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AN INDUCIBLE HYDROLASE FROM *MORTIERELLA ISABELLINA* (BASIDIOMYCETES). THE DEACYLATION OF (+)-USNIC ACID

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Key Word Index—*Mortierella isabellina*; Basidiomycetes; inducible acylhydrolase; (+)-usnic acid; (+)-2-desacetylusnic acid; acetic acid; divalent cations.

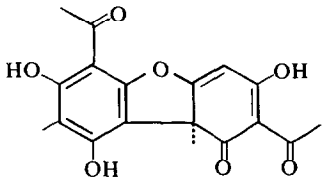
Abstract—An inducible enzyme catalysing the hydrolysis of (+)-usnic acid to (+)-2-desacetylusnic acid and acetic acid has been purified 150-fold from the mycelium of *Mortierella isabellina* grown in the presence of (+)-usnic acid. Purification was achieved by treatment with protamine sulfate, $(NH_4)_2SO_4$ fractionation, negative adsorption on alumina Cγ gel and hydroxylapatite followed by chromatography on DEAE-cellulose and Sephadex G-200. The elution pattern from a Sephadex G-200 column indicated a MW of *ca* 7.6×10^4 for the enzyme. The apparent K_m value for (+)-usnic acid at the pH optimum (pH 7) was 4.0×10^{-5} M. The enzyme was specific for (+)-usnic acid and inactive towards (−)-usnic acid, (+)-isousnic acid or certain phloracetophenone derivatives. Its activity was enhanced in the presence of divalent metal ions such as Co^{2+}, Ni^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+}.

INTRODUCTION

The usnic acids are among the most characteristic and abundant lichen secondary compounds [1, 2]. In addition to (+) and (−)-usnic acids **1**, two other isomers, (+) and (−)-isousnic acids also occur in certain lichens [3]. For a number of years we have been studying the biodegradation of these lichen metabolites and, following a screening of soil samples for microorganisms capable of degrading (+)-usnic acid [4], we selected *Mortierella isabellina* Oudemans & Koning for further study. Among the degradation compounds produced from (+)-usnic acid by this basidiomycete are (+)-2-desacetylusnic acid **2**, (+)-2-acetoxyusnic acid **3** and (+)-1α-hydroxy-2-desacetylusnic acid **4** whose chemical structures have been characterized recently [5]. Bacterial and fungal hydrolytic enzymes acting on a carbon–carbon bond adjacent to a ketone function are reasonably well-known and the biotransformation of (+)-usnic acid to (+)-2-desacetylusnic acid by *M. isabellina* indicated that this fungus was capable of elaborating a similar type of enzyme.

Examples of this class of enzymes are oxaloacetase from *Aspergillus niger* which catalyses the hydrolysis of oxaloacetate to oxalate and acetate [6], L-kynurenine hydrolase which catalyses the formation of anthranilate and L-alanine from L-kynurenine [7] and the acylpyruvase of *Pseudomonas* which converts acetyl pyruvate to acetate and pyruvate [8]. The well established pathways for the degradation of homogentisate and gentisate in animals and microorganisms also involve enzymes of this type e.g., fumarylacetoacetase, catalysing the hydrolysis of 4-fumarylacetoacetate to acetoacetate and fumarate [9, 10].

We report here the partial purification and characterization of an inducible enzyme which catalyses the follow-



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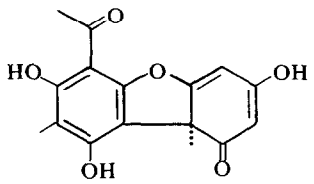

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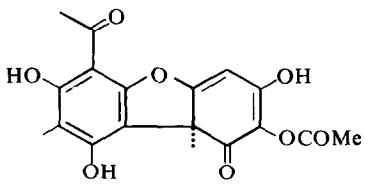

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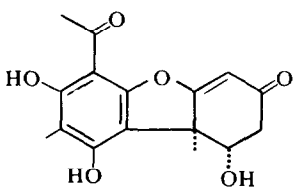

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ing reaction: (+) Usnic acid + H_2O ⇌ (+)-2-Desacetylusnic acid + acetate.

