Perennial peanut (Arachis glabrata Benth.) contains polyphenol oxidase (PPO) and PPO substrates that can reduce post-harvest proteolysis

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Abstract

BACKGROUND: Studies of perennial peanut (Arachis glabrata Benth.) suggest its hay and haylage have greater levels of rumen undegraded protein (RUP) than other legume forages such as alfalfa (Medicago sativa L.). Greater RUP can result in more efficient nitrogen utilization by ruminant animals with positive economic and environmental effects. We sought to determine whether, like red clover (Trifolium pretense L.), perennial peanut contains polyphenol oxidase (PPO) and PPO substrates that might be responsible for increased RUP.

RESULTS: Perennial peanut extracts contain immunologically detectible PPO protein and high levels of PPO activity (>100 nkatal mg\textsuperscript{-1} protein). Addition of caffeic acid (PPO substrate) to perennial peanut extracts depleted of endogenous substrates reduced proteolysis by 90%. Addition of phenolics prepared from perennial peanut leaves to extracts of either transgenic PPO-expressing or control (non-expressing) alfalfa showed peanut phenolics could reduce proteolysis >70% in a PPO-dependent manner. Two abundant likely PPO substrates are present in perennial peanut leaves including caftaric acid.

CONCLUSIONS: Perennial peanut contains PPO and PPO substrates that together are capable of inhibiting post-harvest proteolysis, suggesting a possible mechanism for increased RUP in this forage. Research related to optimizing the PPO system in other forage crops will likely be applicable to perennial peanut.

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Keywords: perennial peanut; polyphenol oxidase; rumen undegradable protein; caftaric acid; post-harvest proteolysis; hydroxycinnamoyl-tartaric acid

INTRODUCTION

Ruminant animals such as sheep (Ovis aries), goats (Capra hircus), and dairy cattle (Bos taurus) poorly utilize protein that is broken down during harvest and storage of forage. In the rumen, protein breakdown products (amino acids and small peptides) are readily utilized by rumen microbes for energy, releasing the amino acid nitrogen to the ruminant's bloodstream where some is recycled back to the rumen but most is excreted into the environment. Since the animal does not get full benefit of the protein being consumed, additional protein must be fed. Thus, the poor utilization of degraded protein has both environmental and economic consequences. It is estimated that it costs US farmers over $100 million annually to supplement rations with the needed true protein because of protein breakdown in alfalfa (Medicago sativa L.),\textsuperscript{1,2} a major forage crop.

It has been shown that red clover (Trifolium pretense L.) experiences substantially less post-harvest proteolysis than alfalfa when ensiled.\textsuperscript{3} This reduced proteolysis is due to the oxidation of abundant endogenous o-diphenolic compounds by polyphenol oxidase (PPO, E.C. 1.10.3.1).\textsuperscript{2} The reduction in proteolysis is presumably by a mechanism that involves reaction of PPO-generated o-quinones with nucleophilic amino acid residues present in endogenous plant proteases and/or proteolytic substrates. Unfortunately, it has been difficult to assess the true potential of the PPO/o-diphenol system in animal nutrition experiments, but several studies suggest improved N use efficiency with diets incorporating PPO and o-diphenol-containing red clover (see Sullivan and Zeller\textsuperscript{4} for a discussion of this).

In the humid, warm environment of the southern United States alfalfa does not persist due to pest and disease issues.\textsuperscript{5} Many alternative legumes exist, including cool-season options such as red clover and warm-season options such as cowpea (Vigna unguiculata [L.] Walp.). Red clover lacks persistence, however, and many of the other options are annuals which require seed bed preparation and planting each year. Perennial peanut (Arachis
glabrata Benth.) is adapted to warm climates, is pest and disease resistant and drought tolerant, and will persist for over 30 years if managed properly. Because there is a one-time establishment cost and minimal input costs and high value, perennial peanut forage is more profitable than other crops in locations such as Florida in the southern United States.6

