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PURIFICATION AND PROPERTIES OF ALCOHOL OXIDASE FROM *TANACETUM VULGARE*

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Key Word Index—*Tanacetum vulgare*; Compositae; alcohol oxidase: enzyme purification; substrate specificity.

Abstract—Alcohol oxidase (alcohol : O_2 oxidoreductase) from leaves of *Tanacetum vulgare* has been purified 5150-fold to homogeneity on disc electrophoresis and gel electrofocussing. The enzyme which is probably flavoprotein, has molecular weight 180 000 daltons and is comprised of two sub-units of 94 000 and 75 000 daltons. It is active over a broad range (pH 5–9) and best accepts primary aliphatic alcohols with 6 to 10 carbons, especially those with a 2-ene group. K_m values for hex-*trans*-2-ene-1-ol, geraniol (3,7-dimethylocta-*trans*-2,6-dien-1-ol) and *n*-octanol were 0.19, 1.56 and 0.49 mM respectively. The significance of the enzyme in the formation of leaf aldehyde (hex-*trans*-2-ene-1-al) and in terpene metabolism is discussed.

INTRODUCTION

Unspecific alcohol oxidases (alcohol : O_2 oxidoreductase; EC 1.1.3.13) that utilise certain saturated and allylic alcohols as substrates occur in fungi [1–3] and bacteria [4, 5] but the only similar enzyme reported in higher plants is an indole-alkanol oxidase from cucumbers [6]. NAD or NADP-dependent dehydrogenases (alcohol : NAD/NADP oxidoreductases; E.C. 1.1.1.1 and 1.1.1.2) that oxidise alk-3-en-1-ols and allylic alcohols do, however, occur in several higher plants [7–9] and bacteria [10], and aliphatic alcohol dehydrogenases with broad substrate specificity have also been reported from several higher plants [10a and references therein]. We now report an alcohol oxidase from foliage of *Tanacetum vulgare* L. (fam. compositae).

RESULTS AND DISCUSSION

Occurrence. Oxidase activity with geraniol (3,7-dimethylocta-*trans*-2,6-dien-1-ol) as substrate was initially discovered in acetone powders prepared for the study of terpene biosynthesis. The activity varied widely over the growing season of *T. vulgare* (Fig. 1) and was essentially non-existent during the winter and low in midsummer at the period of maximum terpene biosynthesis [11]. Concurrent assays of geraniol and H_2O_2 showed that an oxidase (rather than a dehydrogenase) was entirely responsible for the oxidation. Foliage of *Rosa dilecta* cv Lady Seton (assayed in July and September), orange endocarp, cultures of *Saccharomyces cerevisiae* or tomato fruit did not contain detectable quantities of enzyme that oxidised geraniol, but low levels (*ca* 10% of those in *T. vulgare*) were found in orange flavedo and in *Pinus radiata* needles: it was not checked whether the latter were oxidases or dehydrogenases. The level of oxidase in *T. vulgare* did not increase when specimens were kept in simulated daylight at 4° or 10°.

Purification. The purification of the alcohol oxidase is summarised in Table 1 using acetone powders prepared in September. The product, containing 0.1% of the initial protein, represented a purification factor of 5150. The increase in enzyme units recovered during the procedure suggests that inhibitors were initially present: these may have been polyphenols [12] which our work-up was designed [cf. 13] to eliminate. The product was homogeneous to disc electrophoresis giving a single superimposable band of protein and oxidase activity (R_f 0.72, 0.42) when gels containing 7.5 and 5% acrylamide were employed. There was again a superimposable band of protein and oxidase activity at pI 2.85 ± 0.1 on gel electrofocussing: this contained 90 ± 5% of the protein and enzyme activity and was accompanied by a band

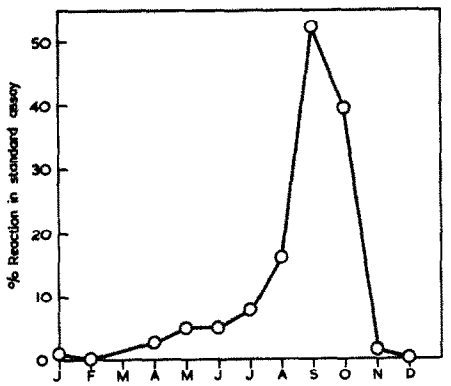

Fig. 1. Seasonal variation of alcohol oxidase in acetone powders from *T. vulgare*. Percentage reaction is yield of aldehyde from geraniol-[^{14}C]. Experiments commenced Jan. 1974. J to D represents Jan.–Dec.

