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POLYPHENOLIC INHIBITORS OF ALPHA-ACID OXIDASE ACTIVITY

ELIZABETH A. WILLIAMS and ROBERT C. MENARY

Faculty of Agricultural Science, University of Tasmania GPO Box 252C, Hobart, Tasmania, Australia

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Key Word Index—*Humulus lupulus*; Cannabaceae; hop; alpha-acids; polyphenols; enzyme inhibition; tolerance.

Abstract—Polyphenolic compounds in the lupulin glands of the female inflorescence of *Humulus lupulus* were identified. It was demonstrated that only those possessing 3′,4′-dihydroxyphenol groups were capable of inducing a lag phase in the enzymic oxidation of alpha-acids during which the phenolic compound was itself altered by the enzyme. Examination of the kinetics of interaction between the resin substrate, phenolic inhibitor-substrate and the enzyme provided a basis for the suggestion that the system may represent a tolerance mechanism (towards a product of secondary metabolism which is stored in an extracytoplasmic compartment) in secretory cells.

INTRODUCTION

During the past decade the breakdown or turnover of a wide variety of secondary plant products has been clearly established. The humulones or alpha-acids are a rare group of isoprenylated acylphloroglucinols produced in glandular scales known as lupulin glands on the inflorescences of the species *Humulus lupulus* L., the common hop. Evidence of enzymic oxidation of these compounds in the presence of manganese and atmospheric oxygen [1] and peroxidation [2] have been reported in the last 5 years. The presence of endogenous inhibitors of alpha-acid oxidation have been noted; these could be removed by treatment of enzyme extracts with insoluble polyvinylpyrrolidone (PVP), suggesting that they were of a polyphenolic nature [1].

Polyphenolic compounds have been reported to be present in mature female inflorescences in a concentration range of 4–14% [3, 4]. Individual components include catechin, epicatechin, *p*-coumaric and caffeic acids, quercetin and kaempferol [5]. McMurrough *et al.* [6] have quantified three different glycosides of both of these flavonols. Vancraenenbroek *et al.* [7] have found procyanidins B-1, B-2, B-3, and B-4 in hops. Analysis of beer haze has identified 16 phenolic monomers [8] and procyanidin B-3 [9] which may have been derived from barley as well as hops.

Interactions of a general nature between proteins and polyphenols are a common problem encountered by plant biochemists [10] and are thought to result from the formation of complexes of *o*-dihydroxyphenolic groups via a bidentate hydrogen bond formation with the keto-imide groups of the protein [11, 12]. Action of *o*-diphenol oxidases results in the formation of *o*-quinones which readily associate into insoluble polymers [13]. Additionally *o*-quinones have been shown to react with nucleophilic groups including lysyl and cysteinyl residues resulting in tanning of proteins with attendant destruction of enzyme activity [10, 14].

More specific interactions with enzyme systems have been reported and may be significant with respect to general plant physiology. Flavonoids and other phenolic compounds affect oxidative phosphorylation in mitochondria in a manner indicative of an uncoupling function [15]. The kinetics of peroxidase mediated oxidation of indole-3-acetic acid (IAA), in the presence of quercetin revealed that both compounds were destroyed and that the presence of each modified the kinetic behaviour with respect to the other in a mutual sparing effect [16]. A mechanism has been proposed for a similar effect in which ferulic acid caused a lag in the oxidation of IAA mediated by wheat peroxidase [17]. Inhibition of lipoxygenase-dependent lipid peroxidation by quercetin in which the flavonol itself was oxidized has led to the suggestion that compounds of this type may act as antioxidants *in vivo* [18].

The present study was undertaken with the objectives of (i) identifying the endogenous inhibitors of alpha-acid oxidase, (ii) determining the nature of this interaction in order to provide a basis for speculation with regard to how this interaction may serve the plant and (iii) providing a means whereby this interaction might be used to improve pre- and post-harvest management of the crop (this third aspect to be reported elsewhere).

RESULTS AND DISCUSSION

Inhibition of alpha-acid oxidase activity

Extracts with alpha-acid oxidase activity were obtained by means of acetone precipitation [1]. When a direct buffer extract was prepared no activity was observed however, treatment of this extract with PVP resulted in observable activity indicating that this had been masked by the presence of inhibitors which may be removed by acetone extraction of the tissue or PVP treatment of the extract. This was confirmed by including an extract of acetone washings in a reaction mixture with acetone powder extract, the activity of which was reduced by 70%.