Recent work by Foster et al.7 indicates that perennial peanut (‘Florigraze’) haylage has greater rumen undegradable protein (RUP) than several other warm-season legume hayages. Lambs fed this perennial peanut haylage exhibited decreased microbial N (estimated from urinary urea nitrogen excretion) than lambs fed the other legume hayages, although perennial peanut haylage had greater digestible organic matter.8 Previous work compared hays of perennial peanut and alfalfa, and although perennial peanut had a slightly lower crude protein concentration than alfalfa, the in situ rate of protein degradation was slower, indicating that the RUP of perennial peanut was greater than that of alfalfa.9 In a study by Gelaye et al., however, comparing alfalfa, perennial peanut, or a combination of the two forages, nitrogen intake and excretion were not different among goats fed these diets, indicating the RUP was not different between alfalfa and perennial peanut.10 The presence of a low concentration of tannin could explain the inconsistent reports of reduced ruminal protein degradation of perennial peanut11 and low levels of tannins have been reported.8,9,12 Additionally, the presence of tannin in perennial peanut has not been consistently reported, with some analyses reporting no tannins present (Muir JP, personal communication). Further, it is presently unclear what types and levels of tannins are required to improve protein utilization efficiency in ruminants, and this is further compounded by the lack of standardized methods for measuring these in forage materials.13 A PPO enzyme and substrate system, similar to that of red clover, in perennial peanut would explain inconsistencies in the literature in regards to RUP and tannin concentration in this forage. Here, we examined perennial peanut for the presence of endogenous PPO and o-diphenol PPO substrates.

**EXPERIMENTAL**

**Plant material**

Perennial peanut (*Arachis glabrata*, Benth., variety not specified) was grown in a field in Beeville, Texas, either in partial shade or in full sun, or in a greenhouse. The fields were planted at least 20 years previously and were cut at least once per year. In the greenhouse, 30 pots were planted by placing one perennial peanut sprig with at least one rhizome and at least 10 cm length at 3 cm depth into a 15-cm diameter pot the spring prior. Plants were fertilized with a custom mix of 5–30–5 monthly and watered at 3 cm depth into a 15-cm diameter pot the spring prior. Plants were fertilized with a custom mix of 5–30–5 monthly and watered at 3 cm depth into a 15-cm diameter pot the spring prior.

To prepare leaf extracts for immunoblotting and PPO assays, mature leaves that were previously harvested, frozen in liquid nitrogen, and stored at −80 °C, were powdered in liquid nitrogen with a mortar and pestle. The material was wrapped in damp paper towels, placed in a −80 °C freezer, and is in the public domain in the USA.

**SDS-PAGE and immunoblotting**

SDS-PAGE (10% acrylamide; 37.5:1 acrylamide-bis-acrylamide) and western transfer were carried out using standard methodologies.16 Leaf extracts for SDS-PAGE were denatured by mixing with an equal volume of SDS-PAGE sample buffer and heating at 70 °C for 15 min. Following electrophoresis proteins were transferred to polyvinylidene fluoride (PVDF; Bio-Rad Laboratories), and the blots were processed and developed according to the manufacturer’s instructions. Detection was accomplished using anti-red clover PPO1 antiserum15 diluted 1:1000, alkaline phosphatase-conjugated goat anti-rabbit antibody diluted 1:3000 as the secondary antibody, and Immun-Star Chemiluminescent Substrate (Bio-Rad Laboratories). Blots were imaged using a FOTO/Analyst FX system (FOTODYNE Incorporated, Hartland, WI, USA).

**Polyphenol oxidase activity assay**

Polyphenol oxidase activity was quantified using the 2-nitro-5-thio-benzoic acid (TNB) quinone trap assay of Esterbauer.17 TNB solution was prepared as previously detailed.15 Reactions consisted of 950 µL of pH 7.0 McIlvaine’s buffer, 20 µL TNB solution, and 20 µL 0.1 mol L−1 caffeic acid in ethanol. To measure total PPO activity, SDS was added to the reaction buffer to a final concentration of 0.25% (w/v). Reactions were initiated by adding 10 µL tissue extract (diluted when appropriate), incubated at 25 °C, and A412nm was measured over time using a Beckman DU50 spectrophotometer and data capture software (Brea, CA, USA). Reaction rates were determined by plotting A412nm versus time, determining the slope of the linear portion of the
reaction by linear regression and converting to nmol substrate using the conversion 91.0 nmol/A_{412nm}. Specific activity of a given extract was calculated by dividing reaction rate by the amount of extract protein present in the reaction, expressed as nkat/mg. −1, and reported as the mean for two independently prepared extracts.

Preparation of phenolic compounds from perennial peanut leaves

Previously frozen perennial peanut leaves were powdered in liquid nitrogen with a mortar and pestle, extracted with 10 mL g−1 0.05 mol L−1 ascorbic acid in methanol–water–acetic acid (50:49:1) for 10 min at room temperature with stirring and the resulting slurry was filtered through Miracloth (Calbiochem, LaJolla, CA, USA). The filtrate was centrifuged at 15 000 × g for 10 min and the supernatant retained. The post-centrifugation supernatant was evaporated to near dryness through rotary evaporation at 45 °C. The resulting residue was dissolved in 50 mL 0.1% acetic acid in water, pH adjusted to 2.5 with HCl and applied to a 5 g ENVI-18 solid phase extraction column (Supelco, St. Louis, MO, USA) pre-equilibrated with 3 × 25 mL of methanol and 3 × 25 mL 0.1% acetic acid in water, pH adjusted to 2.5 with HCl. The column was washed with 4 × 25 mL 0.1% acetic acid in water (pH 2.5 with HCl) and eluted with 25 mL methanol. The eluate was evaporated to dryness through rotary evaporation at 45 °C. The residue was dissolved in 1 mL of methanol and stored at −20 °C.