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Table 1. Purification of alcohol oxidase from *T. vulgare*

Step.	Vol l.	Activ.*[a]	Protein (mg)	Spec. act.†	Yield	Purification
Acetone powder	1.6	3.5	6800	5.1	100	1
Ppt. ex protamine	1.7	6.3	6000	10	88	2
Ppt. ex $(NH_4)_2$ SO_4	0.140	25	532	470	7.8	94
DEAE-Cellulose	0.275	22	27	8150	0.4	1600
Sephadex G-200	0.018	16	6	26270	0.09	5150

* 10^7 kat. with hex-2-*trans*-ene-1-ol in standard assay. † 10^4 activity per mg protein.

at pI 5.05 ± 0.1 comprising 5 ± 2% of protein and enzyme activity. Oxidase activities towards geraniol, *n*-octanol and hex-*trans*-2-ene-1-ol were coincident throughout the column purification steps and each protein band in the above electrophoretic separations stained for all three alcohols as substrates. It seems unlikely that the two well-defined bands on electrofocussing could include an artefact due to complexing of the enzyme with residual phenols [13] and two isoenzymes may exist. Isoenzymes of alcohol dehydrogenases in several plants have been reported [10a].

Properties. Gel filtration on a calibrated column indicated the purified protein to have MW 180000 ± 5000 daltons; no other species was present. Electrophoresis in sodium dodecyl sulphate on polyacrylamide revealed the existence of two sub-units 77500 ± 3000 and 95000 ± 3000 daltons.

The pure oxidase was strongly fluorescent and the intensity emitted from the holoenzyme was *ca.* 62% of that from the prosthetic group (Table 2). This, and the ease of dissociation of the latter by brief heating or treatment with guanidine suggests the prosthetic group was non-covalently bonded. Furthermore the emission and excitation maxima suggest this group to be a flavin [14] although sufficient material was not available to carry out further identification.

Enzyme activity was unaffected by addition of NAD^+, $NADP^+$, FMN or FAD (20 mM) either before or after extended dialysis against buffer at pH 6.0 or 8.5, and was not restored by addition of any of these coenzymes to the apoenzyme obtained after heat or guanidine treatment. It has frequently been observed that the holoenzyme of a flavoprotein may not be readily reconstituted [14].

Potential inhibitors (10 mM) were screened for their effect on oxidation of hex-*trans*-2-ene-1-ol (20 mM) at pH 7.0 and 20°. The values (arbitrary units ± 5) of resulting enzyme activities were: control 100, p-hydroxymercuribenzoate 103; iodoacetate 101; CN^- 93; $MnSO_4$ 100; EDTA 98; α,α′-dipyridyl 95; 1:10 phenanthroline 93; 8-hydroxyquinoline 100. F^- 92; N_3^- 98. Enzyme activity was also unaffected by prolonged dialysis against EDTA. Hence there is no evidence for the involvement of metals. The pure enzyme showed maximum activity at 18 to 22° at pH 6·5 with hex-*trans*-2-ene-1-ol as substrate: the activity dropped to 60% of the maximum at 0°, and to 30% at 45°. A broad plateau of activity occurred at pH 5.5 to 9.0 (falling to half maximal activity at pH 4.8 and 10.0) for the same substrate at concentrations (20 mM = 100 K_m) sufficient to ensure saturation of enzyme.

Table 2. Fluorescent spectra of alcohol oxidase ex *T. vulgare*

	^{I}540(440)*	^{R}540(375,425)†
Holoenzyme	0.74	1.32
Prosthetic group	1.17	1.12

* Intensity of Emission at 540 nm (excitation 440 nm), arbitrary units. † Ratio of emission at 540 nm (excitation at 375 and at 425 nm).