Examination of isolated lupulin glands revealed these structures to be a potent source of inhibitory substances. Inhibitory activity could be removed from lupulin extracts by passing them through small columns of PVP.

The effects of varying concentrations of enzyme, inhibitor, alpha-acids and manganese were investigated using an inhibitory extract prepared from lupulin. Time course studies revealed that, in the presence of inhibitors almost no loss of alpha-acids or oxygen consumption occurred during an initial period (20 min–several hr with increasing volumes of inhibitory extract added) after which oxidation of the resins was observed to proceed at a rate similar to that observed in an otherwise identical mixture which did not include the inhibitory extract (Fig. 1). The duration of this lag phase was shortened by raising the concentration of enzyme. Increasing the concentrations of alpha-acids and manganese also reduced the lag phase duration (Fig. 2). The similarity between the effects of [alpha-acids] and $[Mn^{2+}]$ is consistent with results of kinetic studies which indicate that the actual substrate for alpha-acid oxidase activity consists of Mn-activated resin compounds [19].

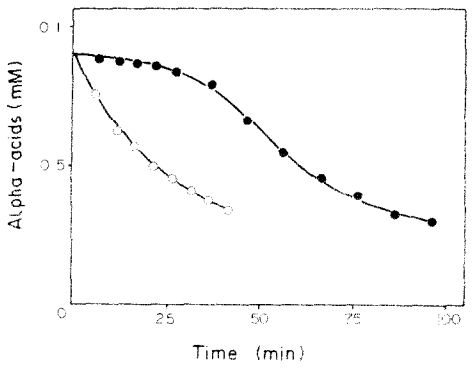

Fig. 1. Time course of the disappearance of alpha-acids from reaction mixtures consisting of crude enzyme extract, 25 μg protein/ml, and alpha-acids in the presence (●) and absence (○) of 0.5 ml inhibitory extract.

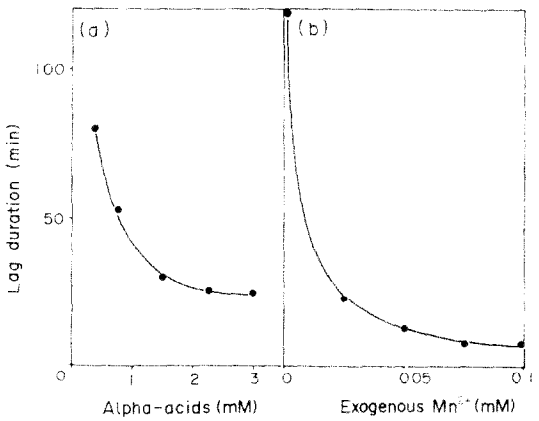

Fig. 2. Effect of [alpha-acids] and $[Mn^{2+}]$ on the duration of the lag in alpha-acids oxidation in manometric assay. Reaction mixtures consisted of crude enzyme extract, 1 ml inhibitory extract of lupulin, endogenous Mn^{2+} in a and 0.75 mM alpha-acids in b.

Identification of lupulin polyphenols

An extract was prepared from lupulin isolated from the cv Nugget and components separated with HPLC. Compounds present in the extract co-chromatographed with gallic, protocatechuic, *p*-hydroxybenzoic, caffeic and *p*-coumaric acids, catechin, epicatechin, phloroglucinol, phloridzin, quercetrin, kaempferol and procyanidin B-7.

One milliliter of an identical preparation was applied to a preparative ODS 2 HPLC column. A peak, the R_t of which was co-incident with that of authentic phloridzin, was collected from three successive injections and dried. This material co-chromatographed with authentic phloridzin on 2D-TLC. The remainder of this fraction was sonicated in glycerol–acetic acid and then analysed by FABMS. In the positive ion mode signals corresponded to the $[M+H]^+$ ions of phloridzin and phloretin, the aglycone of phloridzin. Identification of procyanidin B-7 is tentative pending verification by GCMS and NMR.