RESULTS AND DISCUSSION

Purification of enzyme

By employing a combination of conventional purification procedures the enzyme, (+)-usnic acid 2-acetylhydrolase, was obtained with a 150-fold increase in sp. act. and with a recovery of 21% (Table 1).

When the EtOAc extract of the reaction products from assays with crude enzyme were subjected to TLC and autoradiography, a radioactive spot corresponding to compound **4** always appeared on the autoradiogram. This pattern persisted until the enzyme preparation had been passed through alumina Cγ gel. The hydrolase was eluted from the gel with 0.1 M Pi buffer, whereas, the enzyme with reducing activity, i.e. catalyzing the conversion of compound **2** to **4**, was retained on the gel. Chromatography of the enzyme on DEAE-cellulose with a linear gradient yielded the enzyme in fractions with 0.07 to 0.13 M NaCl in 0.05 M Pi buffer, suggesting that it is a comparatively acidic protein. The active enzyme from a Sephadex G-200 column was resolved into at least 4 protein components by isoelectric focussing over the pH range of 3.5 to 10; the active enzyme was focussed at pH 4.7 As the recovery after this step was only 37%, it was omitted in further studies. Dilute solutions of the enzyme in Na–Pi buffer were stable for at least a month when stored at −80°.

Identification of the product

The product was identified by incubating 50 μmol (+)-usnic acid with an enzyme preparation for 2 hr at 30°. The EtOAc extract of the reaction mixture was purified by TLC and crystallization, and the compound identified by comparing its mp, IR and MS with authentic desacetylusnic acid.

Characteristics of the enzyme

When the organism was grown in a medium without (+)-usnic acid, no hydrolytic activity was detected in the enzyme preparation. Calibration with MW markers on a Sephadex G-200 column gave a MW of 7.6×10^4 for the hydrolase. The apparent K_m for (+)-usnic acid at the optimum pH (pH 7) was 4.0×10^{-5} M.

There was stoichiometry between disappearance of the substrate and formation of desacetylusnic acid. For example, incubation of 0.4 μmol (+)-usnic acid with the enzyme resulted in consumption of 0.132 μmol of the substrate and formation of 0.135 μmol of product. In another experiment, the disappearance of 70.2 mμmol (+)-usnic acid corresponded to the formation of 68.8 mμmol desacetylusnic acid. In a separate experiment, 47.6% of the theoretical amount of HOAc was detected as a product in the ethereal extract of the reaction mixture by GLC. From the above results, the mode of enzyme reaction was deduced to be a cleavage of the C_2–COMe bond of (+)-usnic acid **1** to provide (+)-2-desacetylusnic acid **2**.

Table 1. Purification of (+)-usnic acid 2-acetylhydrolase from *M. isabellina*

	Volume (ml)	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)	Recovery (%)
Crude extract	228	358	72 8	0 203	100
Protamine sulfate	243	349	73 3	0 210	101
$(NH_4)_2SO_4$ precipitation	25	44 8	48 6	1 08	66 8
Alumina Cγ gel	36	17 2	38 1	2 22	52 3
DEAE-cellulose	35	3 17	22 2	7 00	30 5
Hydroxylapatite	4 5	1 07	18 3	17 1	25 2
Sephadex G-200	12 5	0 48	15 2	31 7	20 9

Table 2 shows that Ag^+ and Cu^{2+}, especially Ag^+ at low concn strongly inhibited the reaction. Other ions such as Cd^{2+}, Ca^{2+}, Fe^{2+}, Al^{3+}, Mo^{7+}, W^{6+} also inhibited the reaction to some extent. Certain divalent cations such as Co^{2+}, Ni^{2+}, Mn^{2+} and Zn^{2+} enhanced the activity considerably. The increase in activity was lost when EDTA was added to the enzyme solution prior to the addition of Co^{2+}. The acetyl pyruvate hydrolase from *Pseudomonas putida* is similarly stimulated by several divalent cations including Co^{2+} [8] and Davey and Ribbons have suggested that complex formation between divalent ions and the enol form of the substrate may be involved [8]. The same argument may be used for this hydrolase.