Aliquots of the phenolic preparation were analyzed by HPLC on a Shim-Pack XR-ODS II (C-18) 120 Å column (Shimadzu Scientific Instruments North America, Columbia, MD, USA; 100 × 2.0 mm × 2.2 μm) using a two-solvent system [solvent A, deionized water with 0.1% (v/v) formic acid; solvent B, acetonitrile] at a flow rate of 0.5 mL min−1. The HPLC conditions were 5 min isocratic 2% solvent B, 10 min gradient to 30% solvent B, 3 min gradient to 100% solvent B, 5 min isocratic 100% solvent B, 0.5 min gradient to 2% solvent B, and 3.5 min isocratic re-equilibration at 2% solvent B. Compound elution was monitored (250–500 nm) with a UV–visible photodiode array detector (PDA). In most cases, elution was also monitored with a MS2020 mass spectrometer (Shimadzu Scientific Instruments North America). Caftaric acid was identified by mass spectrometry (MS) and by comparison of HPLC retention time and UV–visible absorption spectrum to a purchased standard (Cerilliant Corporation, Round Rock, TX, USA). Caftaric acid was quantified with LC Solutions Software (Shimadzu Scientific Instruments North America) using the purchased authentic compound as the quantification standard.

To assess utilization of components in the endogenous perennial peanut phenolic preparation by PPO, phenolics equivalent to 33 mg of tissue were incubated with 100 μL control or PPO-alfalfa extracts, perennial peanut extract (all prepared and desalted in MES buffer as described above) or with 0.2 mol L−1 MES, pH 6.5 for 1 h at 37 °C. Following incubation, the reactions were acidified by addition of 10 μL 1 mol L−1 HCl, the phenolics were repurified on a 100 mg ENVI-18 solid phase extraction column as detailed previously and analyzed by HPLC as described above.

In vitro proteolysis assay

Proteolysis reactions were carried out as previously described. Briefly, reactions contained 2 mg mL−1 leaf extract protein (prepared and depleted of endogenous phenolics as described above) in 0.2 mol L−1 MES, pH 6.5 and the purified o-diphenol caffeic acid or the perennial peanut phenolic preparation.

For caffeic acid, 2 μmol mg−1 extract protein was used. For the perennial peanut phenolic preparation, material isolated from 83 mg or 167 mg leaves [fresh weight (FW)] was used per mg extract protein, which we estimate to be one- or two-fold the level found in leaves, respectively, based on recovery of approximately 12 mg soluble protein g−1 FW. Phenolics were pre-measured into the reaction tubes from a stock solution in methanol or ethanol, dried under vacuum, and the residue was dissolved in a small volume [3% (v/v) of the final reaction volume] of ethanol. Control reactions lacking phenolic compounds also contained 3% (v/v) ethanol. Duplicate samples of each reaction were removed at time 0 and at 4 h following incubation at 37 °C. Once removed, the samples were immediately mixed with one-half volume of 15% (w/v) tricholoracetic acid (TCA) and placed on ice for at least 30 min to precipitate proteins and peptides. TCA-insoluble material was removed by centrifugation at 20 000 × g for 5 min and amino acid content of the supernatant was measured using ninhydrin as previously detailed. Amino acid release was determined by subtracting the amino acid concentration of the initial (unincubated proteolysis reactions) from the amino acid concentrations of the reactions following incubation. Results are the mean of three experiments using independently prepared extracts reported with the standard error and expressed relative to the extracts to which no PPO substrate was added.

