Purification and Properties of a New Enzyme from *Evernia prunastri*, which Reduces L-Usnic Acid

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Z. Naturforsch. **36 c**, 35–39 (1981); received September 29, 1980

Evernia prunastri, L-Usnic Acid, Usnic Acid Dehydrogenase, Enzyme Purification

A new enzyme, usnic acid dehydrogenase, which catalyzes the reduction of L-usnic acid, has been isolated from cell-free extract of *Evernia prunastri* thallus. Purification of the enzyme (approx. 150-fold with a recovery of 42%) can be achieved by using gel adsorption and gel filtration techniques. The apparent molecular weight of the enzyme is 450000. Cofactor requirement is restricted to NADH. Kinetics of the enzyme show an allosteric behaviour for both L-usnic acid and NADH. Structure of the reaction product is described.

Usnic acids are lichen substances which are deposited in either the lichen cortex or the medulla as crystals on the outer surface of the hyphae. Its biological role is not well-known though a certain number of hypothetical functions have been suggested in the literature. Usnic acids can be considered as light-screen compounds regulating the solar irradiance reaching the algae zone in the upper cortex [1]. It has been stablished that concentrations of usnic acids vary linearly along light gradients in *Cladonia subtenuis* [2] and that shade populations have a higher rate of CO_2 uptake at light saturation that sun populations [3]. It has been further demonstrated that usnic acids, under laboratory conditions, are able to alter rocks [4] at structural level and to give origin to new materials [5].

Usnic acids, as well as another lichen substances, can be considered as the key compounds in the regulation of the nutrient translocation between lichen symbionts at the level of urease activity in the phycobiont [6, 7].

A very interesting suggestion has been that the lichen acids are storage metabolites which are available for re-mobilization. In this way, Ravinskaya and Vainshtein [8] have described seasonal variabilities in the concentration of usnic acids in several lichens and Vicente *et al.* [9] have reported a mobilization of these compounds in *Evernia prunastri* maintained in harvest conditions.

In this paper, we report the purification and properties of a dehydrogenase which reduces L-usnic acid, from *Evernia prunastri*, considering it as a re-mobilizing enzyme.

Reprint requests to Prof. C. Vicente.

0341-0382/81/0100-0035 $ 01.00/0

Materials and Methods

Biological source. E. prunastri, growing on *Fagus sylvatica* and collected in Montejo de la Sierra (Madrid) has been used throughout this work. Lichen thallus was maintained at 26 °C, in dryness conditions and irradiated with white light (12000 erg · cm^{-2} · s^{-1}) for three days before enzyme assay.

Assay of dehydrogenase activity. Usnic acid dehydrogenase was assayed by following the rate of the enzyme-catalyzed oxidation of NADH by L-usnic acid in a reaction mixture containing 0.5 mg of protein (or 5 µg of purified enzyme), 160 µmol of Tris-HCl buffer, pH 8.0, 82.0 nmol of L-usnic acid and 3.0 µmol of NADH in a final volume of 3.0 ml. A unit of specific activity was defined as the decrease in absorbancy at 340 nm per mg of protein and minute.

Protein determination. Protein concentrations were determined by the Potty's method [10] using bovine serum albumin as standard.

Identification of reaction product. Reaction product was isolated from a reaction mixture maintained for 30 minutes at the optimal pH and temperature conditions. At the end of this period the reaction was stopped by addition of hot ethanol and then centrifuged at 20000 × *g* for 30 minutes at 4 °C. The supernatant was dried in vacuum and then dissolved in methanol-HCl (99:1 v/v). The product was isolated by thin layer chromatography on cellulose MN 300 using as solvent buthanol:acetic acid:water (3:1:1 v/v) [11] and revealing it by fluorescence under ultraviolet light of 254 and 366 nm [12].

The infra-red absorption spectra on the purified product, as well as pure L-usnic acid, were determined in potassium bromide pellets.

