

# Polyphenol Oxidase and *o*-Diphenols Inhibit Post-Harvest Proteolysis in Red Clover and Alfalfa

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Many forages experience significant proteolytic losses when preserved by ensiling. Such losses in alfalfa are especially high, with degradation of 44-87% of the forage protein to ammonia, amino acids, and small peptides. In contrast, red clover experiences up to 90% less proteolysis than alfalfa during ensiling (*Can. J. Plant Sci.* 63:903). Red clover and alfalfa do not differ significantly in inherent proteolytic activity (*Crop Sci.* 35:537), but several observations suggest that post-harvest proteolytic inhibition in red clover is due to the presence of polyphenol oxidase (PPO) and *o*-diphenol PPO substrates (*J. Sci. Food Agric.* 67:329). Whereas PPO and *o*-diphenols are abundant in red clover leaves, we have detected little if any PPO activity or PPO substrates in alfalfa leaves. We have carried out further biochemical experiments in both red clover and transgenic alfalfa expressing a red clover PPO gene to definitively demonstrate the role of PPO and *o*-diphenols in inhibition of post-harvest proteolysis.

We assessed post-harvest proteolysis in leaf extracts by measuring the concentration of 5% TCA-soluble amino acids over time. Following a 4 hour incubation at 37°C, we measured amino acid concentration increases of  $0.15 \pm 0.03$  and  $0.66 \pm 0.04$   $\mu\text{mol}/\text{mg}$  extract protein for red clover and alfalfa extracts, respectively. Removal of low molecular weight compounds from the red clover extract by gel filtration resulted in an increase in amino acid release to a level comparable to that of alfalfa,  $0.73 \pm 0.07$   $\mu\text{mol}/\text{mg}$ . When caffeic acid, an *o*-diphenol, was added back to the desalted red clover extract, amino acid release was reduced to  $0.11 \pm 0.02$   $\mu\text{mol}/\text{mg}$ , a level similar to that seen for crude extracts, indicating that *o*-diphenols are necessary for post-harvest proteolytic inhibition in red clover. We made similar amino acid release measurements using extracts of transgenic alfalfa expressing a red clover PPO gene (PPO-alfalfa) with or without exogenously added caffeic acid. In PPO-alfalfa leaf extracts, amino acid release was reduced by nearly 80% compared to control alfalfa lacking the PPO transgene ( $0.15 \pm 0.02$  and  $0.70 \pm 0.02$   $\mu\text{mol}/\text{mg}$ , respectively), in an *o*-diphenol-dependent manner. Additional experiments using the transgenic alfalfa system revealed that significant proteolytic inhibition ( $\geq 50\%$  at 4 hours) can be achieved with levels of PPO activity 20- to 100-fold lower than that present in red clover, with as little as 0.25 mM (0.125  $\mu\text{mol}/\text{mg}$  protein) caffeic acid substrate, and with diverse *o*-diphenols, including hydrocaffeic acid, chlorogenic acid, catechol, and epicatechin. Preliminary experiments with mini-silos indicate PPO-alfalfa has approximately 20% less proteolysis than control alfalfa when ensiled in the presence of an *o*-diphenol substrate. Together, these results indicate that PPO and *o*-diphenols play a major role in post-harvest proteolytic inhibition in red clover, and that this system has the potential to be exploited in the preservation of other forage crops.