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INHIBITION OF α-ACID OXIDASE BY POLYPHENOLIC COMPOUNDS—A KINETIC MODEL

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Key Word Index—*Humulus lupulus*; Cannabaceae; humulones; *o*-dihydroxy-compounds; chelation; enzyme activation.

Abstract—A model, which is consistent with observed complex kinetic interactions during lags in α-acid oxidase activity induced by the presence of *o*-dihydroxy-compounds, is proposed. According to the model the sigmoidal [substrate] ~ velocity curve is attributable to interaction between the lag-inducing compound and Mn^{2+}, one constituent of the compound substrate. It is shown that this compound substrate for α-acid oxidase activity activates a second activity which degrades the lag-inducing compound and that this second activity is most probably associated with the α-acid oxidase enzymes.

INTRODUCTION

Polyphenolic compounds have been observed to affect a number of metabolic processes in plants including the peroxidase-mediated oxidation of auxin [1] and oxidative phosphorylation [2]. More recently *o*-dihydroxy-compounds have been reported to induce a lag-phase in the α-acid oxidase-mediated oxidation of humulones, the isoprenylated acylphloroglucinols produced and stored in the glandular scales of the female inflorescences of *Humulus lupulus* L., the common hop [3]. The interaction between the enzyme, humulones and the lag-inducing inhibitors was apparently complex; the salient features of the phenomenon are summarized as follows: the duration of the lag was increased by raising the concentration of inhibitor and was decreased by increasing the concentration of either or both components of the Mn–humulone complex (which is the substrate for α-acid oxidase [4]) or by increasing the amount of enzyme present. The duration of the lag was reduced when humulone was added to reaction mixtures prior to the addition of the inhibitor. Williams and Menary [3] demonstrated that the lag-inducing compounds were altered during the lag in humulone oxidation, presumably losing the *o*-dihydroxy-moiety with which the inhibitory activity was associated. This reaction did occur in the absence of humulones, but its rate was greatly enhanced by their presence. Humulone oxidation during the lag followed sigmoidal kinetics whereas simple Michaelis kinetics adequately described the behaviour in the absence of lag-inducing compounds [3].

The model proposed to account for this behaviour can be summarized by the reaction schemes below.

$$Mn^{2+} + \text{Humulone} \rightleftharpoons \text{Mn–Humulone} \quad (1)$$

$$Mn^{2+} + R \rightleftharpoons \text{Mn–R} \quad (2)$$

$$\text{Mn–Humulone} + E \rightleftharpoons \text{E–Mn–Humulone} \rightarrow E + \text{Products} + Mn^{2+} \quad (3)$$

$$+ \text{Humulone} + E \rightleftharpoons \text{E–Mn–Humulone} \rightarrow E + Mn^{2+} + \text{Products} + Mn^{2+}$$
$$+ R \rightleftharpoons \text{Mn–R} \quad (4)$$

$$R + E \underset{K_1}{\overset{k_1}{\rightleftharpoons}} \text{E–R} \rightarrow E + R' \quad (5)$$

$$R + \text{E–Mn–Humulone} \underset{K_2}{\overset{k_2}{\rightleftharpoons}} \text{E–Mn–Humulone-R} \rightarrow \text{E–Mn–Humulone} + R' \quad (6)$$

where R and R′ represent the *o*-dihydroxy-compound and its reaction product respectively, K_1 and K_2 are equilibrium constants and k_1 and k_2 are rate constants.

As shown in Scheme 3 the actual substrate for α-acid oxidase is the complex formed between humulone and manganous ions, which has a stability constant of 10^3 l/mol [4]. *o*-Dihydroxy-compounds also chelate metal ions (Scheme 2). The stability constant of Mn^{2+} chelated by protocatechuic acid (the simplest of the lag-inducing compounds tested by Williams and Menary [3]) was shown by Murakami *et al.* [5] to be of the order of 10^7 l/mol. Thus one effect of including protocatechuic acid in a reaction mixture will be to lower the concentration of the compound substrate of α-acid oxidase because of competition between the two ligands for the available metal ions. This effect can be considered to be similar to that observed by Sluyterman and Wijdenes for the action of benzoylamido acetonitrile on the hydrolysis of benzoylglycine methyl-ester mediated by papain [6]. Segel [7] has described the kinetic behaviour of a general model in which an inhibitor, I, forms a complex with the substrate for an enzymic reaction, the complex, SI, with a

