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# α-ACID DEGRADATION BY SUSPENSION CULTURE CELLS OF *HUMULUS LUPULUS*

R. J. ROBINS, J. M. FURZE and M. J. C. RHODES

AFRC Food Research Institute, Colney Lane, Norwich NR4 7UA, U.K.

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**Key Word Index**—*Humulus lupulus*; Cannabinaceae; suspension culture; α-acids; humulone; cohumulone; peroxidase.

**Abstract**—In a suspension culture of *Humulus lupulus* hop α-acids could not be detected. However, the culture was shown to have an *in vivo* ability to degrade exogenous α-acids and related compounds. It is shown that this is due to peroxidase with a rate constant for α-acid degradation of $2.7 \times 10^4$/M·sec.

## INTRODUCTION

The recognition that when hops (*Humulus lupulus*) are added to wort the beer is much better protected against spoilage dates back to Babylonian times. This technology was apparently revived in Europe in the tenth century but not introduced into England until *ca* 1400. Despite the initial unpopularity of hopped ale the flavour constituents derived from hops are now of prime importance in determining the quality of the product. Many chemicals from the cones contribute to the final flavour but foremost amongst these are the α-acids, principally humulone and cohumulone, which are isomerized to the corresponding bitter *iso*-α-acids during processing [1]. Because considerable variation in the α-acid content of hop cones occurs, it is frequently desirable in large-scale brewing operations to add purified α-acids to the wort in order to obtain a product of consistent flavour [2].

The requirement for additional α-acids, at present extracted from hop cones, has led us to investigate the possibility of producing them by plant cell culture techniques. *Humulus lupulus* has previously only been cultured as callus [3] and, while a number of steroids were identified, no bitter products were produced. Much effort has been expended on ways of determining the optimal time to harvest hops from the vine, since the quantity of α-acids reaches a peak as the cones mature and, subsequently, declines [4]. Despite extensive knowledge of the degradative oxidation during storage of kiln-dried hops [5, 6], it is only recently that an enzyme has been reported that may contribute to the degradation of α-acids in ripe cones [7]. In the present study we report the establishment of a rapidly growing suspension culture of *H. lupulus* and the metabolism of α-acids by this culture.

## RESULTS AND DISCUSSION

### *Cell suspension cultures*

Suspension cultures contained white to light-brown cells, growing singly and as small clumps up to 1 mm diameter, with at least 95% viability [8] throughout the 14-day growth cycle (Fig. 1). An anion analysis showed $H_2PO_4^-$ undetectable after 7 days but, after 14 days, some $Cl^-$ (1%), $NO_3^-$ (20%) and $SO_4^{2-}$ (25%) remained unabsorbed. Growth was readily promoted by chlorinated auxin analogues but not IAA or NAA (Table 1). There was no requirement for exogenous cytokinin. Gibberellins, $GA_3$, $GA_4$, $GA_7$ and $GA_{13}$ did not affect growth between $10^{-7}$ and $10^{-4}$ M but did induce greening of the cultures.

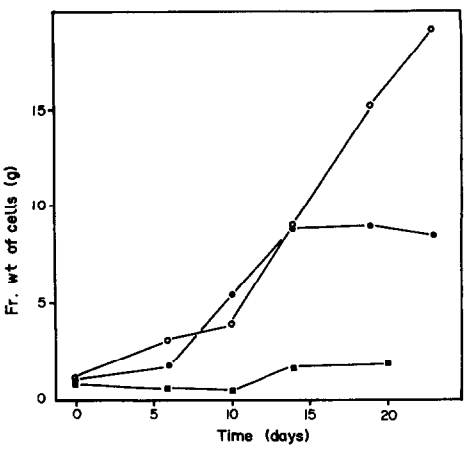


Fig. 1. Time-courses for the growth of *Humulus lupulus* suspension cultures on media formulated according to Gamborg's B5 [9] (●), Schenk and Hildebrandt [10] (○) and Murashige and Skoog [11] (■), supplemented with 54 mM sucrose, 1.0 mg/l. 2,4-D and 0.1 mg/l. kinetin. 50 ml medium was inoculated with 1 g fr. wt cells. Each point represents the analysis of a whole flask.