Experimental design and statistical analysis

The proteolysis experiment was a blocked (three replicates) bifactorial (extract and substrate) design. Data were analyzed by two-way ANOVA and significance determined by Bonferroni post-tests using Prism Software (GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Perennial peanut leaves contain PPO protein and PPO activity

As a first step in examining perennial peanut for components of the PPO system, we prepared leaf extracts and attempted to detect the protein by immunoblotting using antiserum prepared against a major foliar PPO from red clover leaves. Leaf extracts from red clover and alfalfa transformed with the red clover PPO1 gene served as positive controls. Since the endogenous alfalfa PPO gene is not expressed in leaves, extracts of non-transformed alfalfa leaves served as a negative control. It was expected that the antired clover PPO1 antiserum would recognize a perennial peanut PPO since we and others have successfully used this antiserum to detect PPOs from diverse plant species including walnut (Juglans regia L., unpublished result), potato (Solanum tuberosum L.), tomato (Solanum lycopersicum L.), and wheat (Triticum aestivum L.) (Anderson JV, personal communication). As shown in Fig. 1, in perennial peanut the predominant protein band recognized by the anti-PPO antiserum was approximately 61 kDa in size and was present in all three samples although the band intensities varied among the samples. Additionally, several fainter bands migrating as smaller than 50 kDa were also present and may represent proteolytic products of the full-length version. Proteolytic cleavage of PPO, both before and after isolation, has been reported for a number of PPOs, and in some cases truncated versions of the protein appear to be the active form (seeGerdemann et al. and Schmitz et al. and references therein). The control red clover PPO, either from red clover leaves or transgenic PPO-alfalfa, migrated as an approximately 67 kDa protein. Although this is slightly larger than previously reported (65 kDa) for red clover PPO1 and larger.

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produced by available active PPO, are capable of activating latent clover PPO, PPO substrates themselves, presumably as quinones denaturing or protease treatments for full activity. In the case of red without SDS addition. Many PPOs, including red clover, have been (i.e. measured in the absence of SDS). 21 Total PPO activity from measured in the presence of SDS) were similar to those previously are shown in Table 1. Values for total red clover PPO activity (i.e. alfalfa served as a negative control. PPO activities for the extracts transgenic alfalfa served as positive controls, and untransformed alfalfa, nearly all the perennial peanut PPO (i.e. measured (i.e. in the absence of SDS) for the alfalfa-expressed extracts of transgenic alfalfa expressing red clover PPO was well CsVMV promoter. 22 The small fraction (about 1%) of active PPO because PPO expression in transgenic alfalfa is driven by the strong and is in the public domain in the USA.

than predicted based on its sequence,15 the measurement is well within the limits of the technique. All extracts had a faint band migrating at approximately 70 kDa. Since this band was present even in the control-alfalfa lane, which should lack PPO, this may represent some unrelated protein species reacting with antibody present in the antiserum.

To further test whether the cross-reacting proteins present in the perennial peanut extracts represented PPO, we carried out PPO activity assays using caffeic acid as a PPO substrate. The activity assays were carried out in both the presence and absence of 0.25% SDS (w/v) since many PPO enzymes exist in a latent form and require a mild denaturing treatment (such as low concentrations of SDS) to measure the total activity present.19 As in the immunoblotting experiment, red clover and PPO-expressing transgenic alfalfa served as positive controls, and untransformed alfalfa served as a negative control. PPO activities for the extracts are shown in Table 1. Values for total red clover PPO activity (i.e. measured in the presence of SDS) were similar to those previously measured as was the fraction (approximately 30%) of active PPO (i.e. measured in the absence of SDS).21 Total PPO activity from extracts of transgenic alfalfa expressing red clover PPO was well above the background measured in extracts of control-alfalfa and greater than that measured for red clover extracts, presumably because PPO expression in transgenic alfalfa is driven by the strong CsVMVM promoter.22 The small fraction (about 1%) of active PPO measured (i.e. in the absence of SDS) for the alfalfa-expressed protein is similar to previous observations.20 The perennial peanut extract had levels of total PPO activity greater than red clover extract and similar to that of the transgenic alfalfa PPO extract. PPO activities from the different perennial peanut tissue samples varied by about two-fold. It is not clear whether this reflects growing conditions of the plant material or some other variable since this study was not designed to address such a question. In contrast to the red clover PPO, either from red clover or transgenic alfalfa, nearly all the perennial peanut PPO (>90%) was active without SDS addition. Many PPOs, including red clover, have been reported to be isolated in a mostly inactive form, requiring mild denaturing or protease treatments for full activity. In the case of red clover PPO, PPO substrates themselves, presumably as quinones produced by available active PPO, are capable of activating latent PPO as well.23 Together, these observations have suggested a general model of PPO activation being a result of conformational changes in the PPO protein.21,23 That most perennial peanut PPO was isolated in the active form might indicate the perennial peanut protein more easily folds into the active conformation on its own or has undergone an activating proteolytic cleavage prior to or during isolation. Because the different perennial peanut leaf samples all showed high levels of PPO, the experiments described below used only the material grown in partial shade, as limited amounts of the other tissues were available for analysis.