As shown in Table 3, metal chelating agents such as EDTA, *o*-phenanthroline and α,α′-dipyridyl as well as BAL (2,3-dimercaptopropan-1-ol) were inhibitory. Fumaryl acetoacetate fumarylhydrolase from beef liver, is reported to be completely inactivated by sulfhydryl-specific inhibitors [12], but neither thiolalkylating nor mercaptide-forming agents inhibited the (+)-usnic hydrolase reaction suggesting that the active site of the enzyme does not involve SH-groups.

Table 2. Effect of metal ions on the rate of (+)-usnic acid hydrolysis

	Final concentration	Relative activity
No addition	—	100%
$HgCl_2$	0.02 mM	99 1
$AgNO_3$	0.02	1.32
$AgNO_3$	0.005	4.70
$CdSO_4$	1.0	81.9
$CoSO_4$	1.0	179
$CoSO_4$	0.5	183
$CoSO_4$	0.2	145
EDTA (1.0 mM) + $CoSO_4$	0.5	77.3
EDTA (1.0 mM) + $CoSO_4$	0.2	74.0
$CuCl_2$	1.0	45.4
$CaCl_2$	1.0	86.2
$ZnCl_2$	1.0	112
$NiSO_4$	1.0	144
$MgCl_2$	1.0	119
$MnCl_2$	1.0	123
$FeSO_4$	1 0	99.9
$FeCl_3$	1.0	85 6
$Al_2(SO_4)_3$	1 0	79.9
$NaMoO_4$	1.0	89.5
Na_2WO_4	1.0	71.8

The reaction mixture consisted of 0.1 ml of enzyme soln, 0.8 ml 0.1 M sodium phosphate buffer, pH 7, and 0.1 ml metal ions in a total vol. of 1 ml. After 5 min preincubation, the reaction was initiated by adding 0.2 μmol of (+)-usnic acid-[6-$CO^{14}CH_3$] in 0.1 ml ethyleneglycol monomethylether, and proceeded for 1 hr. For EDTA inhibition EDTA 0.1 ml was added prior to the addition of metal ion. Relative activity was expressed in per cent against the sample with no ion added

Table 3. Effect of enzyme inhibitors on the rate of (+)-usnic acid hydrolase

	Final concentration	Relative activity
No addition	—	100%
p-Chloromercuribenzoate	0.05 mM	97.7
p-Chloromercuribenzoate	0.1	95.5
$NaAsO_2$	1.0	95.0
Iodoacetate	0.5	94.3
GSH	1.0	98.0
L-Cysteine	0.5	93.6
BAL	0.5	54.3
Diethyldithiocarbamate	0.5	106.1
α,α′-Dipyridyl	0.5	83.0
o-Phenanthroline	0.5	79.1
EDTA	1.0	79.9
Thiourea	0.5	94.9
Phenylmethylsufonylfluoride	0.5	99.5
p-Chloromercuribenzene-sulphonic acid	0.01	106.1
2-Mercaptopyridine	0.5	83.2
Phenazinemethosulfate	0.5	80.0

The reaction mixture consisted of 0.1 ml of enzyme soln in 0.8 ml 0.1 M Na phosphate buffer, pH 7, and 0.1 ml test compound in a total vol. of 1 ml. The mixture was preincubated for 5 min before the addition of the substrate.