Substitution of Gamborg's B5 [9] basal medium with that of either Schenk and Hildebrandt [10] or Murashige and Skoog [11] had a marked effect on growth (Fig. 1). On the former medium, a growth phase of extended duration was attained, the cell mass reaching 40% of the total volume of culture. In contrast, growth was totally inhibited in Murashige and Skoog's medium although, after several months, a culture adapted to the greater $NH_4^+$ concentration of this medium could be obtained.

A number of other substances were studied (Table 2) all

Table 1. Effect of various plant growth factor combinations on growth of *Humulus lupulus* suspension cultures

| Auxin (1 mg/l.)* | Cytokinin (0.1 mg/l.) None | Kinetin | Zeatin | 6-Benzyladenine |
|---|---|---|---|---|
| None | 0 | 0 | 0 | 0 |
| 2,4-D | 91 | 100† | 76 | 57 |
| 4CPA | 103 | 109 | 92 | 59 |
| 4C2MPA | 77 | 78 | 79 | 72 |
| IAA | <5 | 18 | <5 | <5 |
| NAA | 22 | <5 | <5 | <5 |

Growth was measured over six successive subcultures as the fr. wt per flask and is expressed as a percentage of the growth under control conditions (1 mg/l. 2,4-D; 0.1 mg/l. kinetin) during the relevant subculture.

*2,4-D, 2,4-Dichlorophenoxyacetic acid; 4CPA, 4-chlorophenoxyacetic acid; 4C2MPA, 4-chloro-2-methylphenoxyacetic acid; IAA, indoleacetic acid; NAA, 1-naphthylacetic acid.

†Mean growth per flask of 9.1 ± 1.7 g in 14 days.

of which, with the exception of L-arginine, showed some inhibition of growth. Increasing the sucrose concentration appeared to prevent cell expansion and water accumulation as the effect was much less marked when measured on a dry wt basis. L-Amino acids above a concentration of 1 mM have been found previously to inhibit growth [12, 13] and the same effect was found here, with the notable exception of L-arginine. We have shown by $^{31}$P NMR that inorganic phosphate accumulates in the vacuole of *H. lupulus* cells [14], the cells growing normally at phosphate concentrations up to 20 mM, much higher than those previously reported [11]. L-Arginine is known to accumulate in vacuoles [15, 16] and it would appear that the degree of toxicity of these compounds may reflect the extent to which the cells are vacuolated or to which the substance is sequestered by the vacuole.

No stimulation of α-acid accumulation was detected when the precursors of α-acid biosynthesis, L-valine, L-leucine, acetate or malonate [17], were presented (Table 2). Neither was phloroglucinol, closely related to natural intermediates, effective. In an attempt to divert polyketide precursors away from flavonoid biosynthesis by decreasing the availability of the phenolic component of the pathway, the potent inhibitors of phenylalanine ammonia-lyase (EC 4.3.1.5), amino-oxyacetic acid and amino-oxyphenylpropionic acid were used, but without success. We, therefore, examined the stability of α-acids in the culture system.

*Degradation of exogenous α-acids* in vivo

The addition of exogenous α-acids to cultures resulted in their rapid disappearance from the medium without any concurrent accumulation within the cells (Fig. 2). Degradation occurred equally rapidly (Table 3) if cells were resuspended in fresh medium or buffer (5 mM MES, pH 5.5, containing 54 mM sucrose), but degradative activity was largely destroyed by boiling the cells for a few minutes prior to resuspension. The slow background rate of degradation in fresh medium or buffer was not enhanced by the addition of hydrogen peroxide. In contrast, although the rate in spent medium was comparable to that in fresh medium (Fig. 2), the addition of cells (Fig. 2) or hydrogen peroxide (Table 3) caused a marked increase in the rate of degradation of α-acids. The stimulation by hydrogen peroxide was completely destroyed when the spent medium was boiled prior to incubation with the substrates.