Verification of polyphenols as alpha-acid oxidase inhibitors

Authentic samples of gallic, protocatechuic, *p*-hydroxybenzoic, caffeic and *p*-coumaric acids, phloroglucinol, catechin, phloridzin, quercetin, quercetrin, rutin and kaempferol were each included in reaction mixtures (1.86 μM) and the time courses of the disappearance of 0.1 mM alpha-acids were recorded. Lag phases were observed in those mixtures containing compounds possessing the *o*-dihydroxyphenolic group (Fig. 3), i.e. protocatechuic and caffeic acids, catechin, quercetin and its glycosides quercetrin and rutin. Mixtures containing the other compounds displayed time courses identical to that of a mixture containing only enzyme extract and alpha-acids.

To test this apparent structural requirement for lag phase induction and to investigate the effects of different substitution patterns the following compounds were included in reaction mixtures: gossypetin-3-galactoside, homoprotocatechuic acid and fisetin all possessing the 3',4'-dihydroxyphenolic grouping, morin with hydroxyl groups in positions 2' and 4', ferulic acid with the 3'-hydroxyl group methylated and cyanidin-3-galactoside possessing only one hydroxyl group at the 4' position. Consistent with the previous result only those compounds with the structure represented in Fig. 3 caused a lag phase, the others being inactive.

Examination of the lag phase

In order to determine whether precipitation of enzyme–polyphenol complexes of the type discussed in ref. [11] was a factor in the lag phenomenon, 1.5 mg bovine serum albumin (BSA) was added to a mixture containing an estimated 0.3 mg alpha-acid oxidase, 1.86 μM quercetrin and 0.1 mM alpha-acids. The time course of alpha-acids disappearance was indistinguishable

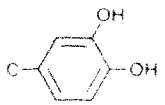

Fig. 3. The structural component common to all substances which induced a lag in enzymic oxidation of alpha-acids.

from that of an identical mixture not containing any BSA indicating that the lag phase was not a result of impaired enzyme activity due to precipitation of enzyme–polyphenol complexes.

Incubation of inhibitory extract with enzyme prior to the addition of alpha-acids resulted in a shortening of the lag phase, longer pre-incubation achieving greater reduction of the lag and an increased effect evident with higher enzyme concentration suggesting that interaction with the enzyme resulted in alteration of the inhibitors with attendant destruction of their ability to inhibit alpha-acid oxidation.

UV spectra of mixtures containing buffer plus quercetrin, enzyme extract plus quercetrin and enzyme extract alone measured prior to and following 24 hr incubation at 36° (Fig. 4) support the conclusion that the polyphenol is altered by the enzyme as does the colour change from pale yellow to reddish brown observed during this incubation.

Confirmation was obtained using analytical HPLC to follow changes in quercetrin levels in reaction mixtures consisting of 2 ml crude enzyme extract and 0.4 mg quercetrin (added in 0.5 ml methanolic solution) in the presence and absence of alpha-acids, 2.0 mM. Chromatograms indicated disappearance of quercetrin and the appearance of peaks with shorter R_ts. The disappearance of quercetrin was considerably faster in the presence of alpha-acids (2–3 hr) than in their absence (> 24 hr). Following the disappearance of quercetrin peaks from the chromatograms, oxidation of alpha-acids was observed to proceed rapidly. Incubation of extracts containing protocatechuic acid resulted in the total loss of this peak from the chromatograms with no product peaks detectable at either 254 or 280 nm, which, considered with the spectral change observed for quercetrin, suggests that aromatic ring cleavage of the phenolic inhibitors occurred during the lag phase in alpha-acid oxidation.

Kinetics of alpha-acid oxidation during lag phase

Two protein peaks with alpha-acid oxidase activity were separated by eluting a crude extract through a 60 × 2.5 cm column of DEAE-Sephadex A50 with 0.1 M phosphate buffer, pH 7.5. These were equivalent to the pI 3 and pI 10 bands obtained by isoelectric focussing [1], the pI 3 fraction consisting of three small proteins with pIs 3.0, 3.3 and 3.6 and the pI 10 fraction containing one larger protein in a clearly symmetrical peak separated from the other fraction by 150 ml eluate devoid of protein. Anion exchange proved to be a powerful preparative procedure because endogenous inhibitors were retained by the gel and the desalting effect yielded proteins free from endogenous manganese. The velocity versus [substrate] response was determined for each fraction in the presence and absence of quercetrin. All mixtures containing quercetrin exhibited biphasic time courses; the reaction velocities reported here are those observed during the initial/lag phase. Double reciprocal plots revealed simple Henri–Michaelis–Menten kinetics in the absence of quercetrin. In the presence of quercetrin the relationship between initial velocity and alpha-acids substrate concentration was sigmoidal indicating positive homotropic co-operativity with respect to alpha-acids.

