of alfalfa: good digestibility and high protein levels. However, unlike alfalfa and most grasses, when red clover is ensiled, protein degradation is typically limited to less than 15−20%, no matter the final pH. The lack of excessive proteolysis is due to the action of a polyphenol oxidase and appropriate *o*-diphenol substrates, both found in relatively high levels in red clover leaves (*10*). Polyphenol oxidase (PPO, EC 1.10.3.1) catalyzes the double oxidation of *o*-diphenols to produce *o*-quinones that are highly reactive molecules, binding to nucleophilic sites on other molecules, including proteins. Though the exact mechanism of proteolytic inhibition by PPO activity has not been fully established, it most likely involves the binding of *o*-quinones directly to proteases or to substrate proteins, resulting in decreased total protein degradation.

Polyphenol oxidases are widely distributed in the plant kingdom (*11*) and have been identified in numerous cash crops, such as sugar cane (*12*), tomato (*13*), wheat (*14*), potato (*15*), and pear (*16*). Early on, both PPO and peroxidase were implicated in enzymic browning of plant tissue (*17*). The browning reaction, typically associated with the discoloration of fruit and vegetables, has been well studied as a polyphenol reaction in food (*11*, *18*−*20*). As a result, many studies use the browning reaction as an indication of PPO activity (*10*, *12*, *21*). Polyphenol oxidases can catalyze (1) oxidation of *o*-diphenols to *o*-quinones and/or (2) hydroxylation of monophenols to *o*-diphenols followed by the oxidation to *o*-benzoquinones. The latter reaction occurs at a lower frequency in plants, where the ratio of monophenol to diphenol oxidase activity is in the range of 1:40 to 1:10, depending on the plant sources (*17*, *22*, *23*).

Testing for PPO activity among typical forage legumes revealed only red clover contained appreciable levels of PPO (*24*). There are several temperate grasses commonly harvested and ensiled as forage for animal production in the cooler-humid regions of the U.S. and similar climatic regions of the world. Orchardgrass and fescues are among these and occupy around 30% of pastoral land in the United Kingdom and continental Europe (*25*). Cereals (i.e., winter wheat) provide winter grazing and are frequently used as cover crops in mixtures with legumes (*32*). Cereals (i.e., oats, rye) are also used to provide flexibility in meeting short-term changes in the nutrient demand of a given ruminant production system. There have been some studies to investigate PPO activity in C3 grasses (*26*, *27*). In addition, work has been carried out to identify substrates of the PPO enzyme in grass species such as orchardgrass, timothy, meadow fescue, and ryegrasses (*6*, *27*). However, a broader approach evaluating the vegetative portions of a wider range of temperate grasses and cereals for both PPO activity and the presence of appropriate *o*-diphenol substrates has not been undertaken. Both PPO activity and appropriate *o*-diphenol substrate(s) are required in order to effectively inhibit proteolysis during the ensiling process. This project was undertaken to determine the potential role of the PPO enzyme in proteolytic inhibition in the presence of the *o*-diphenol caffeic acid, to determine the levels of PPO activity, if a subset of PPO substrates are solubilized in grass extracts, and to examine the browning reaction relative to PPO activity in the ten grasses and four cereals presented below.

MATERIALS AND METHODS

Plant Protein Extraction and in Vitro Proteolysis Assay. Ten perennial grass species, which included smooth bromegrass, meadow fescue, ryegrass, reed canarygrass, orchardgrass, Kentucky bluegrass (*Poa pratensis* L.), tall fescue (soft leaf), tall fescue, timothy, and quackgrass (*Elymus repens* L.), were established in 3 gallon pots in the greenhouse under a 14/10 h (day/night) lighting regime and also established in 20 ft by 15 ft field plots at Arlington, WI. Cereal grasses, which included winter wheat (*Triticum aestivum* L.), two varieties of oats (*Avena sativa* L.), rye (*Secale cereale* L.), and two varieties of spring wheat (*Triticum aestivum* L.), were established in 3 gallon pots in the greenhouse under the same growing conditions listed above. Leaf blades were harvested from plants at the vegetative stage, frozen in liquid nitrogen, and stored at −80 °C. To determine the potential impact of PPO and *o*-diphenols on proteolytic activity, leaf blades were ground/homogenized in the Mini-beadbeater (Biospec Products Inc.) mill for 20 s at 5000 rpm and placed back in liquid nitrogen, and beater-milling was repeated two more times. Ground samples were suspended in 50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5, beater-milled for 10 s at 3800 rpm, and set on ice for 15 min before centrifuging for 3 min at 14000*g*. An aliquot (100 μL) of the supernatant after centrifugation was taken and used for protein determination by trichloroacetic acid (TCA) precipitation, followed by a bicinchoninic acid (BCA) protein assay (Pierce). Three treatments were analyzed in replicate: (1) homogenized, (2) homogenized and desalted, and (3) homogenized and desalted with an *o*-diphenol substrate added. Under treatments 2 and 3, supernatants were processed through a G-25 Sephadex spin column (gel filtration media) for desalting and clarifying plant extracts. At time zero under treatment 3, caffeic acid (3 mM final concentration) was added to each sample. Aliquots (80 μL) were removed from each replicate at specific time intervals (t0, t1, t2, t4, and t24 h), and 40 μL of 15% TCA (5% w/v final concentration) was added to precipitate nondegraded proteins. Samples were immediately mixed before placing at 4 °C for a minimum of 30 min. Samples were centrifuged at 14000*g* for 5 min to pellet the TCA-insoluble material. Soluble amino acids and small peptide concentrations of the 5% TCA supernatants were quantified using Nin-Sol AF ninhydrin reagent with glycine as the standard. The amino acid released, assessing the degree of proteolysis following the 37 °C incubation, was calculated by subtracting the amino acid concentration at t0 from the amino acid concentration at subsequent time points. Results were expressed as micromoles of amino acids released per milligram of sample protein.