The rate of degradation showed a dependence on the age of cells since the last subculture, being maximal at 6–8 days (Fig. 3). It was also influenced by the pH of the buffer, showing optimal activity at pH 6.2. The activity in green, light-grown cells was no greater than that in dark-grown cells of the same age (Table 3).

If, in addition to α-acids, cells were incubated with the β-acid, colupulone, or the structurally closely related

Table 2. Levels of various effectors inhibiting growth of *Humulus lupulus* suspension culture cells

| Effector | Maximum concentration giving <5% inhibition (mM) | Concentration giving 50% inhibition (mM) | Concentration giving 80% inhibition (mM) |
|---|---|---|---|
| Sucrose | 64 | 164 | — |
| Phosphate | 20 | 45 | — |
| L-Arginine | 10 | — | — |
| L-Lysine | 0.5 | 4 | 10 |
| L-Valine | 0.5 | 3.5 | 10 |
| L-Leucine | 0.5 | 2.5 | 5 |
| Acetate | 1.0 | 3.5 | 5 |
| Malonate | 5 | 16 | 25 |
| Phloroglucinol | 0.1 | 3.5 | 7 |
| Amino-oxyacetic acid | 0.08 | 0.4 | 0.8 |
| Amino-oxyphenyl propionic acid | 0.001 | 0.1 | — |

Growth was measured as fr. wt over three successive subcultures (one for sucrose, phosphate and amino-oxyphenylpropionic acid) in normal medium containing various concentrations of the relevant effector.

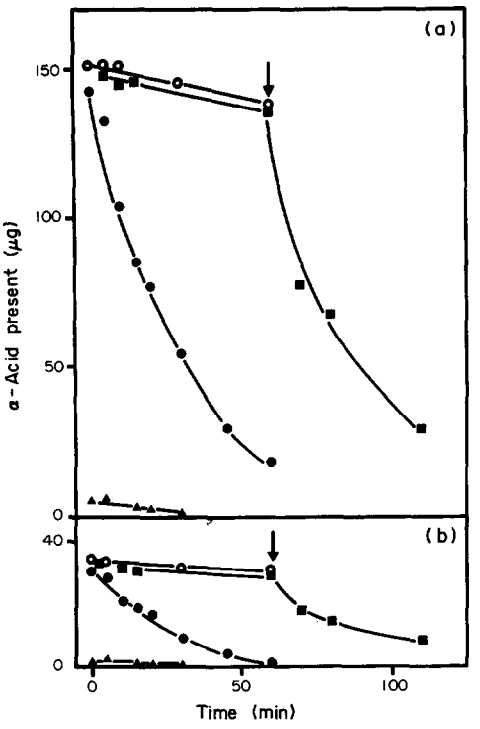


Fig. 2. Time-courses showing the effect of incubating (a) humulone and (b) cohumulone with: cells in 5 mM MES, pH 5.5, plus 54 mM sucrose (●); spent medium (■); fresh medium (○). The intra-cellular content is shown (▲). At the time indicated by the arrow, 0.5 g fr. wt cells was added to the spent medium.

compound 3-allylketoisobutylphloroglucinol, the rates of degradation of the α-acids were not inhibited. Both additives were, however, degraded at rates comparable to, or greater than, that of humulone (Table 3).

That the degradative activity is not specific to *H. lupulus* was shown by incubating α-acids with suspension culture cells of *Beta vulgaris*. Comparable rates of degradation were found in both species (Table 3).

From these results with whole cells it was apparent that the cultures possessed an ability to degrade α-acids at a considerable rate and that this required the presence of cells. The degradative apparatus was, however, also present in medium but required added hydrogen peroxide for activity if cells were absent. The total peroxide-dependent activity in the medium was comparable to the total in the cells. As it has previously been reported that cell suspension cultures readily secrete peroxidase [18] and the degradation of α-acids observed here required hydrogen peroxide we further investigated the degradation using a cell-free extract.

### *Degradation by a cell-free extract*

In order further to characterize the degradative activity of intact cells of *H. lupulus* towards α-acids, the total protein fraction obtained by Sephadex G-25 chromatography was used.