formation constant K_0, not being a substrate. He derived the expression for the actual substrate concentration to be

$$[S] = [S]_t - \frac{\sqrt{([I]_t - [S]_t + K_0)^2 + 4K_0[S]_t} - ([I]_t - [S]_t + K_0)}{2}$$

where $[I]_t$ and $[S]_t$ are the total concentrations of all molecular species containing inhibitor and substrate respectively. Substitution of this expression into the Michaelis equation

$$v/V_{max} = [S]/(K_m + [S])$$

results in a sigmoidal relationship between reaction velocity and $[S]_t$ in the presence of the inhibitor (see Fig. 1 for the theoretical curves). In the case of α-acid oxidase the reaction scheme may be represented by (4) and although the expression for velocity will be more complex than in the system described by Segel (including terms for the formation of both manganese chelates) the gross effect on the kinetics will be the same.

Such a model for the inhibition of α-acid oxidase activity during lags induced by the presence of *o*-dihydroxy-compounds does account for the observed kinetics of humulone oxidation during the lag, Fig. 5 of ref. [3] being strikingly similar to Fig. 1.

Interaction between the enzyme and the phenolic compound or its Mn-complex is not necessary to explain this aspect of the observed behaviour; however it is clear from the results described in [3], and from the fact that the lag phase ends, that the lag-inducing compounds are altered by interaction with some component of the enzymic reaction mixture and that this interaction destroys their ability to inhibit the enzyme's activity towards humulone.

The non-enzymic couple, R + Products → R′, must be considered as a possible mechanism for the disappearance of the lag-inducing compounds. The chemistry is yet to be established but the similarity of the products of enzymic oxidation of humulones with the products of their autoxidation [8] suggests that, as with autoxidation [9], the enzymic reaction may proceed via-free-radical intermediates. Polyphenolic compounds, which have been shown to be scavengers of free-radicals [10], could be expected to disappear from reaction mixtures in which these radicals are generated. However, the ability to donate protons in the scavenging of radicals is not restricted to *o*-dihydroxyphenols but it is only these compounds which have been observed to disappear from reaction mixtures during the lag phase in humulone oxidation. This, along with the fact that the *o*-dihydroxy compounds disappeared from mixtures in which they were incubated with the enzyme extract in the absence of humulones (and thus in the absence of Products), the rate of this disappearance increasing with increased enzyme concentration [3], and the spectral differences between the R′ compounds observed in these investigations [3] and those products of the non-enzymic couple reported by other workers [11, 12] form the basis for rejecting the possibility of non-enzymic destruction of the lag-inducing compounds.

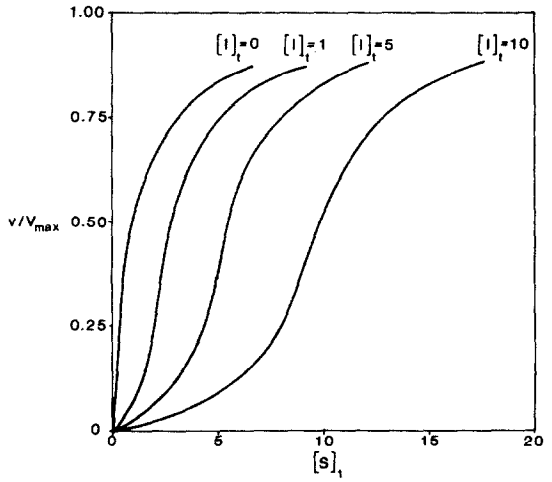

Fig. 1. Theoretical curves for the velocity response to $[substrate]_{total}$ in the presence of an inhibitor which combines with substrate with an association constant, $K_0 = 0.1$, $K_m = 1.0$ (after Segel [7]).