PPO Activity Assay. Frozen leaf blades were further processed and analyzed for PPO activity following the modified methods of Sullivan et al. (*28*). Briefly, samples were ground/homogenized in a Mini-beadbeater in a process similar to that above, except that the buffer was 50 mM TRIS-acetate buffer, pH 7.0. An aliquot (100 μL) was taken and used for protein determination by TCA precipitation followed by a BCA protein assay (Pierce). Small molecular components were removed by passing the remaining enzyme supernatant through a G-25 spin column (3 mL). PPO activity was determined in duplicate by a spectrophotometric assay using 5-thio(2-nitrobenzoate) (TNB) as a chromophore to monitor PPO activity. The supplied substrate, when oxidized by PPO to an *o*-quinone, reacts with the sulfhydryl group on TNB, forming a substrate−sulfhydryl conjugate that has decreased absorbance at 412 nm. PPO activity was calculated from the rate of color change (412 nm) due to the reaction of PPO generated *o*-quinones with reduced TNB. The assay reaction mixture consisted of 20 μL of *o*-diphenol substrate (concentration of 100 mM caffeic acid, chlorogenic acid, or catechol dissolved in 80% ethyl alcohol; EtOH), 20 μL of TNB solution (prepared as described by Esterbauer et al. (*29*)), 940 μL of 50 mM tris(hydroxymethyl)aminomethane (TRIS)-acetate buffer pH 7.0, 10 μL of catalase (1 unit of activity per reaction), and 10 μL of the enzyme solution. PPO activity was defined as millimole of *o*-quinones formed per milligram of protein per minute.

Soluble Phenolics. Frozen samples were ground in liquid nitrogen by mortar and pestle and extracted with milli-Q H_2O/methanol (MeOH) (80:20, 3 mL of buffer per gram of fresh weight). Samples were placed in an incubator shaker (45 °C) for 20 min and then centrifuged (24500*g*). Supernatants were collected, acidified to pH 2.0 with 0.1 M hydrochloric acid (HCl), and pulled through prepared C-18 solid phase extraction columns (Supelco ENVI-18, 20 mL). Columns were prepared by washing with 2 vol of 100% MeOH and 2 vol of acidified Milli-Q H_2O, pH 2.0. Phenolics bound to C-18 columns were eluted with 15 mL of 100% MeOH. Aliquots (5000 μL) were dried down, and an internal standard (50 μg of 4,4′-ethylidenebisphenyl) was added to each sample before derivatization with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (40 μL) and pyridine (10 μL). Trimethylsilylated products were separated by gas liquid chromatography (GLC) on a Zebron ZB-5 ms column (Phenomenex;

30 m × 0.25 mm, 0.25 μm film) and identified by their electron impact mass collected via Thermoquest GCQ ion-trap mass spectrometry (MS). The GLC conditions were as follows: injector, 315 °C; detector, 300 °C; and a temperature program of 200 °C for 1 min, 4 °C/min to 248 °C, 30 °C/min to 300 °C, and hold for 20 min. Conditions were run at a constant pressure of 18 psi.

Browning Reaction. Frozen leaf blades were further processed and analyzed for browning associated with PPO activity. Samples were ground/homogenized in a Mini-beadbeater and processed in a manner similar to that for samples prepared for the PPO activity assay (see the PPO Activity Assay section). At time zero, 20 μL of *o*-diphenol substrate (concentration of 100 mM caffeic acid, chlorogenic acid, or catechol dissolved in 80% ethyl alcohol; EtOH) was added to desalted clarified extracts and monitored over 12 h for browning.

RESULTS AND DISCUSSION

Red clover might be considered the perfect forage for ensiling based on the amount of total protein preserved as true protein during the ensiling process (*30*). The mechanism behind protein preservation during red clover ensiling is the presence of PPO and the abundance of its *o*-diphenol substrates (*24*, *28*). Despite the ubiquitous presence of PPOs among plants, there are notable exceptions, particularly among forages that may be harvested and stored by an ensiling process. Prominent among these forages is alfalfa that lacks PPO in its vegetative tissue (*31*). This raises the question of whether other forages, especially grasses, contain PPO activity and appropriate *o*-diphenol substrates. This work was undertaken to evaluate PPO in leaf tissue from a wide range of temperate grasses and cereals grown in the field and greenhouse and to help determine whether smaller greenhouse trials could be representative of comparable field trials.

Proteolytic inhibition in the different grasses and cereals was determined using caffeic acid as the primary *o*-diphenol substrate. Caffeic acid was selected because it outperformed other *o*-diphenols as the preferred substrate in previously published forage experiments (*28*). PPO activities were determined utilizing three representative *o*-diphenols (caffeic acid, chlorogenic acid, and catechol). Soluble phenolics in extracts were examined to determine the potential of a grass to be used as a natural source of an *o*-diphenol substrate. Lastly, timed experiments were performed to determine whether browning could be used as an indicator for PPO activity in these grasses.