Stoichiometry. The reaction products after incubation with pure enzyme to ensure complete disappearance of geraniol, hex-*trans*-2-ene-1-ol or *n*-octanol as substrate were shown by TLC and GLC to be solely the corresponding aldehydes. At 50% reaction the disappearance of hex-*trans*-2-ene-1-ol (12 μmol) was shown to be accompanied by the formation of the aldehyde (11.5 μmol) and H_2O_2 (11.6 μmol) and the uptake of oxygen (12.3 μmol). Similar results were obtained for geraniol and *n*-octanol.

Specificity. The extents of oxidation of various alcohols by the pure enzyme in a standard assay system (see Experimental) are shown in Table 3. An excellent discussion of possible artefacts arising in the determination of substrate specificity has been given [10a]. We found that Tris (a trihydric alcohol) was not a substrate for the oxidase and that substrates that were sparingly soluble in the solutions used for assay could be introduced in acetone (5% v/v of the total solution) without imparing the enzyme activity. All substrates used were glc and tlc pure (> 99·5% on 3 systems) and so low measured enzyme activities are unlikely to be due to unsuspected reactive contaminants in the alcohols that were screened. The following conclusions may be drawn: (a) The optimum chain lengths for unbranched alkanols are C8 to C10; (b) The introduction of a 2-ene group causes a large increase in the rate of oxidation (cf. entries 3 and 4; 5 and 7; 10 and 11) whereas a 3-ene group has little effect (cf. 5 and 6; 10 and 12); (c) a methyl at C_3 reduces the rate of oxidation (cf 7 and 8); (d) geraniol (*trans*-2-ene, 16) is a more effective substrate than its *cis*-isomer, 17); (e) Diols (15) and various terpenols (22 to 25) are poor substrates or are unreactive, and secondary and tertiary alkanols (C3 to C5; not shown in Table) and aromatic alcohols (benzyl alcohol; 1- and 2-phenylethanol) are completely unreactive.

The pure enzyme exhibited Michaelis–Menten kinetics and the parameters were evaluated for hex-*trans*-2-ene-1-ol (at pH 6.5) and geraniol and *n*-octanol (at pH 8.0) over a 10 to 20-fold range of substrate concentration: viz. K_m 0.19, 1.56 and 0.49 mM; v_{max} 8.9, 6.4, 3.2 μmol mg^{-1} min^{-1}. Hex-*trans*-2-ene-1-ol was clearly the best substrate of those screened.

Discussion. It seems probable that the oxidase is responsible for the formation of leaf aldehyde (hex-*trans*-2-ene-1-al) which together with leaf alcohol (hex-*cis*-3-ene-1-ol; Table 3, No. 12) is widely distributed in foliage

Table 3. Specificity of alcohol oxidase from *T. vulgare*

Substrate	Rel. activ.*	Substrate	Rel. activ.*
1) MeOH	0	14) *n*-Octanol	40
2) EtOH	0	15) 1,8-Octandiol	6
3) *n*-PrOH	0	16) Geraniol	24
4) CH_2=$CHCH_2$ OH	7	17) Nerol	5
5) *n*-BuOH	1	18) Citronellol	1
6) But-3-ene-1-ol	1	19) *n*-Nonanol	45
7) But-2-ene-1-ol	20	20) *n*-Decanol	74
8) But-3-ene-3-methyl-1-ol	1	21) *n*-Dodecanol	19
9) *n*-Pentanol	2	22) 2-*trans*-6-*trans*-Farnesol	10
10) *n*-Hexanol	15	23) Menthol	0
11) Hex-*trans*-2-ene-1-ol	100	24) Artemisia alcohol	0
12) Hex-*cis*-3-ene-1-ol	22	25) Phytol	0
13) *n*-Heptanol	41	26) Geranylgeraniol	0
		27) Glycollic acid	0

* Relative values for production of H_2O_2 using the incubation conditions detailed in the Experimental.