The effect of increasing quercetrin concentration in a reaction mixture with crude enzyme extract was to shift this sigmoidal relationship to the right along the [substrate] abcissa (Fig. 5). Positive homotropic co-operativity is effectively a switching mechanism with regard to enzyme activity and the effect of raising the level of quercetrin in the mixture raises the alpha-acid concentration required to switch on the alpha-acid oxidation function of the enzyme.

In all the studies reported above inhibitory substances were added to reaction mixtures prior to initiation of alpha-acids oxidation by addition of resin substrate. When the order of addition of the two components was reversed the lag phase was of significantly shorter duration. Macháčková *et al.* [17] observed a similar effect with ferulic acid induced lags in IAA -oxidase activity for which they have proposed a mechanism.

The evidence supporting the proposition that the activity towards 3′,4′-dihydroxyphenols may be attributed to the alpha-acid oxidases is two-fold. Firstly, crude extracts have been shown not to possess phenolase

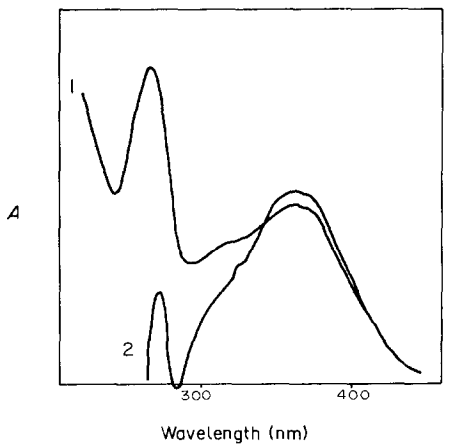

Fig. 4. UV-spectra of quercetrin (1) prior to and (2) following 24 h incubation at 36°, buffer and enzyme blanks subtracted.

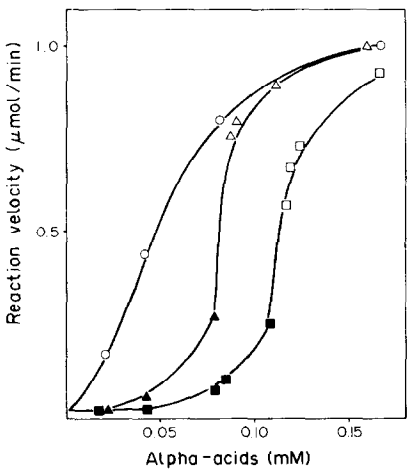

Fig. 5. Response of reaction velocity of a crude enzyme extract, 25 μg protein/ml, to alpha-acids concentration in the presence of 1.82 (▲) and 3.63 μM (■) authentic quercetrin. Note that the crude extract (○) contained some endogenous inhibitors. Open symbols represent reaction mixtures for which no lag phase was observed.

activity, this being retained in the acetone powder residue during rehydration. Similarly crude extracts do not display peroxidase activity. The second, and perhaps the stronger argument is to be found in the kinetic observations; were the two functions attributable to separate enzymes the reduction in the lag duration effected by increased alpha-acid and Mn^{2+} concentrations would be entirely unexpected, and if the disappearance of inhibitory compounds was due to binding to the nucleophilic residues of the protein addition of BSA to reaction mixtures could be expected to shorten the lag.

In the absence of a convenient procedure to assay for alpha-acids and dihydroxyphenols simultaneously the mechanism of lag induction remains unclear. The ligand exclusion model proposed by Fisher *et al.* [20] which predicts hyperbolic response to [substrate] in the absence of inhibitor and sigmoidal response with inhibitor present is perhaps the most appropriate to the data. This model requires the presence of two active sites for the substrate, a situation which has been observed in respect of the pI 10 protein [19].

Possible significance of lag phase inhibition

Takahama has proposed that flavonols may act as antioxidants *in vivo* [18]. This study tends to support the view that some polyphenols act as very specific antioxidants effectively protecting the hop resins from enzymic oxidation. The presence of phenolic inhibitors introduces a period of time during which the alpha-acids are spared, the magnitude and duration of the effect being determined by the concentrations of the 3',4'-dihydroxyphenolics, alpha-acids and manganese. If this interaction occurs *in vivo* it may represent a mechanism whereby the resins are protected during the time interval between their synthesis and sequestration into the subcuticular space of the gland, while the cell retains a measure of control of cytoplasmic concentrations of alpha-acids which appear to travel to the plasmalemma unprotected by any enclosing membrane [21] in a manner resembling that thought to occur in the case of the cannabinoid resins in the closely related *Cannabis sativa* L. [22].