Proteolytic Activity. A clarified extract and desalted clarified extracts of grass samples were prepared. Clarified extracts without desalting and without substrate added were monitored to see if desalting had an affect on residual proteolysis. Caffeic acid was added to one desalted clarified extract and compared to the desalted clarified extract without substrate added to determine the potential degree of proteolytic inhibition for each grass. In experiments with red clover and PPO modified alfalfa, caffeic was found to be the most effective in inhibiting proteolysis in leaf extracts (*10*, *21*).

Graphs of the rate of protein degradation (μmol of amino acid released per mg of protein) over 24 h for all three treatments (clarified, clarified desalted, and clarified desalted + caffeic acid) were all similar to those for orchardgrass presented in **Figure 1**. These differences in rates were typical of all grasses and cereals examined, with clarified desalted extracts exhibiting the highest degree of protein degradation (defined as the potential degree of proteolytic inhibition for a given extract). The rate of protein degradation in clarified (not desalted) extracts was always between those for clarified desalted extracts with and without substrate rates. Clarified desalted extracts with caffeic acid added consistently exhibited the lowest levels of protein degradation, indicating proteolytic inhibition increases with the addition of caffeic acid (oat excluded with < 25% proteolytic inhibition),

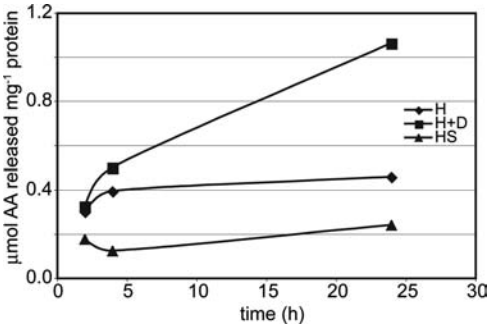

Figure 1. Rate of proteolysis in orchardgrass as represented by μmol of amino acid (AA) released per mg of protein over 24 h (H, clarified extract; H+D, clarified desalted extract; HS, clarified desalted extract with caffeic acid added).

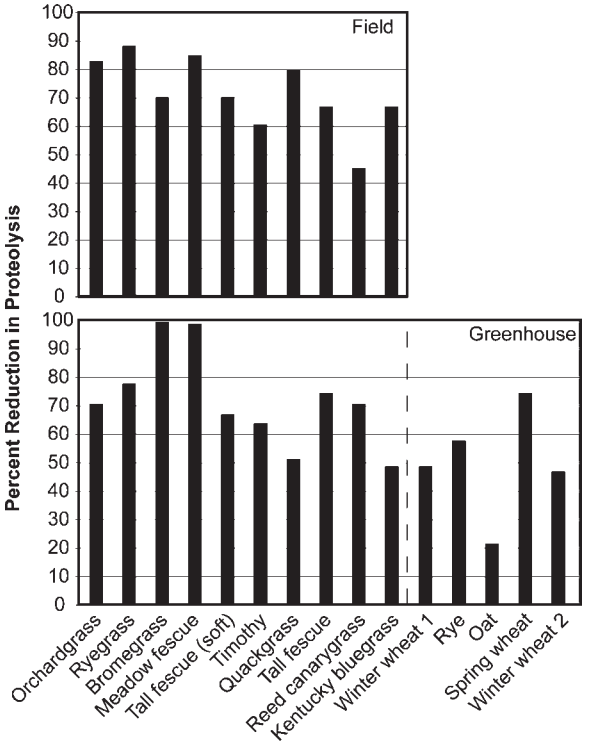

Figure 2. Percent proteolytic inhibition due to polyphenol oxidase (PPO) activity when caffeic acid is added to clarified desalted grass and cereal (1, Glacier; 2, Cardinal; oat, Gem) extracts.

regardless of the environment in which the grasses were grown (**Figure 2**). The decrease in the rates of proteolysis in clarified extracts could be due to the presence of metabolites interfering with the ninhydrin reaction and in turn skewing the degree of proteolysis detected, or other protease inhibitors could be present in the form of oxidases or peroxidases which utilize endogenous oxidase−enzyme substrates present in the clarified extracts. Therefore, to determine the potential degree of proteolytic inhibition for each grass that could be attributed to endogenous PPO, low molecular weight compounds (e.g., metabolites, *o*-diphenols) were removed by gel filtration of clarified extracts (i.e., clarified desalted extracts).

The desalted clarified extracts obtained after gel filtration were incubated over 24 h in the absence and presence of an *o*-diphenol substrate, caffeic acid. Protein degradation was calculated as micromoles of amino acids released per milligram of protein, and the extent of proteolytic inhibition was determined as the