Perennial peanut PPO inhibits proteolysis in the presence of phenolic compounds

To test whether the perennial peanut PPO activity present in its leaves was capable of inhibiting proteolysis in the presence of PPO substrates, extract was prepared from perennial peanut leaves in the presence of ascorbic acid to prevent PPO-mediated oxidation of endogenous substrates and subjected to gel filtration to remove endogenous phenolic compounds, including any potential PPO substrates. Proteolysis in the extract was then measured as amino acid release over time and the impact of added PPO substrate on extract proteolysis was assessed. Substrates used included caffeic acid, since we had already shown this to be a good substrate for the perennial peanut PPO, and also an endogenous phenolic mixture that had been extracted from perennial peanut leaves. Caffeic acid was used at 2 μmol mg⁻¹ soluble protein, an amount comparable to that found in red clover.4 The peanut phenolic preparation was used at levels we estimated to be one- or two-fold that present in peanut leaves (see Experimental section). As shown in Table 2, addition of caffeic acid resulted in an approximately ten-fold reduction in proteolysis in the perennial peanut extract (P < 0.001). This was accompanied by extract browning characteristic of PPO-mediated oxidation of PPO substrates (data not shown). Similarly, addition of the peanut phenolic preparation resulted in both a browning reaction and reduced proteolysis in the peanut extract by three- to four-fold relative to no phenolic addition (P < 0.001), although the reduction was not as great as that seen with caffeic acid (P < 0.05). That the peanut phenolics were not as effective as caffeic acid in inhibiting proteolysis (P < 0.05 and P < 0.01 for the 1× and 2× levels, respectively) may be the effect of both the type and amount of phenolics present in the peanut extract, which we have only partially characterized to date (see below). We have previously shown that both the types and levels of PPO substrates

![Image](http://www.soci.org/mlsullivanandjlfsullivan/doi/10.1002/jsfa.2424/f1.png)

**Figure 1.** Immunological detection of PPO in perennial peanut. Protein (5 μg) from leaf extracts of perennial peanut grown in a greenhouse (G), or in the field under partial sun (P) or full sun (F), red clover, control-alfalfa (Control-Alf), or alfalfa expressing the red clover PPO1 transgene (PPO-Alf) were resolved by SDS-PAGE, transferred to a PVDF membrane, and PPO was detected with an anti-red clover PPO antibody. An arrowhead indicates the ∼60 kDa cross-reacting protein presumed to be perennial peanut PPO. The migration position of a cross-reacting band present in all lanes (including control-alfalfa) is indicated with an asterisk.

![Table 1](http://www.soci.org/mlsullivanandjlfsullivan/doi/10.1002/jsfa.2424/t1.png)

<table>
<thead>
<tr>
<th>Leaf source</th>
<th>Polyphenol oxidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Active (−SDS)</td>
</tr>
<tr>
<td>Perennial peanut</td>
<td>197 ± 28.6</td>
</tr>
<tr>
<td>Partial sun</td>
<td>299 ± 35.6</td>
</tr>
<tr>
<td>Full sun</td>
<td>124 ± 11.5</td>
</tr>
<tr>
<td>Red clover</td>
<td>10 ± 5.5</td>
</tr>
<tr>
<td>Control-alfalfa</td>
<td>&lt;1</td>
</tr>
<tr>
<td>PPO-alfalfa</td>
<td>2 ± 0.2</td>
</tr>
</tbody>
</table>

The polyphenol oxidase activity is reported as mean ± SEM for two independently prepared extracts.
levels of peanut phenolics (the 2× level) appeared to result in a slightly greater level of proteolytic inhibition, although the effect was not significant (P > 0.05) in this experiment. This observation is consistent with previous findings that near maximal proteolytic inhibition can be achieved with relatively low levels of PPO substrate beyond which increases in substrate concentration have only a modest impact on proteolytic inhibition.4