This can be considered reasonable only if the presence of alpha-acids compromises the functions of the cell and all three components of the system are present in the secretory structure. Addressing the first of these points, alpha-acids were shown to inhibit the growth of soy bean root callus cultured on Miller's medium [23] (Table 1). In respect of the second, anion exchange chromatography of crude acetone powder extracts of isolated lupulin glands [1] resulted in the isolation of active alpha-acid oxidase proteins, the activity of which presumably had been masked by the presence of the inhibitors in the crude extract. Thus it is conceivable that this complex interaction between enzyme, its resin substrate and phenolic inhibitor-substrate may represent a tolerance mechanism in secretory tissue while non-secretory tissues, by virtue of a lesser content of inhibitor-substrates, display a much lower level of tolerance mediated by the same enzymes [19].

Table 1. Effect of alpha-acids on the growth of soy bean root callus

[Alpha-acids] (M)	Fresh weight of callus (g)
0	1.49
10^{-7}	1.23
10^{-6}	1.26
10^{-5}	0.68
10^{-4}	0.17

s.e. = 0.17.

EXPERIMENTAL

Alpha-acid oxidase activity. Where this activity was measured in terms of oxygen consumed gas uptake was measured in a Warburg Respirometer according to the method described by Menary *et al.* [1]. Time courses of alpha-acid disappearance from reaction mixtures were observed using a dual beam spectrophotometer. The absorbance at 355 nm was measured and the alpha-acids concentration calculated using the absorption coefficient which was determined to be 0.0097 units absorbance per μmol resin. Reactions were conducted at pH 7.5 and 36°. Protein content of extracts was determined according to the method of ref. [24] using ovalbumin as the standard protein. Alpha-acid substrate was a mixture of humulone, cohumulone and adhumulone prepared as described in [1].

Alpha-acid oxidase preparation. This preparation was a further development of procedures reported in [1], modifications being rehydration of Me_2CO powders in 0.1 M Na-Pi buffer, pH 7.5, for spectrophotometric assay and pH 6.5 for manometric assay, in the presence of PVP (1:2, PVP: Me_2CO powder) and further PVP treatment of extracts obtained by filtration of this rehydration mixture.

Lupulin isolation. Lupulin glands were separated from the other floral tissues by macerating the hop cones in H_2O in a slow speed blender and sieving the resultant slurry with the aid of a jet of cold water according to the method of ref. [25].

Extraction of inhibitors. 1 g lupulin was sonicated for 10 min with 30 ml toluene and filtered to remove resins. This was repeated twice and followed by sonication with 3 × 30 ml MeOH. The MeOH extract was dried *in vacuo* and the residue washed with 1 ml 20% aq. MeOH. 20μl of these washings were subjected to analytical HPLC.

HPLC. Authentic phenolic compounds and extracts were analysed on an Alltech C 18, 25 × 0.46 cm column. Solvents A and B were H_2O propionic acid (10:1) and H_2O EtOH (1:1) respectively. The linear gradient programme was initially 100% solvent A to 30% solvent A in 40 mins and 100% solvent B after 50 mins with a flow rate of 2 ml per min. throughout. The absorbance of the eluant was monitored at 280 nm.

Reaction mixtures were chromatographed on a Waters 10 μ, C 18 Radpac column in a radial compression unit. Solvents and gradients were as described above except that the solvent was changed to 100% EtOH at the end of the programme in order to elute alpha-acids and their oxidation products. Detection was at 254 and 280 nm.

Extracts of lupulin were fractionated on a Whatman M20, 25 cm ODS 2 column. The linear gradient programme was 40% 60% MeOH in 30 mins. Column temp. was 40°, flow rate 10 ml per min. and detection at 325 nm.

TLC. Silica thin layer plates were developed with 6% aq. HOAc and then in a perpendicular direction with *t*BuOH HOAc H_2O (3:1:1).

FABMS. Samples were sonicated in glycerol HOAc (10:1) and analysed in a Varian MAT CH7 mass spectrometer with an ion source housing adapted to fit an Ion Tech atom gun. The gun was operated at 7 kV and the reagent gas was xenon.

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