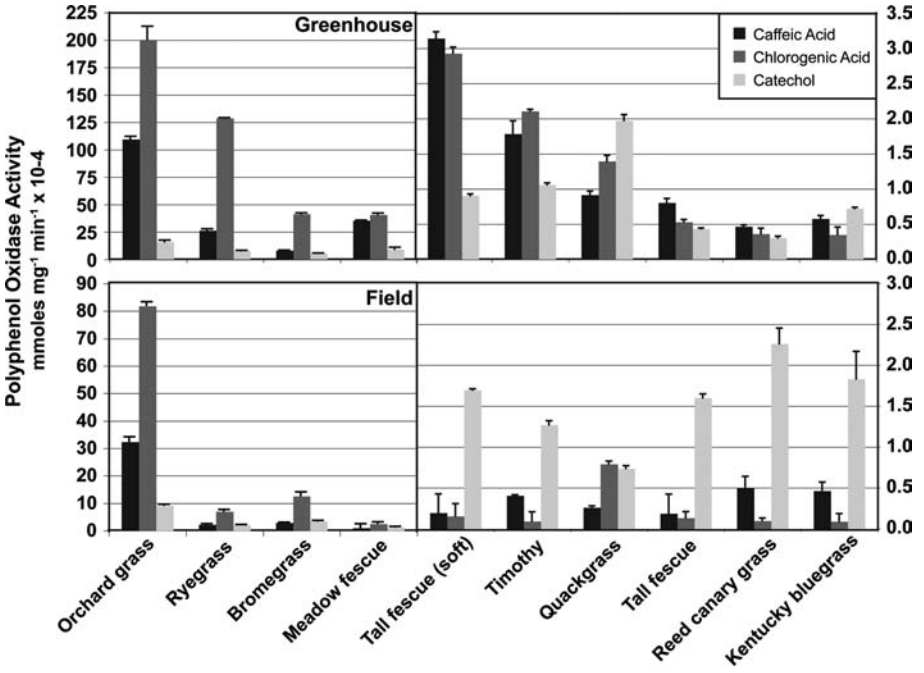

Figure 3. Comparison of polyphenol oxidase (PPO) activities (mmol mg^{-1} min^{-1}; mean ± SD) in different grass species using caffeic acid, chlorogenic acid, and catechol as substrates.

proportional difference between desalted clarified extracts with and without substrate after 24 h. All grasses exhibited some degree of proteolytic inhibition (**Figure 2**). The percent of proteolytic inhibition for a given grass species was dependent on the environment in which the crystals were grown. Consistently, there was an observed increase in proteolytic inhibition after supplying an *o*-diphenol, suggesting that several of the more important grass species that were examined could contain PPO activity at sufficient levels to produce *o*-quinones capable of binding to proteins and inhibiting proteolysis. However, since there were lower levels of protein degradation in clarified extracts compared to clarified desalted extracts, the decrease in protein degradation observed in all clarified desalted extracts with caffeic acid added could not result solely from PPO type reactions. Lee et al. (*26*) found that substantial proteolytic inhibition occurred in orchardgrass extracts without the addition of *o*-diphenol substrates. It was not reported in these studies what *o*-diphenols were present and at what levels. Both results combined suggest that proteolysis may be effectively inhibited by an alternate mechanism to PPO in grass extracts that may utilize *in vivo* phenolic type substrates present in sufficient concentrations. Parveen et al. (*6*) identified chlorogenic acid, 2-*O*-caffeoylisocitric acid, 2-*O*-caffeoylisocitric acid 6-methyl ester, 2-*O*-caffeoylhydroxycitric acid, and 2-*O*-caffeoyltheronic acid as the most common *o*-diphenols in orchardgrass. Two of these *o*-diphenols, 2-*O*-caffeoylisocitric acid and 2-*O*-caffeoylisocitric acid 6-methyl ester, accounted for over 75% of the total *o*-diphenols identified, and their concentration levels in fresh tissue appeared to be influenced by environmental growing conditions. In this study, the *o*-diphenols observed by Parveen et al. (*6*) were not detected in any of the grasses examined (see Soluble Phenolics section), independent of either environment these plants were grown in. The proteolytic inhibition results suggest a reduction in proteolysis can be achieved if adequate *o*-diphenol substrate, such as caffeic acid, is available in grasses or supplied.

Polyphenol Oxidase and *o*-Diphenols. Polyphenol oxidase activity has been analyzed spectrophotometrically with methylcatechol as substrate and reported in temperate grass species such as orchardgrass, perennial ryegrass, Italian ryegrass, hybrid ryegrass, tall fescue, and timothy (*26*). The levels of PPO activity reported varied depending upon the grass species, but orchardgrass outperformed the other grasses 2- to 10-fold. In this study, a wider range of grasses and cereals were examined to determine their PPO activity. Analysis for the PPO activity of leaf tissue from greenhouse grown spring wheat, winter wheat, oat, and rye was included. These represent cereal crops typically grown in the Midwestern U.S. that have been used as cover crops to supplement farm forage output (*32*), so it was of interest to determine the PPO activity in the vegetative portion of these plants. The amount of PPO activity (mmol mg^{-1} protein min^{-1}) measured in each plant extract (**Figure 3**) varied significantly depending upon the species and upon the chemical characteristics of specific *o*-diphenols used as the primary PPO substrate. Caffeic acid and chlorogenic acid (**Figure 4a**) were chosen to test as substrates for PPO activity because they worked well as substrates for PPOs in previously investigated forages (*6*, *24*, *27*, *30*). Catechol (**Figure 4a**) was selected as a substrate because of its availability and simple *o*-diphenol chemical structure. Even though there was a broad range in PPO activity among the grasses and cereals, all those tested contained some level of PPO activity. These results help expand the knowledge base of grasses with known PPO activity.