*pH-dependence.* In 5–50 mM MES, the buffer normally used, optimal activity was obtained at pH 6.0, as with intact cells. When acetate (50 mM) or succinate (50 mM) buffers were used, however, a two-fold enhancement of the degradation rate was found at pH 6.0, while the optima shifted to pH 5.0 or 5.5, respectively.

*Temperature-dependence.* The rate of degradation increased steadily from 10 to 50°, giving a linear Arrhenius plot from which an $E_s = 4.93 \pm 0.08 \times 10^4$ J/mol was obtained.

*Effect of hydrogen peroxide concentration.* Considerable substrate inhibition was found with hydrogen peroxide over the entire range at which activity could be measured (Fig. 4). The relationship between concentration and rate followed a first-order quadratic function. This effect of hydrogen peroxide supported the initial conclusion that the enzyme activity responsible for degradation is a peroxidase.

Table 3. Degradation of α-acids *in vivo* under various incubation conditions

| Incubation conditions | Rate of degradation (pkat/g fr. wt) | | |
|---|---|---|---|
| | Humulone | Cohumulone | Additive |
| Complete culture | 137 | 37 | — |
| Cells in fresh medium | 203 | 60 | — |
| Spent medium (per ml) | 0.3 | 0.09 | — |
| Spent medium + 17.6 μM Hydrogen peroxide (per ml) | 24 | 7 | — |
| Cells in buffer,* pH 5.5 | 235 | 54 | — |
| Cells in buffer,* pH 6.2 | 249 | 89 | — |
| Cells in buffer,* pH 7.0 | 179 | 54 | — |
| Dark-grown cells in buffer,* pH 5.5 | 129 | 33 | — |
| High light (2500 lux) grown cells in buffer,* pH 5.5 | 147 | 38 | — |
| Cells in buffer,* pH 5.5, + 19 μM 3-allylketoisobutylphloroglucinol | — | — | 878 |
| Cells in buffer,* pH 5.5, + 12 μM colupulone | — | — | 469 |
| *Beta vulgaris* cells in buffer,* pH 5.5 | 449 | 114 | — |

Incubations were conducted using 0.5 g fr. wt of cells with initial concentrations of 19.7 μM humulone and 4.5 μM cohumulone.

*Buffer, 5 mM MES containing 54 mM sucrose.

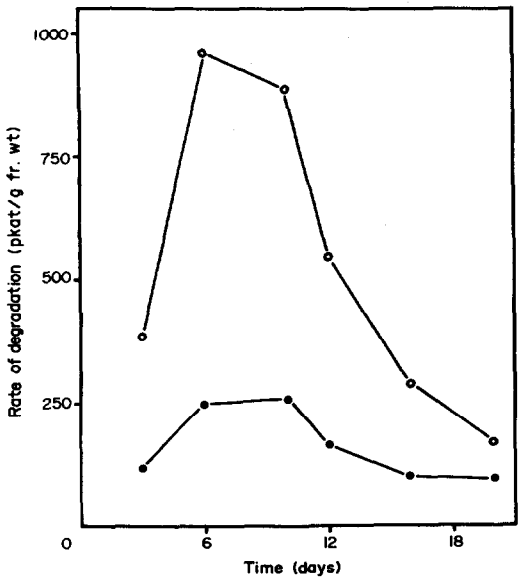


Fig. 3. Influence of the age from subculture on the humulone (○) and cohumulone (●) degrading activities of suspension culture cells of *Humulus lupulus*.

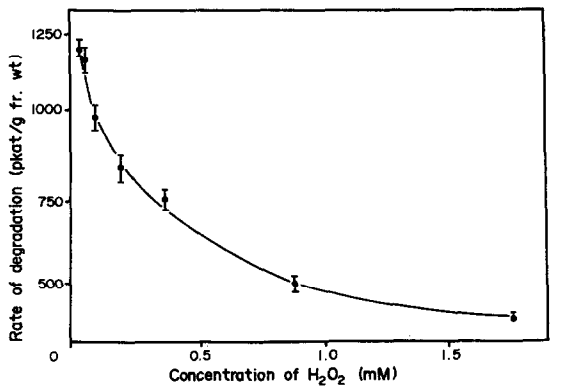


Fig. 4. Effect of varying the concentration of hydrogen peroxide on the rate of degradation of α-acids by a cell-free extract of *Humulus lupulus* suspension culture cells. Each point is the mean of at least triplicate determinations ± s.d. Best fit was calculated by non-linear regression using a first-order quadratic function. Rate at zero added hydrogen peroxide was 3.3 pkat/g fr. wt.