Substrate specificity

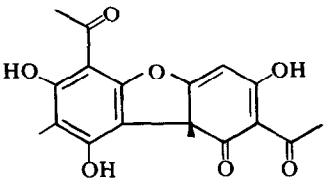
(+)-Usnic acid was the only compound tested which served as a substrate for the enzyme. Potential substrates that were not hydrolysed included (−)-usnic acid **5**, (+)-isousnic acid **6**, 3-methylphloracetophenone **7** (R = Me), phloracetophenone **7** (R = H), 2,4-dihydroxyacetophenone **8** (R^1 = H, R = OH), phloropropiophenone **9** and phloretin **10**. The last 6 compounds are readily attacked by an inducible hydrolase found in many fungi [13].

It would be of interest to know whether a similar enzyme is elaborated for (−)-usnic acid which is also a fairly common lichen constituent.

EXPERIMENTAL

Organism and culture conditions. Stock cultures of *M. isabellina* (UBC #129) were maintained on modified malt agar slants (7 g malt extract, 1 g soytone, 0.5 g yeast extract, 15 g agar and H_2O to 1 l. The culture medium contained 5 g D-glucose, 2 g asparagine, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.2 ml vitamin stock soln, 0.2 ml mineral stock soln and H_2O to 1 l. Vitamin stock soln contained 2 μg biotin, 0.5 mg thiamine, 0.5 mg pyridoxal, 25 μg inositol and 40% EtOH to 100 ml. Mineral stock soln contained 98 mg $FeCl_3.6H_2O$, 78.5 mg $CuSO_4.5H_2O$, 40.5 mg $MnSO_4.4H_2O$, 88 mg $ZnSO_4.7H_2O$ and H_2O to 250 ml. Finely powdered (+)-usnic acid was added to a final conc of 0.1 %(w/v). Conical flasks (4 l.), each containing 1.5 l. of the medium were inoculated with a suspension of spores and the cultures kept on a rotary shaker (100–120 rpm) at room temp.

Enzyme purification. The mycelium from 8 flasks (190 g wet wt) was frozen in liquid N_2 and ground to a fine powder in a mortar. The powder was suspended in 300 ml 0.1 Na–Pi buffer, pH 7, containing 1 mM EDTA, 0.5 mM GSH (Buffer A) and 18 g PVP. The suspension was stirred occasionally for 30 min, squeezed through cheese cloth and the filtrate centrifuged at 10000 *g* for 15 min. To the supernatant (225 ml) 25 ml 2% protamine sulfate in 0.1 M Pi buffer, pH 7, was added. After stirring for 20 min, the mixture was centrifuged for 15 min at 20000 *g*. The protein in the supernatant was pptd between 35–55% $(NH_4)_2SO_4$ satn and redissolved in 12 ml of 0.05 M Pi buffer, pH 7, containing 0.5 mM EDTA and 0.25 mM GSH



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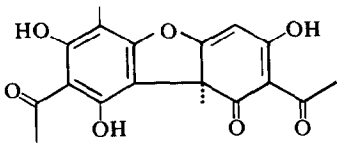

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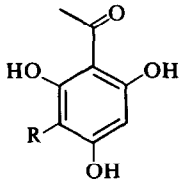

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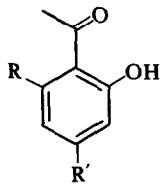

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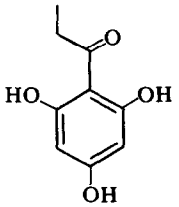

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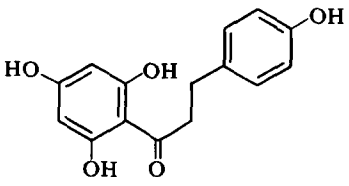