In both greenhouse and field environments, orchardgrass, ryegrass, smooth bromegrass, and meadow fescue exhibited the highest PPO activities with 10- to 1000-fold higher activity compared to the remaining species, depending on the PPO substrate available. All four grasses utilized the supplied substrates more efficiently than any of the remaining grasses. Orchardgrass outperformed all of the grasses in both greenhouse and field trials with levels of activity similar to what had previously been reported (*26*). Although chlorogenic acid and/or caffeic acid were the preferred substrates for the four grasses with the highest PPO activity regardless of where the plants were grown, their individual PPO activities ranged 2- to 10-fold less in the field grown trial. In greenhouse grown grasses, using caffeic acid as a substrate resulted in less than half the level of PPO

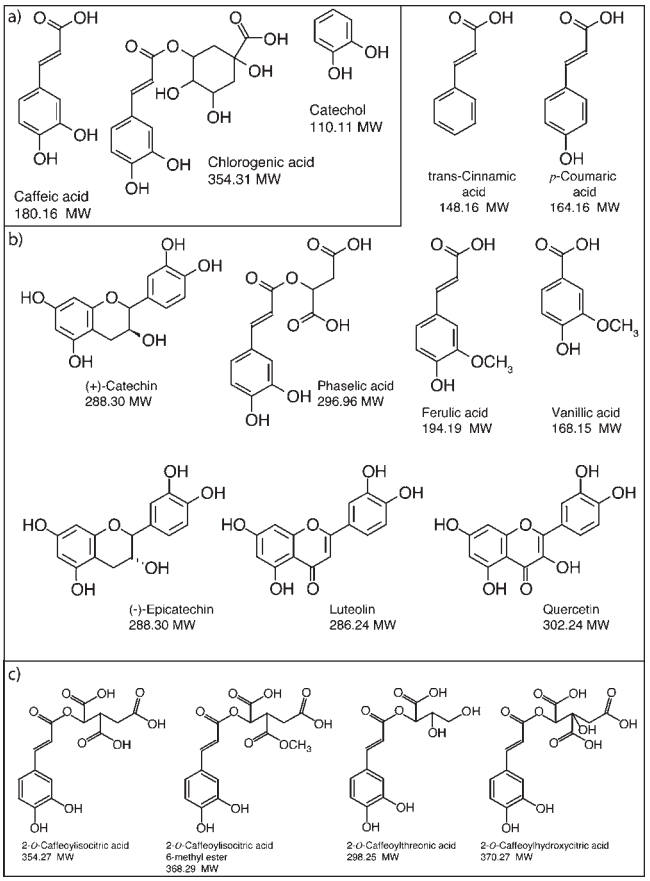

Figure 4. Chemical structures of (**a**) three common *o*-diphenols utilized in this study, (**b**) potential *o*-diphenol substrates and related phenolics, and (**c**) most abundant *o*-diphenols previously reported, utilized by polyphenol oxidases (PPO) in plants.

activity compared to the chlorogenic acid substrate among the four highest performing grasses. When catechol was the *o*-diphenol substrate, these same four grasses exhibited similar levels of PPO activity, but with approximately 10−50-fold less activity compared to the preferred *o*-diphenols, chlorogenic acid, or caffeic acid. Among grasses and cereals exhibiting limited PPO activity (**Figure 3**; **Table 1**), there were differences as to which substrate was the best utilized depending on the environment they were grown in. In some cases, regardless of the environment grown (Kentucky bluegrass, winter wheat, quackgrass, and rye), catechol was the preferred substrate. The variability observed between substrate effects on the ability of the remaining grass PPOs to oxidize the *o*-diphenols was not as pronounced, since the overall levels of PPO activities were low (0.2−3.2 mmol mg^{-1} of protein min^{-1}). Grasses with little or negligible PPO activity may be indicative of grasses without a PPO enzyme or grasses that have a PPO enzyme utilizing a substrate with a significantly different molecular structure than those of the three compounds used in this study. If warranted, this hypothesis can be tested using other *o*-diphenols as the primary PPO substrate (e.g., catechin, epicatechin). In general, these results suggest potential differences in PPO enzymes among the individual grass species. The PPOs in the four grasses with the highest PPO activity may be different at the gene level (i.e., gene expression level or structural gene sequence) from the PPOs in the grasses with little PPO activity and as a result different in their substrate utilization. In addition, the environment in which grasses are grown may affect expression levels.

The variability of PPO activity in wheat grain has been well documented (*14*, *21*); however, the results reported here are on the

Table 1. Comparison of Polyphenol Oxidase (PPO) Activities (mmol mg^{-1} $min^{-1} \times 10^{-4}$; mean ± SD) in the Vegetative Portion of Cereals Using Caffeic Acid, Chlorogenic Acid, and Catechol as Substrates

	caffeic acid	chlorogenic acid	catechol
spring wheat	0.21 ± 0.02	0.11 ± 0.04	0.25 ± 0.01
winter wheat—Cardinal	0.24 ± 0.01	0.17 ± 0.01	0.21 ± 0.01
winter wheat—Glacier	0.12 ± 0.06	0.11 ± 0.04	0.21 ± 0.00
rye—Spooner	0.27 ± 0.13	0.17 ± 0.05	0.41 ± 0.01
oats—Botte	0.24 ± 0.01	0.10 ± 0.04	0.20 ± 0.00
oats—Gem	0.26 ± 0.01	0.17 ± 0.03	0.19 ± 0.01

levels of PPO activity specifically in the vegetative portion of the cereal plants. The vegetative portions examined here had PPO levels lower than any of the grasses examined here or previously reported (**Table 1**). It is possible that the environmental conditions where the cereals were grown did not enhance the expression of PPO enzymes in vegetative tissues. Because PPO enzymes are expressed in the grain of cereals such as wheat (*14*, *21*), the genes for PPO are present within their plant genome. Further study would be needed to determine if there are environmental conditions that would enhance their expression in vegetative tissues and/or in the grain.