*Effect of α-acid concentration.* The insolubility of α-acids in aqueous solution at pH 6.0 made it possible to examine this effect only over the range 5.5–69 μM. The extremely high affinity of peroxidase for its primary substrate prevents the activity being described theoretically by Michaelis–Menten kinetics. Nevertheless, the affinity shown by the oxidized enzyme towards α-acids is fitted satisfactorily by a rectangular hyperbola giving a value for half-saturation of 12.2 μM and a maximal rate of 1130 pkat/g fr. wt.

In order to obtain a value of $k_4$ for the peroxidation of α-acids, the effect of enzyme concentration on both α-acid and guaiacol peroxidation was compared under optimal conditions for each substrate at a standard concentration of hydrogen peroxide. Using a $k_4$ for guaiacol of $3.33 \times 10^5/M \cdot sec$ [19] a $k_4 = 2.7 \times 10^4/M \cdot sec$ is obtained for α-acids. Thus, α-acids may be considered a moderately good acceptor, comparable in affinity to ascorbate, urate, aniline and dihydroxymaleate [20].

*Effect of inhibitors.* The rate of degradation was examined in the presence of a number of inhibitors for peroxidase (Table 4). A decrease in the rate was observed with inhibitors that competed for the hydrogen peroxide, those that bound to the haem of peroxidase and alternative reductants of the oxidized enzyme. The relationship between inhibitor concentration and α-acid concentration was examined for one inhibitor of each of the second and third types, potassium cyanide (Fig. 5) and ascorbate (Fig. 6), respectively. As expected, potassium cyanide, competing with the primary oxidant for the haem site, showed non-competitive inhibition with a concentration of 16.2 ± 1.1 μM giving 50% inhibition of activity.

Ascorbate, competing for the substrate binding site, showed characteristic uncompetitive inhibition of α-acid degradation, with 50% inhibition occurring at 9.6 ± 0.8 μM. α-Acids, however, were found not to inhibit ascorbate peroxidation. It appeared, therefore, that α-acids were not degraded equally well by all isoenzymes of peroxidase.

*Relationship of α-acid and guaiacol peroxidation.* Figure 7 shows the elution profiles for the peroxidation of α-acids and guaiacol from an Ultragel AcA-34 column. The peroxidation of guaiacol showed three peaks, none of which corresponded to the peak of α-acid peroxidation. The degradation of α-acids is found, however, across the entire range of elution of guaiacol peroxidation and appears to be a general property of peroxidase, not that of any particular isoenzyme.

*Identification of products.* During the progress of enzyme reaction, as the *A* maximum at 330 nm (due to humulone and cohumulone) decreased in intensity an *A* maximum appeared at 269 nm, characteristic of humulinone. When examined by HPLC, humulone and cohumulone were each converted to one major product which was more polar than the substrate and than the iso-α-acids produced by boiling in dilute alkali. From these properties we tentatively identified them as humulinone and cohumulinone, products known to result from the peroxidation of these substrates [1].

## CONCLUSION

Suspension cultures of *H. lupulus* are able to degrade rapidly α-acids in an enzyme-catalysed reaction involving their peroxidation to more polar products. This activity differs substantially from the oxidation reported for hop cones [7], which involves molecular oxygen, has a much lower affinity for the substrate ($K_m$ = 3.0 mM) and produces a product of intermediate polarity, corresponding to those obtained by auto-oxidation, the δ-acids.

The degradation of a desired product by plant cell cultures is also known for some alkaloids [21] and may present a serious problem to the biotechnological production of certain compounds. It may prove possible, however, to select cell lines deficient in the degradative activity which will be net accumulators of product.