It seemed likely that the observed proteolytic inhibition in the peanut extract to which caffeic acid was added is due to caffeic acid oxidation by PPO, since, at least in alfalfa, caffeic acid itself appears to have little impact on endogenous proteolytic activity2,4 (and see also Table 2, control-alfalfa). For the perennial peanut phenolics, the presence of PPO activity in perennial peanut leaves and the observed browning upon addition of the phenolics to leaf extracts suggest proteolytic inhibition involved PPO-mediated oxidation of substrates in this case as well. Still, it could not be ruled out that components in the peanut leaf phenolic preparation might be inhibiting proteolysis in a PPO-independent manner. In order to test the PPO requirement of the peanut phenolic preparation in proteolytic inhibition, we used leaf extracts from transgenic PPO-alfalfa and nontransgenic control-alfalfa in the proteolysis assay (Table 2). Although peanut phenolics at both tested levels did lead to some proteolytic inhibition independent of PPO (control-alfalfa, P < 0.01 and P < 0.001 for the 1× and 2× level of phenolics, respectively), significantly more proteolytic inhibition was seen in the presence of PPO (PPO-alfalfa versus control-alfalfa, P < 0.001 for caffeic acid or peanut phenolic at either level). Proteolytic inhibition was not significantly different in peanut extract compared to PPO-alfalfa extract for any of the tested phenolic conditions (P > 0.05) but was significantly different from the control-alfalfa (perennial peanut versus control-alfalfa, P < 0.001 for caffeic acid and 1× peanut phenolics, P < 0.01 for 2× peanut phenolics). Together, these results indicate that while the peanut phenolic preparation may have some potential for PPO-independent proteolytic inhibition, a significant and substantial portion of the observed reduction in proteolysis is due to PPO-mediated oxidation of phenolic substrates. It should be noted that in a previous study,4 a preparation of phenolics from red clover leaves also exhibited some level of PPO-independent proteolytic inhibition. In that study, some of the o-diphenolic components extracted from red clover appeared to be easily oxidized without PPO (e.g. clovamide) and were able to inhibit proteolysis in a PPO-independent but oxygen-dependent manner. Other phenolic components of the red clover mixture (e.g. p-coumaroyl-malate) inhibited proteolysis in a manner that was both PPO- and oxygen-independent, indicating some phenolic compounds can act as general proteolytic inhibitors. Given the modest level of proteolytic inhibition observed in the control-alfalfa extract to which perennial peanut phenolics were added (Table 2), the perennial peanut phenolic mixture may contain compounds capable of inhibiting proteolysis by either of the PPO-independent mechanisms described above for red clover phenolics (i.e. by being easily oxidized o-diphenols or by acting as general proteolytic inhibitors).

**Perennial peanut leaves contain PPO-utilizable phenolics including caftaric acid**

To better characterize the phenolic preparation from perennial peanut leaves, it was resolved by reversed phase HPLC with both PDA detection (250–500 nm) and negative ion mass spectrometry. Several major compound peaks were detected by the PDA, many of which had strong negative ion signals in MS analysis (Fig. 2 and Table 3). To determine which of these compounds might be substrates for the perennial peanut PPO, a portion of the phenolic mixture was incubated with desalted perennial peanut extract and then the phenolics repurified by C-18 solid phase extraction. o-Quinones generated from o-diphenol PPO substrates should react with nucleophilic sites on extract proteins making the original o-diphenol no longer detectable following repurification and separation by reversed phase HPLC. Previously this approach was successfully used to identify PPO substrates in red clover phenolic preparations.6 Following incubation with the peanut extract, two major peaks (a and j) and several minor peaks (e, k, l, m, n) disappeared from the peanut phenolic preparation. Most of the remaining peaks showed some decrease in area following the incubation, but the areas of these peaks were generally >70% that of the untreated phenolics and the observed decrease may be due to general losses associated with incubation in the presence of plant extract proteins. Interestingly, two new peaks (o and p) appeared upon incubation, suggesting enzymatic activities besides PPO could be metabolizing compounds in the preparation to generate new ones (discussed in more detail below). Incubation of the peanut phenolics in buffer alone did not alter their HPLC elution profile (data not shown), indicating the changes observed were due to the peanut extract. In an attempt to identify peaks that disappear in a PPO-specific manner, we incubated the perennial peanut phenolic preparation with leaf extracts of control-alfalfa or transgenic PPO-alfalfa. Incubation of the peanut phenolics with control-alfalfa extract had relatively little effect on the LC elution profile (although there might have been some loss of peaks l, m and n), and the new peaks that were seen upon incubation with the peanut extract did not appear with the alfalfa extract, suggesting some enzymatic differences in the extracts. Similar to what was seen with the peanut extract, areas of most peaks were

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### Table 2. PPO-mediated proteolytic inhibition by caffeic acid and perennial peanut phenolics

<table>
<thead>
<tr>
<th>Substrate†</th>
<th>Perennial peanut</th>
<th>Control-alfalfa</th>
<th>PPO-alfalfa</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>1.00 ± 0.034(^A)</td>
<td>1.00 ± 0.061(^A)</td>
<td>1.00 ± 0.119(^A)</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.09 ± 0.005(^A)</td>
<td>0.92 ± 0.049(^A)</td>
<td>0.10 ± 0.009(^A)</td>
</tr>
<tr>
<td>1× peanut phenolics</td>
<td>0.34 ± 0.015(^A)</td>
<td>0.74 ± 0.048(^A)</td>
<td>0.26 ± 0.012(^A)</td>
</tr>
<tr>
<td>2× peanut phenolics</td>
<td>0.26 ± 0.011(^A)</td>
<td>0.53 ± 0.029(^A)</td>
<td>0.20 ± 0.009(^A)</td>
</tr>
</tbody>
</table>

† Caffeic acid was present at 2 µmol per mg extract protein present in the reactions. 1× and 2× corresponds to phenolics extracted from 83 mg and 167 mg (fresh weight) perennial peanut leaves, respectively, used per mg of extract protein present in the reactions. The 1× amount approximates the level of phenolic relative to soluble protein present in perennial peanut leaves.