[15, 16 and refs. therein] and the levels of which are increased on injury [15, 17, 18] or ageing [18]. We have, in fact, detected (GLC–MS) low levels (6 mg kg^{-1}) of the aldehyde in autumnal and necrotic (frost-damaged) foliage of *T. vulgare*. The compound could have a protective role as it is thought to endow fungal resistance on some plant species [19]. We intend to screen other species for a similar oxidase, for although there is some circumstantial evidence that an oxidase (rather than a dehydrogenase) is involved in formation of leaf aldehyde in several species [20] a recent investigation shows that it, together with hex-3-*cis*-ene-1-al, is formed from hex-*cis*-3-ene-1-ol in *Thea sinensis* by the action of a NAD-dependent dehydrogenase [21].

We have not been able to demonstrate a geraniol dehydrogenase in *T. vulgare*, and this, coupled with the existence of the oxidase capable of accepting geraniol as substrate has significance for terpene biosynthesis. One route for conversion of geraniol into nerol (its *cis*-2-ene isomer) *in vivo* is believed to be by means of a redox system involving the corresponding aldehydes, and a geraniol dehydrogenase presumably implicated has been found in cell-free extracts from rose [22] and orange [23]*: there is also evidence for the corresponding farnesol dehydrogenase in orange extracts [24] and *in vivo* in the fungi [25]. However, in *T. vulgare* we have previously shown [11] that nerol is not formed from geraniol (or *vicé-versâ*) but rather two separate routes to the two monoterpenols from the hemiterpenoid precursors may be inferred. This is as expected if the oxidase provides the only pathway of oxidation of geraniol in *T. vulgare*, for unlike a dehydrogenase this enzyme would be unlikely to be involved in the reduction step under physiological conditions.

EXPERIMENTAL

Materials. Foliage was collected in mid-September from plants grown outdoors in central London from seed provided by the Royal Botanic Gardens, Kew, England, and acetone powders were immediately prepared [26]. *P. radiata* was similarly grown and *S. cerevisiae* was cultured and harvested using standard procedures [27]. All reagents used were analytical grade or the equivalent and all operations described below were carried out at 4° unless otherwise stated. Geraniol-[2-^{14}C] (4.7 mCi $mmol^{-1}$) was prepared from ethyl bromoacetate-[^{14}C] and was purified by TLC [28, 29]. Geraniol-[1-$^3H_1$] (22 mCi $mmol^{-1}$) was obtained by reduction of geraniol with $NaBH_4$-[$^3H_4$] [cf. 30]. Buffers used for construction of pH-activity profiles were citrate-P, (pH 2.7 to 6.5); P, (pH 5.5–9.0); Tris–HCl (pH 7.0–9.0) and borate (pH 8.0–10.5).

Enzyme assay. These were made at 20° by measurement of production of H_2O_2. The enzyme soln (4.0 ml) was added to horse radish peroxidase (100 μg), *o*-dianisidine (200 μg) and the substrate (6.5 μmol) in Tris–HCl (pH 8.0; 1 ml; 0.2 M): insoluble substrates were introduced in Me_2CO (5% w/v). After incubation (10 min) the reaction was stopped by addition of HCl (4N; 0.2 ml) and the colour developed was measured at 400 nm. The stoichiometry of oxygen uptake was measured using a glass electrode or by micromanometry [31]: aldehyde formation was monitored by GLC. Protein determinations were by the modified Lowry procedure [32]. In early experiments geraniol-[^{14}C] or [^{3}H] was substrate: the reaction products were separated by TLC on Si gel G, eluted with C_6H_6–EtOAc (9 : 1; geraniol, geranial R_fs: 0.26, 0.47) and scanned for tracer by autoradiochromatography.