At present no convenient technique is available whereby the progress curves for the disappearance of lag-inducing compounds can be observed directly in the presence of humulone resins; the discussion below seeks to consider possible explanations of the dihydroxyphenol-degradation reaction on the basis of observed progress curves for humulone oxidation. In order to do this it is necessary to make a number of assumptions: (i) that the duration of the lag in humulone oxidation reflects the concentration of the lag-inducing compounds, and (ii) that for a given concentration of lag-inducing compound, differences in the duration of the lag in humulone oxidation reflect different rates of disappearance of the lag-inducing compound. The first possible explanation is that uncomplexed lag-inducing compound is the substrate for this second activity in the extract and that it reacts at an active site which is independent of the site for alpha-acid oxidase activity, possibly on a different enzyme. Then, for fixed total concentrations of enzyme extract, *o*-dihydroxy-compound and Mn^{2+}, increases in the humulone concentration would be expected to result in increases in the concentration of free R by causing a shift in the equilibrium

$$R + Mn^{2+} \rightleftharpoons Mn\text{–}R$$

away from complex formation. As a consequence the rate at which R disappears from the mixture would be expected to increase with increasing concentrations of humulone and this would be observed as a shorter inhibited phase in the progress curves of humulone oxidation. Logically, increasing the concentration of Mn^{2+} would be expected to result in longer lag phases. The order in which R and humulones were added to the Mn^{2+} containing enzyme extract would not be expected to have any effect on the duration of the lag. The latter two predictions are clearly at odds with the experimental observations [3] so this postulate with respect to the dihydroxyphenol-degradation reaction must be discounted.

The second possible mechanism is that the complex formed between R and Mn^{2+} is the substrate for this reaction, and the activity is independent of alpha-acid oxidase activity. Raising the total humulone concentration would be expected to lengthen the lag in humulone oxidation, contrary to the observed effect.

Thus the conclusion that these two activities in the extracts of hop cones are not independent of each other is inescapable. Now, if the relationship between the two is such that it is described by Schemes 5 and 6 and either $K_1 << K_2$ and or $k_1 << k_2$ then the presence of humulone and Mn^{2+} in the reaction mixture would promote the disappearance of R. The observed relationships between the duration of the lag in humulone oxidation and $[\text{humulone}]^{-1}_{\text{total}}$ and $[Mn^{2+}]^{-1}_{\text{total}}$ are shown in Fig. 2, which reflects activation of free enzyme or enzyme-R complex by a tight-binding activator [13]. If the active site for humulone oxidation is the activation site for the R reaction then Mn-humulone can be expected to behave as a tight-binding activator since the K_m for this substrate is of the order of 10^{-7} M [4].

As a consequence of this activation, the order in which humulone and R are added to the reaction mixtures might be expected to affect the duration of the lag in humulone oxidation since shifts in the equilibrium between enzyme–Mn–humulone and unbound enzyme may result in transient phases in the progress curves for the disappearance of R from these mixtures. The effect of such transients on the duration of lag phases in humulone oxidation is illustrated in Fig. 3. Clearly, without direct measurements of the rates of disappearance of lag-inducing compounds any consideration of the mechanism of this activation can only be speculative, however, for the

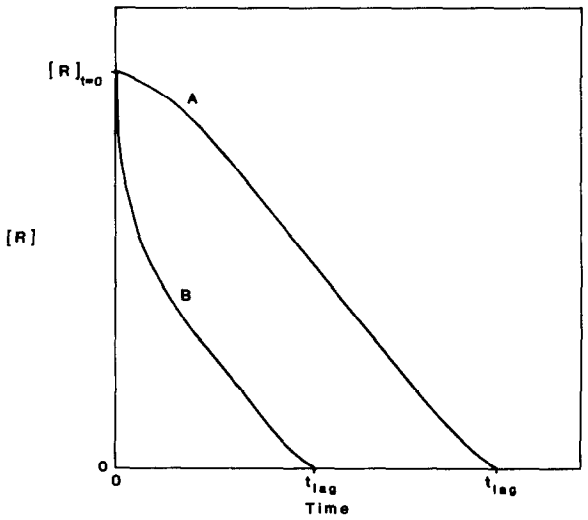

Fig. 3. The effect of the order of addition of humulone and a lag-inducing compound, R, on theoretical progress curves for the disappearance of R from reaction mixtures and the concomitant effect on the duration of the lag in humulone oxidation. Curve A: R added first, curve B: humulone added first.