‡ Values for each extract are expressed relative to the respective no addition control and are the mean ± SEM for three experiments using independently prepared extracts. Amino acid release in the absence of PPO substrate was 1.16 ± 0.040, 1.26 ± 0.076 and 1.03 ± 0.123 mmol g\(^{-1}\) soluble protein for perennial peanut, control-alfalfa, and PPO-alfalfa extracts, respectively.

\(^A\) Different lower-case letters within a column indicate significant difference (P < 0.05).

\(^B\) Different upper-case letters within a row indicate significant difference (P < 0.05).
Figure 2. Effect of PPO on perennial peanut phenolics. Phenolic compounds were isolated from perennial peanut. The phenolics were treated with desalted perennial peanut, control-alfalfa, or PPO-alfalfa extracts and repurified as detailed in Experimental section. Untreated or treated phenolics as indicated were resolved by reversed phase HPLC and detected with a photodiode array detector (250–500 nm) and negative ion mass spectrometry. Major peaks are indicated with lower-case letters and spectral characteristics and tentative identifications are provided in Table 3.

somewhat lower than untreated or buffer treated phenolics, but were still present at levels >70% that of the untreated phenolics. In contrast to the control-alfalfa extract, incubation with the PPO-alfalfa extract led to near complete disappearance of the same peaks that disappeared upon incubation with the peanut extract (major peaks a and j; minor peaks e, k, l, m, n). We have identified major peak a as trans-caftaric acid (2-caffeoyl-L-tartaric acid, Fig. 3) based on comparison of its retention time on reversed phase HPLC, UV–visible absorption spectrum, and MS profile with a purchased standard (data not shown) and estimate its abundance in the starting leaf material to be on the order of 1 mmol kg$^{-1}$ fresh weight. Although caftaric acid and other hydroxycinnamoyltartaric acid derivatives have been described in other species, particularly *Vitas* species$^{24}$ and *Echinacia purpurea* L.$^{25}$ to our knowledge this class of compounds has not been previously detected in legumes. Snook et al.$^{26}$ reported the presence of
chicoric acid (dicaffeoyl-tartaric acid), but not caftaric acid, in annual peanut (*Arachis hypogaea* L.). We have been unable to identify major peak j, whose predicted molecular weight is 356, but its PPO-specific disappearance strongly suggests it is a substrate for the red clover, and presumably perennial peanut, PPO. Identification of this compound may require accurate mass determination and/or purification of sufficient amounts for analysis by nuclear magnetic resonance. Minor peak e also appears to be a substrate for PPO, and its predicted molecular mass suggests it could be a caftaric acid isomer. Preliminary UV irradiation of the other compounds that were lost upon incubation with control-alfalfa extract (k, l, m and n) are also currently not known, although abundance of l, m and n seem somewhat diminished by incubation with control-alfalfa extract (to < 30% that of the untreated phenolics), so their loss may not be totally attributable to PPO. We did not detect chicoric acid (2,3-dicaffeoyl-L-tartaric acid) in perennial peanut leaves.

Several additional major peaks present in the perennial peanut phenolic preparation do not appear to be substrates for PPO.