Enzyme purification. (A) Acetone powder (230 g) was stirred into MES (0.1 M; pH 7.0, 21) containing 2-thioethanol (1 mM), KCN (15 mM), sodium metabisulphite (10 mM) and Dowex 1-X8 resin (5% w/w; previously equilibrated with the buffer). The mixture was allowed to stand (0°/30 min; occasional stirring), filtered through gauze and centrifuged (15 min; 3500 *g*). (B) The supernatent (1.8 l) was treated with protamine sulphate (107 ml; 2% w/w; in buffer as above but omitting resin), stirred (10 min) and centrifuged (15 min; 3500 *g*). (C) The supernatent (1·7 e.) was taken to 50% saturation with $(NH_4)_2$ SO_4, stirred (25 min), and centrifuged (15 min; 3500 *g*). The resulting supernatent was taken to 90% saturation in the same way and worked up to give a pellet which was dissolved in Tris–HCl (10 mM; pH 8.0 containing 2-thioethanol 1 mM) and dialysed overnight against the same buffer. (D) The dialysate (120 ml) was centrifuged (20 min; 6000 *g*) and the supernatent was diluted with buffer (150 ml) and passed through DEAE-cellulose (50 × 5 cm; flow rate 1.6 ml min^{-1}) that had been equilibrated with the Tris–HCl. Elution was successively made with Tris–HCl (pH 8.0, containing 2-thioethanol 1 mM) at successively 50 mM, 100 mM, 100 mM + NaCl (100 mM) and 100 mM + NaCl (200 mM) with volumes 0.5, 1.6, 0.5 and 1.6 l respectively. Oxidase activity eluted in the last buffer and the active fractions were pooled and concentrated by forced dialysis vs Tris–HCl (10 mM; pH 8.0 containing 2-thioethanol, 1 mM). (E) This concentrate (5 ml) was passed through Sephadex G-200 (55 × 2.5 cm; flow rate 4 ml hr^{-1}) that had been equilibrated with the buffer

* A geraniol dehydrogenase has also been reported in potato tubers [10a] but it seems unlikely that conversion of geraniol to nerol is physiologically necessary in this tissue.

used in dialysis to give the oxidase with an elution volume of 125–135 ml. This fraction was lyophilised, dissolved in water (2.5 ml) and stored at −30°.

Electrophoresis methods. Disc electrophoresis was carried out at 5.1 mA per tube/15 min [33] on polyacrylamide gels (5 or 7.5% w/w) in *Tris*-HCl at pH 8.1. Proteins were stained with Naphthalene black 1B (1% in 7% aq. AcOH) and oxidase activity was detected with a peroxidase-carbazole reagent [34] using either geraniol, *n*-octanol or hex-*trans*-2-ene-1-ol as substrate. Gel electrofocussing on carrier ampholytes at pH 3–10 followed standard procedures [35].

MW determinations. These were made [35] on a column of Sephadex G-200 (2.5 × 65 cm) with catalase (elution vol 132 ml; MW 232000); beef heart lactic dehydrogenase (148 ml; 140000) and horse radish peroxidase (177 ml; 43000) as markers in *tris*–HCl (pH 8.0; 0.01 M + 1 mM 2-thioethanol). Sodium dodecyl sulphate-gel electrophoresis followed standard recipes [36] with old yellow enzyme (partially dissociated in preincubation; R_f 0.19, 0.36; MW 52000, 104000 [37]); bovine serum albumin (Rf 0.27; MW 68000 [38]); and ovalbumin (R_f 0.38 MW 43000 [39]) as standards.

Miscellaneous. Fluorescence measurements on protein solutions (1.2 mg ml^{-1}) in Pi buffer (0.1M; pH 7.7) at 25° were made on a ratio-recording spectrofluorimeter incorporating a rhodium B quantum counter constructed by Dr. D. Ballou and Mr. G. Ford in Dept. Biol. Chem., Ann Arbor, Michigan. In order to release the prosthetic group the soln was boiled (3 min) and ultrafiltered (all procedures in dark). ^{14}C-Assays were as previously described [40]. pH activity profiles were obtained by the standard assay in Pi buffer (0.1 M; pH 6.5; 3.6 ml) aftes the enzyme (5 μg) had been preincubated in the appropriate buffer (0.2 ml; 0.1 M) at 20°/2 hr. Stability and inhibition studies were performed similarly. The seasonal variation of oxidase activity was determined using specimens of *T. vulgare* cultivated outdoors April to November. During the winter, greenhouse plants (20°; natural illumination) were employed. Percentage conversions of substrate were recorded after incubation (20°/2 hr) in the standard assay system.

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