Substrate-dependent activation of polyphenol oxidase in red clover

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Introduction Polyphenol oxidases (PPO) are copper metaloproteins that catalyse the oxidation of *o*-diphenols to quinones, highly reactive molecules which readily bind to nucleophilic sites on cellular components and proteins. Red clover protein, due to this enzyme is resistant to protease degradation during. Theses enzymes (*circa*. 60-65 kDa) are located in the thylakoid lumen and can be converted to a 40-45 kDa form by proteolysis both *in vitro* and *in vivo* (Gelder *et al.,* 1997). Conversion to the smaller form has been demonstrated to confer activity at neutral pH. Other treatments, such as the presence of lipids or detergents *eg.* SDS, can also confer activity at pH7 (Gelder *et al.,* 1997). Here we describe studies on treatments that affect red clover PPO activity at neutral pH, which is equivalent to the physiological pH of macerated/homogenised leaf extracts.

Materials and methods Red clover leaf material (0.5g approx.) was extracted in McIlvaine buffer, pH7 with (+asc) or without (-asc) 50mM ascorbic acid. Extracts were desalted to remove ascorbic acid and endogenous substrates as descibed by Winters *et al.* (2003). After pre-treatment with the *o*-diphenols, methylcatechol, caffeic acid and chlorogenic acid, PPO activity was analysed in extracts and visualised in gels following separation of proteins by SDS-PAGE according to Winters *et al.* (2003).

Results and discussion Table 1 shows red clover PPO activity, following extraction with ascorbic acid (+asc) after 0 and 20h following treatment of extracts with *o*-diphenol compounds. Additional results are for leaves extracted in the absence of ascorbic acid (0h –asc), so that PPO was active during the extraction process. Results are presented as a percentage of maximum PPO activity of 0h +asc extracts, measured in the presence of SDS (491 ΔOD/min per g FW). Incubation of +asc extracts for 20h increased activity by approximately 2.5 fold. Extraction in the absence of ascorbic acid and treatment of +asc extracts with caffeic acid, chlorogenic acid and methylcatechol also increased PPO activity by between 1.8 and 3.6 fold. These findings suggest that the presence of endogenous or applied phenolic substrates can activate the PPO enzyme in the absence of ascorbic acid.

Table 1 PPO activity of red clover leaf extracts, prepared with and without ascorbic acid and following treatment with a range of *o*-diphenols. Results are presented as % of maximum potential activity

Treatments*					
0h +asc	20h +asc	0h -asc	Caffeic acid	Chlorogenic acid	Methylcatechol
28	70	100	93	44	48

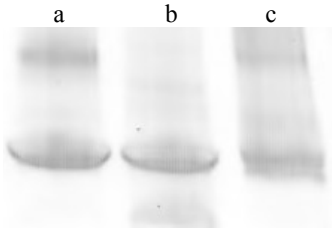

Figure 1 PPO activity following separation of proteins by SDS-PAGE. a) 0h +asc extract, b) 20h extract +asc and c) 0h –asc extract. Relative PPO activities were 28, 70 and 100% for a, b and c respectively.

Figure 1 shows that two size classes of PPO (*circa.* 63k Da and 45 kDa) occur in red clover and are evident in 0h +asc and 0h –asc extracts. The upper form had disappeared after incubation of +asc extracts for 20h. This was concomitant with an increase from 28 to 70% of maximal activity and is consistent with proteolytic cleavage of an inactive form to an active one. The two forms were still present in the –asc extracts, suggesting that the increased activity observed with this treatment is not due to cleavage of an inactive form. We propose that activation following incubation with *o*-diphenols is due to the interaction of quinones with an inactive, larger form of PPO. This results in altered conformation of the PPO protein, conferring activity in a similar manner to SDS treatment.

References

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