## EXPERIMENTAL

*Plant material and cell cultures.* Leaf and stem explants from *H. lupulus* L. var. Northern Brewer were surface sterilized. Callus

Table 4. Effect of various inhibitors on the rate of α-acid degradation in a cell-free extract of *Humulus lupulus* suspension culture cells

| Action | Inhibitor | Concentration (μM) | % inhibition |
|---|---|---|---|
| Competition for hydrogen peroxide | Catalase, pH 5.5 | 0.001 | 17 |
| | Catalase, pH 7.0 | 0.001 | 24 |
| Competition with hydrogen peroxide for haem binding site | Potassium cyanide | 2760 | 81 |
| | Potassium cyanide | 27.6 | 16 |
| Competitive reductants of peroxidase II complex | *o*-Diaminobenzidine | 1000 | 100 |
| | 2,4-Dichlorophenol | 61 | 98 |
| | Phloroglucinol | 175 | 89 |
| | Guaiacol | 180 | 87 |
| | Guaiacol | 18 | 35 |
| | 2,6-Di-*tert*-butyl-4-methylphenol | 45 | 37 |
| | Ascorbate | 28 | 26 |

Initial α-acid concentration, 27.6 μM.

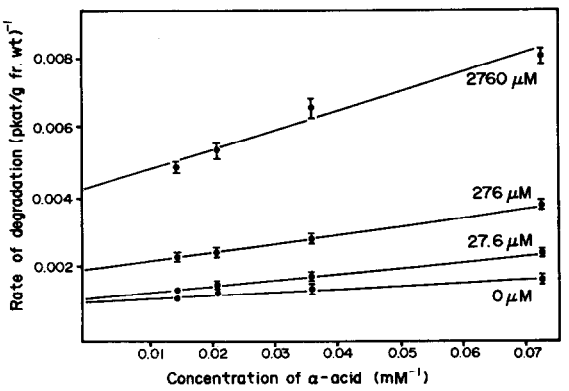


Fig. 5. Double-reciprocal plot showing the effect of increasing concentrations of potassium cyanide on the relationship between α-acid concentration and the rate of degradation. Each point is the mean of at least triplicate determinations ± s.d. Best-fit was calculated by linear regression.

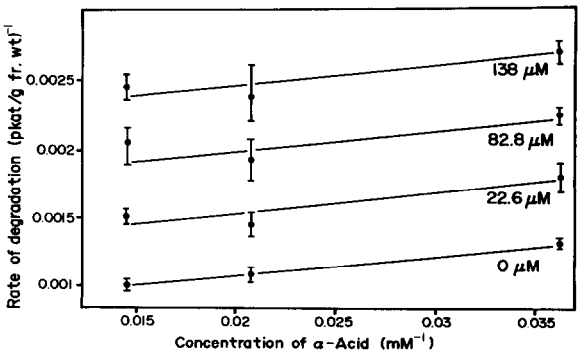


Fig. 6. Double-reciprocal plot showing the effect of increasing concentrations of ascorbate on the relationship between α-acid concentration and the rate of degradation. Each point is the mean of at least triplicate determinations ± s.d. Best-fit was calculated by linear regression.

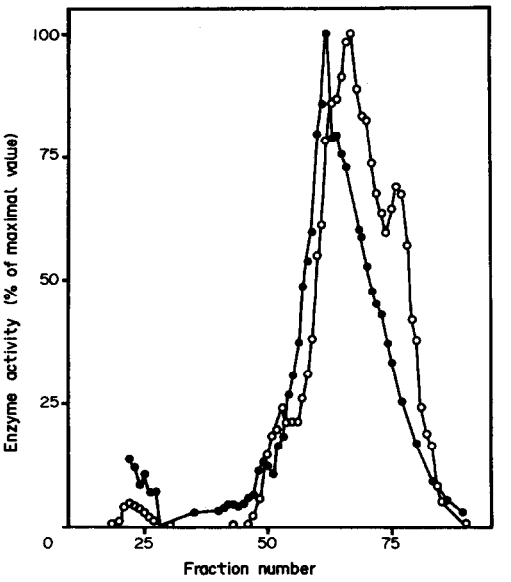


Fig. 7. Elution profiles for α-acid (●) and guaiacol (○) peroxidation from a column of Ultragel AcA-34 eluted as described in the text. Maximal activities were 164 and 9730 pkat/ml for α-acid and guaiacol peroxidations, respectively.