The proteolytic inhibition data showed the addition of caffeic acid to all clarified desalted grass and cereal extracts decreased protein degradation, while the PPO activity data showed only certain grasses contained high levels of activity. These are seemingly contradictory results, as in oat with low PPO activity but 70% proteolytic inhibition or in orchardgrass with the highest PPO activity but "average" proteolytic inhibition compared to the other grasses. In conjunction, these results suggest the presence of an additional mechanism of protein protection in the grass and cereal extracts. Depending on the proteases present in individual extracts, some might be more susceptible to inhibition by the *o*-quinones formed. Certain classes of proteases with active sites may contain specific amino acids (e.g., serine verses cysteine), and as a result their interaction with *o*-quinones would be variable, influencing the degree of protein inhibition. So, what needs to be considered in future work and is outside the scope of this study are what other mechanisms are present besides PPO in grasses and cereals that help prevent protein degradation, what types of proteases are present, what are their activities, and (how) do they interact with *o*-quinones.

To determine the utilization of *o*-diphenols over time in grasses with the most active PPO, GLC-MS was used to monitor the oxidation of supplied *o*-diphenols. **Figure 5** shows the presence of chlorogenic acid in ryegrass extract after adding the substrate at time zero and the lack of chlorogenic acid in the same extract after 24 h. Trimethylsilylated chlorogenic acid was separated by GC, and its identity was confirmed by selective and total ion chromatograms of its molecular ion(s) from electron impact mass data (**Figure 5c** and **d**). This indicated that the PPO readily oxidized chlorogenic acid and the generated *o*-quinone readily reacted with other components in the extract, including proteins. Spectra of the other three grasses with the greatest PPO activity provided the same evidence of chlorogenic acid utilization.

In Situ *o*-Diphenols. Since PPO enzymes require *o*-diphenol substrates for activity, soluble phenolics were extracted from each grass species and examined by GC-MS for caffeic acid, chlorogenic acid, catechol (**Figure 4a**), and other soluble phenolics considered potential PPO substrates (**Figure 4b**). Total ion chromatographs of grass extracts were also evaluated for 2-*O*-caffeoylisocitric acid, 2-*O*-caffeoylisocitric acid 6-methyl ester, 2-*O*-caffeoylhydroxycitric acid, and 2-*O*-caffeoyltheronic acid, the major *o*-diphenols (**Figure 4c**), as reported present in