### Table 3. Major peaks identified in HPLC-MS analysis of perennial peanut leaf extract

<table>
<thead>
<tr>
<th>Peak</th>
<th>λ_{max} (nm)</th>
<th>[M − H]^−</th>
<th>Identity^†</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>327</td>
<td>311</td>
<td>trans-Caftaric acid</td>
</tr>
<tr>
<td>b</td>
<td>313</td>
<td>295</td>
<td>trans-(p-Coumaroyl)-L-tartaric acid (?)</td>
</tr>
<tr>
<td>c</td>
<td>275</td>
<td>239</td>
<td>—</td>
</tr>
<tr>
<td>d</td>
<td>308</td>
<td>295</td>
<td>cis-(p-Coumaroyl)-tartaric acid (?)</td>
</tr>
<tr>
<td>e</td>
<td>323</td>
<td>311</td>
<td>Caftaric acid isomer (?)</td>
</tr>
<tr>
<td>f</td>
<td>258</td>
<td>431</td>
<td>—</td>
</tr>
<tr>
<td>g</td>
<td>327</td>
<td>325</td>
<td>trans-Feruloyl-tartaric acid (?)</td>
</tr>
<tr>
<td>h</td>
<td>323</td>
<td>325</td>
<td>Feruloyl-tartaric acid isomer (?)</td>
</tr>
<tr>
<td>i</td>
<td>318</td>
<td>325</td>
<td>Feruloyl-tartaric acid isomer (?)</td>
</tr>
<tr>
<td>j</td>
<td>328</td>
<td>355</td>
<td>—</td>
</tr>
<tr>
<td>k</td>
<td>255</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>l</td>
<td>326</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>m</td>
<td>318</td>
<td>471</td>
<td>—</td>
</tr>
<tr>
<td>n</td>
<td>329</td>
<td>471</td>
<td>—</td>
</tr>
<tr>
<td>o</td>
<td>309</td>
<td>ND</td>
<td>p-Coumaric acid (?)</td>
</tr>
<tr>
<td>p</td>
<td>321</td>
<td>ND</td>
<td>Ferulic (?)</td>
</tr>
</tbody>
</table>

^† Molecular weight of major negative ion detected; ND: no major ion detected.

^‡ A definitive identification is in bold, tentative identifications are indicated with (?)

![Figure 3](image_url). Structure of hydroxycinnamoyl-tartaric acid esters.

Interestingly, peaks b and g have predicted molecular weights that suggest they are trans-2-(p-coumaroyl)-L-tartaric acid and trans-2-feruloyl-L-tartaric acid (Fig. 3). Further, UV–visible absorption spectra of b and g are consistent with their being trans-(p-coumaroyl) and trans-feruloyl derivatives, respectively, and relative retention times of peaks a, b and g are consistent with those of the comparable malate esters of caffeic, p-coumaric and ferulic acid, respectively (unpublished data, and Sullivan and Zarnowski). If peaks b and g are p-coumaroyl- and feruloyl-tartaric acid, respectively, that might explain the appearance of peaks o and p upon incubation with the peanut extract, as peaks o and p have retention times and UV–visible absorption spectra indistinguishable from p-coumaric and ferulic acids, respectively, and could be produced by an esterase activity in the perennial peanut extract. Notably, the free acids of these hydroxycinnamates ionize poorly under the conditions used here, consistent with the lack of detection of negative ions for o and p by MS. If peak b is trans-(p-coumaroyl)-tartaric acid, then given its molecular weight, peak d seems likely to be the cis version. Further supporting this tentative assignment, the UV–visible absorption spectrum and relative retention time of peak d compared to peak b are consistent with our observations of cis- versus trans-hydroxycinnamoylmalate esters (unpublished data). Similarly, given their predicted molecular weights, peaks i and h may be isomers of feruloyl-tartaric acid (e.g. the cis isomer and perhaps an isomer of the tartaric acid moiety). Unfortunately, a current lack of authentic standards makes these identifications tentative. Still, along with the definitive identification of peak a as trans-coumaroyl-L-tartaric acid, the tentative identification of b and g as trans-(p-coumaroyl) and -feruloyl esters with tartaric acid is appealing since in red clover a hydroxycinnamoyl-CoA:malate hydroxycinnamoyl transferase (HCT2) capable of making a similar series of hydroxycinnamoyl-CoA:malate esters has been identified. It will be interesting to see whether perennial peanut leaves contain a comparable hydroxycinnamoyl-CoA:tartarate hydroxycinnamoyl transferase. If so, comparison of red clover’s hydroxycinnamoyl-malate transferase with a potential perennial peanut hydroxycinnamoyl-tartarate transferase could provide important information about the structure/function relationships of this class of enzymes.

### CONCLUSIONS

Perennial peanut contains levels of a caffeic acid-oxidizing PPO activity greater than that found in red clover as well as PPO o-diphenol substrates. Together, these are able to inhibit proteolysis in plant extracts similar to what has been reported for red clover. The presence of a PPO/o-diphenol system in perennial peanut may account for the improved nitrogen utilization efficiency in ruminants reported for this forage crop. Thus, research related to adapting and optimizing the PPO system in other forage crops will likely be applicable to perennial peanut. Two major phenolic compounds in perennial peanut leaves appear to be PPO substrates, one of which has been definitively identified as caftaric acid. Other major compounds in perennial peanut leaves not oxidized by PPO appear to be related hydroxycinnamoyl-tartaric acid esters.

### ACKNOWLEDGEMENTS

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

REFERENCES