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(Buffer B). This soln was desalted on a Sephadex G-25 column. The protein fraction was collected (22 ml) and stirred with alumina C_γ gel (4 g, solid content 5%) for 15 min. The suspension was centrifuged at 10000 *g* for 10 min, the supernatant decanted and the gel washed with Buffer A (8 ml) and centrifuged again. The two supernatants were combined to give 30 ml of soln which was applied to a DEAE-cellulose column equilibrated with 0.05 M Pi buffer, pH 7, containing 0.25 mM GSH (Buffer C). The column was first washed with the same buffer (100 ml) and the adsorbed protein eluted with a linear gradient prepared with 250 ml each of Buffer C and 0.4 M NaCl in Buffer C. The combined active fractions (35 ml) were treated with $(NH_4)_2SO_4$ to 40% satn, the ppt. dissolved in Buffer C (2 ml) and desalted on a Sephadex G-25 column. The desalted soln (3 ml) was passed through a column of hydroxylapatite in Buffer C. The active enzyme was eluted after the void vol. and freeze-dried. The resulting residue was dissolved in H_2O (1 ml) and chromatographed on Sephadex G-200 equilibrated with 0.02 M Pi buffer, pH 7. Isoelectric focussing was carried out in a LKB-8101 ampholine column with 1% (w/v) carrier Ampholyte of pH 3.5–10. The enzyme prepn from the previous step (20 ml accumulated from several runs) was applied to the middle part of the column. An initial voltage of 100 V for 4 hr and 300 V for 16 hr was used. At the end of the run, 3 ml fractions were collected and monitored for pH and A_{280}.

Enzyme assay. The standard assay mixture consisted of 0.2 μmol 6-$CO^{14}CH_3$-(+)-usnic acid (8.46×10^4 dpm) in 0.1 ml ethylene glycol monoMeether, the enzyme (usually 0.1 ml) and an appropriate amount of 0.1 M Na–Pi buffer, pH 7, to give a total vol. of 1.1 ml. The reaction was allowed to proceed for 1 hr at 30° and terminated by adding 0.1 ml M HCl. The acidified micture was extracted with EtOAc (3 × 3 ml). After removal of the solvent, the residue was dissolved in MeOH and used for TLC. Bands corresponding to the substrate and products were detected under UV, eluted from the chromagram and the radioactivities determined. To each counting vial was added 1 ml MeOH and 14 ml scintillation fluid (4 g PPO and 50 g POPOP in 1 l. toluene). Controls, without enzyme or with enzyme added at the termination of the expt, were always carried out. Protein concn was measured by the method of ref. [11] with BSA as standard. MW was calibrated with the following markers: Apo-ferritin, horse, 4.8×10^5; aldolase, 1.58×10^5; ovalbumin, 4.5×10^4; and myoglobin, 1.78×10^4.

Synthesis of (+)-usnic acid-[6-$CO^{14}CH_3$]. To a soln of $AlCl_3$ (63.1 mg) and AcCl-2-^{14}C (29.5 μl, 1 mCi) in dry nitrobenzene (1.2 ml) was added (+)-6-desacetylusnic acid (30.3 mg) [5] and the mixture stirred for 1.5 hr at 60° under N_2. After cooling at 0°, the mixture was diluted with $CHCl_3$ (5 ml) and M NaOH (5 ml), and stirred for another 0.5 hr at room temp. The two layers were separated and the organic phase extracted with M NaOH (5 ml). The combined aq. layers were carefully acidified with M HCl and extracted with EtOAc (3 × 15 ml). The EtOAc extract was washed with dil. NaCl, H_2O and dried over Na_2SO_4. The solvent was evapd under red. pres. and the residue kept under vacuum for 7 hr. From the crude product (25.6 mg), pure (+)-usnic acid-[6-$CO^{14}CH_3$] (11 mg) was obtained by PLC and by recrystallization from EtOH–$CHCl_3$. This labelled (+)-usnic acid was diluted with inactive (+)-usnic acid (100 mg) and recrystallized to give a product (74 mg) with a sp. act. of 1.23×10^6 dpm/mg.

TLC and GLC. TLC was carried out on Si gel sheets with fluorescence indicator (Eastman chromagram) with solvent system A: C_6H_6–Me_2CO–HOAc (16:3:1). For PLC, Si gel impregnated with 2% oxalic acid was used with solvent system B: C_6H_6–Me_2CO (19:1). Chromosorb 101 columns were used for GLC: carrier gas, He, temp. programmed 150° to 250° at 5°/min; FID.

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