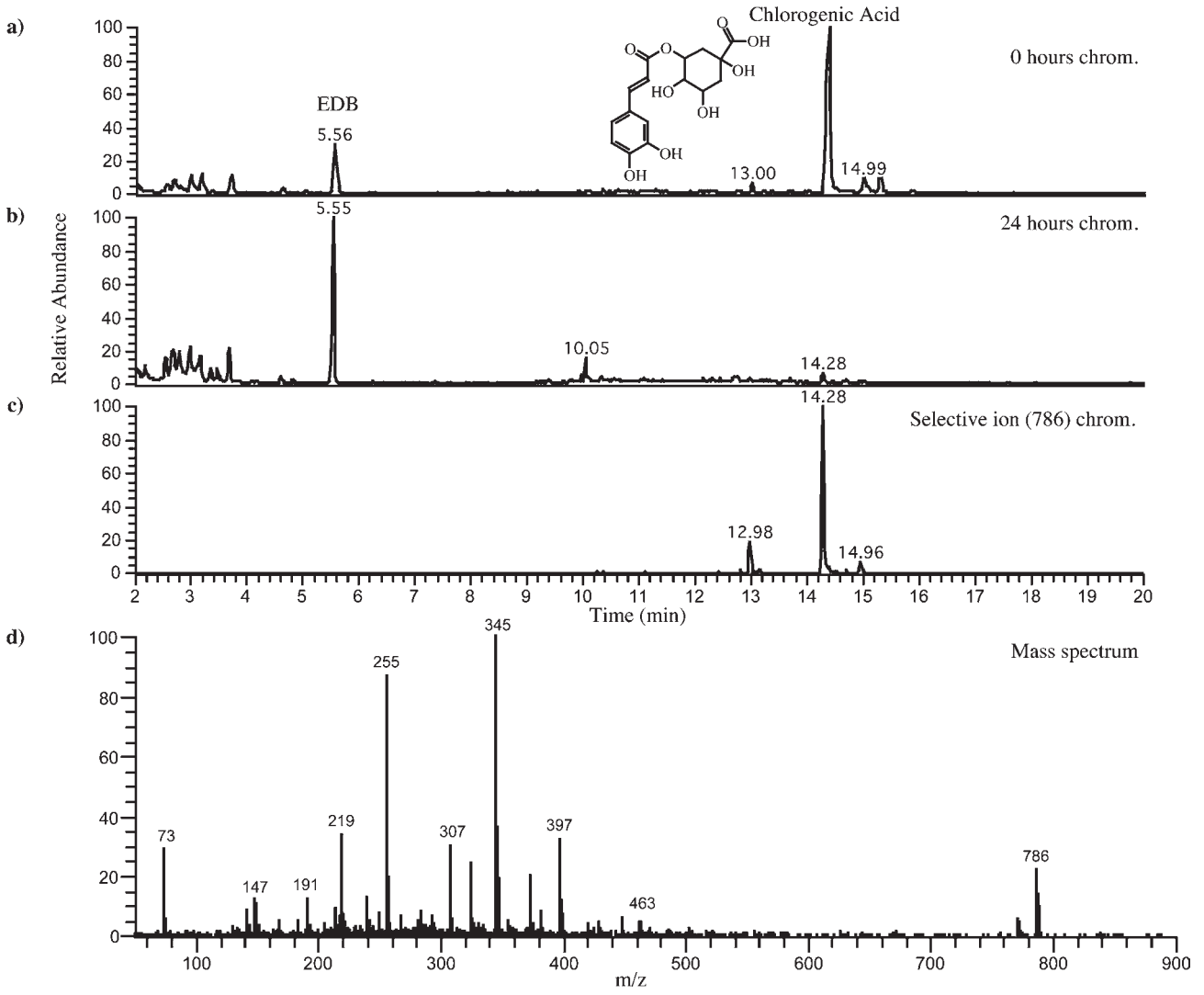

Figure 5. Mass spectrometry surveying substrate utilization over 24 h in ryegrass extract with polyphenol oxidase (PPO) activity. Trimethylsilylated chlorogenic acid was separated by GC and identified by its electron impact mass data collected on a Thermoquest GCQ ion-trap MS. (**a**) GLC-MS total ion chromatogram of the ryegrass extract at time zero after chlorogenic acid was added; (**b**) GLC-MS total ion chromatogram of the ryegrass extract after 24 h; (**c**) selective ion chromatogram (786 for chlorogenic acid); and (**d**) GLC-MS total ion chromatogram of ryegrass extract at time 14.28 min.

orchardgrass by Parveen et al. (*6*). Chlorogenic acid and caffeic acid were the two abundant soluble *o*-diphenols present in any grass extract, with measurable levels resulting in a range of μmol g^{-1} FW for replicated grass extracts. Tall fescue and timothy contained the highest levels of chlorogenic acid (3−11 μmol g^{-1} FW) compared to all other grasses (**Table 2**). Most grass extracts contained only trace amounts ($\leq$0.005 μmol g^{-1} FW) of caffeic acid (**Table 2**). The lack of detection of free caffeic acid may not be unexpected, as it appears that caffeic acid is typically combined with other components to make a caffeoyl conjugate, typically through an ester or amide linkage (*33*). No measurable amounts of screened for soluble phenolics were detected in any of the grasses. Interestingly, none of the major caffeoyl conjugates previously reported by Parveen et al. (*6*) in orchardgrass were detected, suggesting the presence of soluble *o*-diphenols in orchardgrass (and most likely other grasses) is variable and may be dependent upon the environment grown. Neither tall fescue nor timothy exhibited high levels of PPO activity, although they contained high levels of chlorogenic acid. The lower levels of PPO activities may result because the PPO enzyme in these two grasses was inactivated, since measurable *o*-diphenols (e.g., chlorogenic acid) were already present and available. As a result, adding caffeic acid, chlorogenic acid, or catechol would have less of an effect on the PPO activity being measured.

Table 2. Most Abundant Soluble *o*-Diphenols (μmol g^{-1} FW) Found in Grass Extracts[a]

grass species	caffeic acid	chlorogenic acid
orchardgrass	trace	trace
smooth bromegrass	$\leq$0.02	$\leq$0.5
meadow fescue	trace	$\leq$0.5
perennial ryegrass	ND	trace
reed canary grass	trace	trace
Kentucky bluegrass	ND	$\leq$0.5
tall fescue	trace	$\geq$3
timothy	ND	$\geq$3
quackgrass	trace	trace

[a] "ND" = none detected, and "trace" indicates $\leq$0.005 μmol g^{-1} FW. Other potential soluble PPO substrates were screened for (**Figure 4**).

Browning Reaction. Browning of plant extracts has been indicative of PPO activity when an appropriate substrate is available (*10*, *12*, *21*). Desalted clarified extracts were prepared by gel filtration as discussed above. The desalting was done to prevent browning prior to any *o*-diphenols being added and to