was initiated and maintained on agar (1%) containing Gamborg's B5 basal medium [9] supplemented with 54 mM sucrose, 1 mg/l. 2,4-D and 0.1 mg/l. kinetin. Suspension cultures were induced from well-established callus using the same medium lacking agar. They were maintained, with subculture (six-fold dilution) every 14 days, in 1 l. conical flasks containing 300 ml medium agitated on a rotary shaker (*ca* 100 strokes/min) at 26° with 600 lux illumination (Growlux red-enhanced) for 16 hr/day. Expts were conducted in 250 ml flasks containing 50 ml medium to which additions were made as filter sterile solns.

*Materials.* A pure preparation of hop α-acids was the kind gift of the Brewing Research Foundation, U.K. and contained (by HPLC [22]) 76% humulone, 22% cohumulone and 2% adhumulone. Colupulone and 3-allylketoisobutylphloroglucinol were generously given by Dr. P. V. R. Shannon, University

College, Cardiff, U.K. Gamborg's B5 [9] and Murashige and Skoog's [11] basal media were purchased from Flowlabs, Irvine, Scotland.

*Extraction of cell cultures for α-acids.* Cells were separated from medium by filtration (GF/A paper) under vacuum, lyophilized and ground in 2–3 vols $Et_2O$. Media were acidified and extracted with $Et_2O$. The $Et_2O$ fraction was taken to dryness and the residue resuspended in 200 μl MeOH. Insol material was removed by centrifugation and the supernatant analysed by HPLC [22].

*Preparation of enzyme extracts.* Cells, 6–8 days old, were disrupted (Ultra-turrax) in 3–5 vols ice-cold 50 mM HEPES, pH 7.6, containing 1 mM $EDTAK_2$, 0.5 mM DTE and 1% Polyclar AT fines. Following the removal of residue by filtration (Miracloth) and clarification of the filtrate by centrifugation ($K_{av}$ = 15000 *g*; 20 min) the extract was eluted from a column of Sephadex G-25 (fine) with 5 mM MES, pH 6, at 0.5 ml/min. Protein was determined from *A* at 280 nm and the relevant fractions pooled for the determination of enzyme activity. Further purification, on Ultragel AcA-34, was conducted using 5 mM MES, pH 5.5, containing 100 mM KCl and eluting at a flow rate of 10 ml/hr. Fractions of 2.0 ml were collected.

*Degradation of α-acids.* In vivo. Cells (0.5 g fr. wt) were resuspended in 20 ml 5 mM MES, pH 5.5, containing 54 mM sucrose and left to equilibrate at 25°. α-Acid (200 μl of a 1 mg/ml soln in MeOH) was added and the time-course of degradation followed by injecting samples directly onto the HPLC.

In vitro. Assays were performed at 30°. Typically, 0.5 ml of Sephadex G-25 eluent (diluted to give an *A* at 280 nm of 1.3), 0.39 ml 50 mM MES, pH 6, and 10 μl $H_2O_2$ (1.76 mM) were placed in both cuvettes. After 2 min, 0.1 ml buffer diluted 10% with MeOH was added to the reference cell and 0.1 ml 276 μM α-acid soln (1 mg/ml stock in MeOH diluted 10-fold in buffer) mixed into the substrate cell. The initial rate of reaction was determined by *A* at 310 nm and was pseudo-linear for several min.

*Other enzymes.* Peroxidase was determined with guaiacol [23] and ascorbate peroxidation by loss in *A* at 255 nm.

*HPLC anion analysis.* Media samples (10 μl) were injected, without pretreatment, onto a Vydac 3021C column eluted isocratically at room temp. with 2 mM KHphthalate, pH 4.91, at 3 ml/min, with *A* at 255 nm monitored.

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