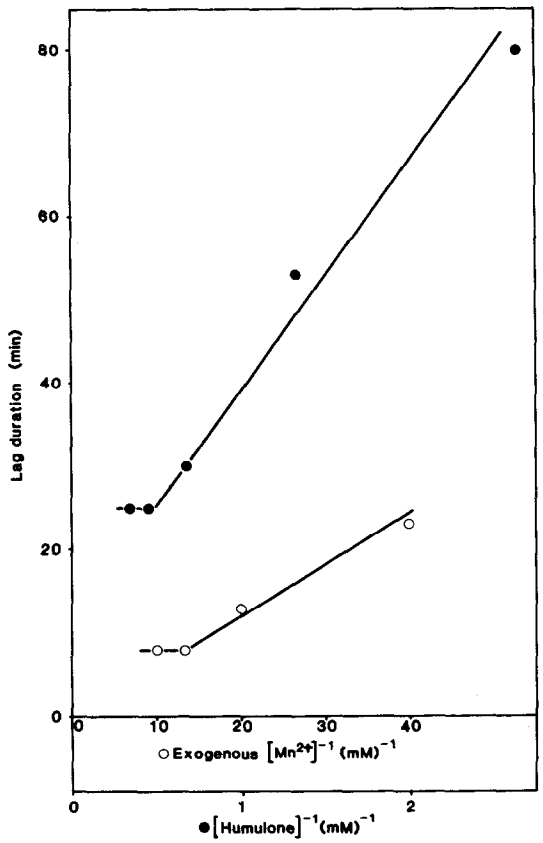

Fig. 2. Plots of lag-phase duration $\sim [\text{Humulone}]^{-1}$ and $\sim [Mn^{2+}]^{-1}$ (Data from ref. [3]).

effect shown in Fig. 3 to occur within the time scale of the experimentally observed phenomenon the activation would probably need to involve hysteretic changes in the enzyme.

According to the model the lag can never be completely eliminated because the removal of R will always require a finite time interval, even when $R \rightarrow R'$ procedes at rates approaching V_{max}. Experimentally the lag does appear to be eliminated in certain circumstances, e.g. when Mn–humulone concentrations are such that the rate of humulone oxidation during the lag is indistinguishable from the subsequent post-lag rate and when the concentration of lag-inducing compounds is sufficiently low for the lower limit of the lag duration to be less that the sampling interval of the assay. In the latter case the presence of an undetectable lag has been confirmed by observing identical reaction mixtures at a temperature 10° lower, at which the rate of $R \rightarrow R'$ is reduced and the lag is evident.

Although it can be argued in this way that the promotion of the reaction degrading the *o*-dihydroxy-compound depends on the substrate for alpha-acid oxidase activity binding to the dihydroxyphenol-degrading enzyme, humulone oxidation as a consequence of this binding has not been established. Since most of the investigations utilized crude extracts, the possibility that the humulone substrate for alpha-acid oxidase is an activator for the activity of another enzyme which catalyses the degradation of *o*-dihydroxy-compounds remains. However, since all preparations of alpha-acid oxidase did possess dihydroxyphenol-degrading activity [3], and activation of this activity by Mn–humulone reflects binding of a similar order to the K_m for alpha-acid oxidase activity, the possibility that both activities are functions of the same enzyme seems likely.

REFERENCES

1. Gelinas, D. A. (1973) *Plant Physiol.* **51**, 967.
2. Stenlid, G. (1970) *Phytochemistry* **9**, 2251.
3. Williams, E. A. and Menary, R. C. (1988) *Phytochemistry* **27**, 35.
4. Williams, E. A. (1988) University of Tasmania Dissertation, (in preparation).
5. Murakami, Y., Nakamura, K. and Tokunaga, M. (1963) *Bull. Chem. Soc. Jpn* **36**, 669.
6. Sluyterman, L. A. and Wijdenes, J. (1973) *Biochim. Biophys. Acta* **321**, 697.
7. Segel, I. H. (1975) *Enzyme Kinetics.* John Wiley, New York.
8. Menary, R. C., Williams, E. A. and Doe, P. E. (1983) *J. Inst. Brew.* **89**, 200.
9. Verzele, M. (1986) *J Inst. Brew.* **92**, 32.
10. Torel, J., Cillard, J. and Cillard, P. (1986) *Phytochemistry* **25**, 383.
11. Sorata, Y., Takahama, U. and Kimura, M. (1984) *Biochem. Biophys. Acta* **799**, 313.
12. Takahama, U. (1985) *Phytochemistry* **24**, 1443.
13. Reiner, J. M. (1969) *Behaviour of Enzyme Systems.* Van Nostrand Reinhold, New York.