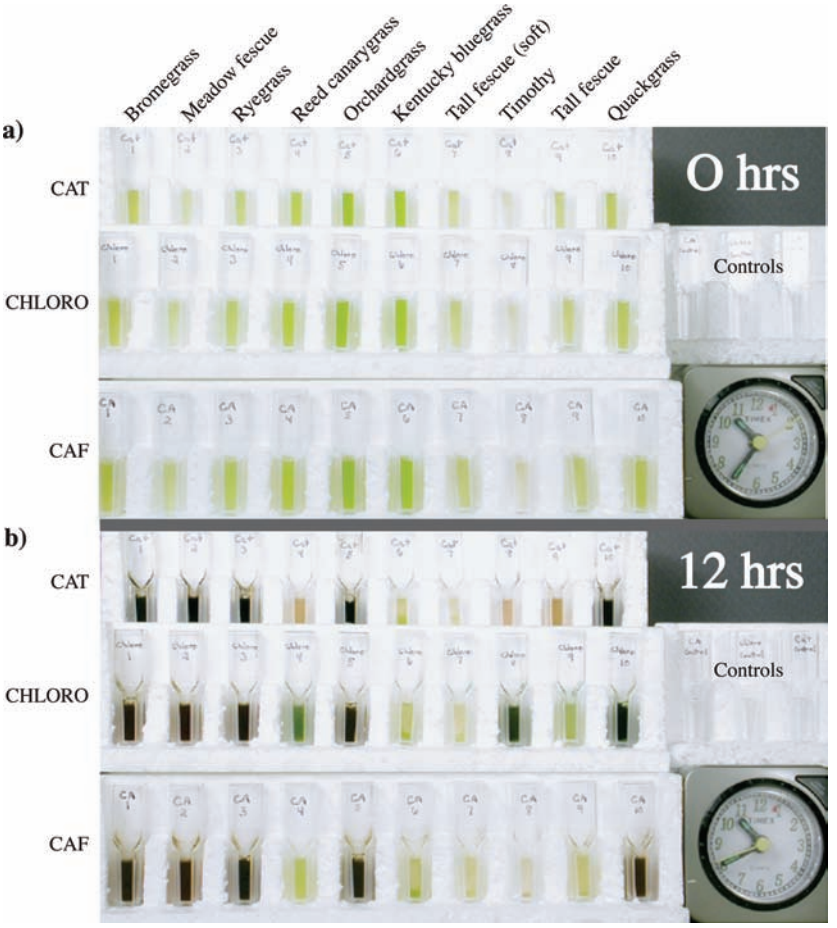

Figure 6. Differences in browning of clarified desalted grass extracts (a) at time zero and (b) after 12 h at room temperature in the presence of catechol (CAT), chlorogenic acid (CHLORO), and caffeic acid (CAF). Controls are buffer extracts with each substrate added.

distinguish browning that may be due to some other type of reaction instead of a PPO based reaction. Gel filtration removes any native phenolics present. However, gel filtration does not remove larger compounds such as peroxidases or peroxidase-like compounds that have been implicated in browning reactions (*17*). Equivalent amounts of catechol, chlorogenic acid, and caffeic acid were added to the desalted clarified grass extracts. Timed photos were taken to monitor the degree of browning over 12 h (**Figure 6**). The results of the experiment, as indicated by the degree of browning, suggested that smooth bromegrass, meadow fescue, ryegrass, orchardgrass, and quackgrass had the greatest PPO activity in the presence of any individual substrate supplied. The browning experiment results corroborated the PPO assay results, with the exception of quackgrass and timothy. There was a greater degree of browning of the timothy extract with chlorogenic acid added over caffeic acid or catechol. Why quackgrass and timothy exhibited browning but did not have any measurable PPO activity is still unknown. This could be related to the presence of other oxidases or peroxidases in plant extracts, as discussed above (see the Proteolytic Activity section) and cautions the use of browning as a rapid screening method for indicating PPO activity in grasses.

Conclusions. PPO activity was present in all varieties of grass species and cereals examined; however, the level of PPO activity varied up to 1000-fold depending on the *o*-diphenol substrate supplied. *In vitro* proteolytic assays indicated that there is a potential for inhibition of proteases when specific *o*-diphenols are supplied to grass extracts and that a mechanism other than PPO may be present in these grasses and cereals (i.e., peroxidases or oxidases) that also help inhibit proteolysis. Orchardgrass, meadow fescue, smooth bromegrass, and ryegrass consistently had 70% or greater proteolytic inhibition in the presence of caffeic acid. The same grasses had the greatest PPO activity regardless of substrate supplied (chlorogenic acid, caffeic acid, or catechol) or environment in which they were grown (greenhouse or field). Together, these results suggest a potential for improvement in ensiling of specific grass species with the addition of an appropriate *o*-diphenol substrate.

There seems to be variability in *o*-diphenols in PPO containing grasses such as orchardgrass based on these results and those previously published. Regardless of whether this variability is due to environmental affects or genetic differences in plant material examined, it may make it difficult to develop management practices for ensiling that give consistent results to improve animal performance. The variability in available *o*-diphenols in some forages (e.g., orchardgrass) does offer a potential for selection to improve total *o*-diphenol production providing more consistent and higher levels of PPO substrate in such forages. Alternatively, some forages (e.g., tall fescue and timothy) may already have an *o*-diphenol production system in place producing high levels of soluble *o*-diphenols (**Table 2**), and it may be possible to utilize these natural sources of substrate for PPOs in grasses with low or any endogenous substrate. This would require coensiling the substrate source forage with the PPO source forage. One would presume higher levels of conditioning to disrupt cells would allow adequate mixing of substrate with PPO enzyme. These results suggest there exists flexibility in grasses that can be exploited to help decrease protein losses during ensiling, to

decrease production costs, and to decrease nitrogen losses to the environment.

ABBREVIATIONS USED

PPO, polyphenol oxidase; TCA, trichloroacetic acid; BCA, bicinchoninic acid; TNB, 5-thio(2-nitrobenzoate); EtOH, ethyl alcohol; TRIS, tris(hydroxymethyl)aminomethane; MeOH, methanol; HCl, hydrochloric acid; GLC, gas liquid chromatography; MS, mass spectrometry.

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