Enzyme purification. Samples of thallus were washed with distilled water and then were macerated with enough 0.1 M Tris-HCl, pH 8.0. Crude extract was centrifuged at 27 000 × *g* for 20 minutes at 4 °C. Supernatant was filtered through a Millipore GS filter with pores of 0.22 μm in diameter. This cell-free extract was brought up to 60 per cent saturation with ammonium sulfate and kept for 2 hours at 4 °C. At the end of this time the precipitate was recovered by centrifugation at 27 000 × *g* for 30 minutes, dissolved in 10.0 ml 0.1 M Tris-HCl, pH 8.0, and dialysed against 2.0 l of the same buffer for 24 h at 4 °C. The dialysate was mixed with calcium phosphate gel (for each mg of protein, 75.0 mg of calcium phosphate gel in dry weight was added). The gel was collected by centrifugation and dehydrogenase was eluted from it by increasing the molarity of the buffer. The eluate from 1 M Tris-HCl was filtered through a Sephadex G-200 column, 21 cm in height and 3 cm in diameter, equilibrated with the same buffer.

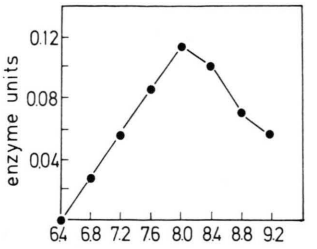

Fig. 1. Dependence of usnic acid dehydrogenase activity on the pH values of the incubation mixture. The assay is conducted in the presence of 0.1 M Tris-HCl buffer.

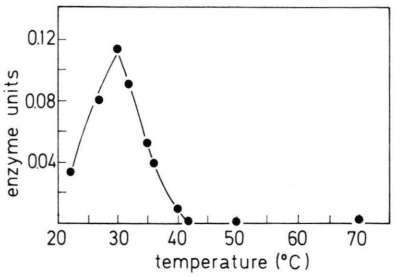

Fig. 2. Dependence of usnic acid dehydrogenase activity on the temperature.

Results

Usnic acid dehydrogenase has been purified 150-fold with an overall yield of 42 per cent from *E. prunastri* crude extract (Table I). The enzyme prepared by standard procedure has been used for enzyme analysis.

The activity of the enzyme has been measured at a range of pH values 6.4–9.2 in Tris-HCl buffer as shown in Fig. 1. The dehydrogenase from *E. prunastri* shows a clear optimum at pH 8.0. The enzyme exhibits a temperature optimum at 30 °C, being extremely termolabile (Fig. 2).

The substrate saturation kinetics for usnic acid dehydrogenase are sigmoidal for both L-usnic acid (Fig. 3) and NADH (Fig. 4). The v_{max} values for L-usnic acid and NADH are 4×10^{-3} and 1×10^{-3} ΔOD at 340 nm per minute respectively. The enzyme has a strict requirement for NADH. This pyridine nucleotide is not replaced with NADPH. However, L-usnic acid can be replaced with D-usnic acid (Table II).

The molecular weight of usnic acid dehydrogenase is estimated to be 450 000 by gel filtration on Sephadex G-200 (Fig. 5). The ratios of V_e to V_0 are

Table I. Purification procedure of usnic acid dehydrogenase of *Evernia prunastri*.

Step	Volume [ml]	Protein [mg/ml]	Total protein [mg]	Total activity [units]	Specific activity [units]	Recovery [%]	Purification (-fold)
I. Cell-free extract	50	0.48	24.0	0.048	0.002	100.0	–
II. Ammonium sulfate precipitation (60% saturation)	10	1.2	12.0	0.048	0.004	100.0	2.0
III. Calcium phosphate gel eluate	9	0.2	1.8	0.045	0.025	93,75	12.5
IV. Sephadex G-200 eluate	6	0.014	0.084	0.019	0.23	39.58	115.0

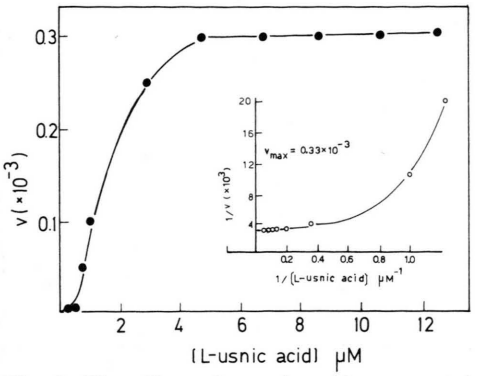

Fig. 3. The effect of L-usnic acid concentration on the dehydrogenase reaction. The insert shows the double-reciprocal plot.

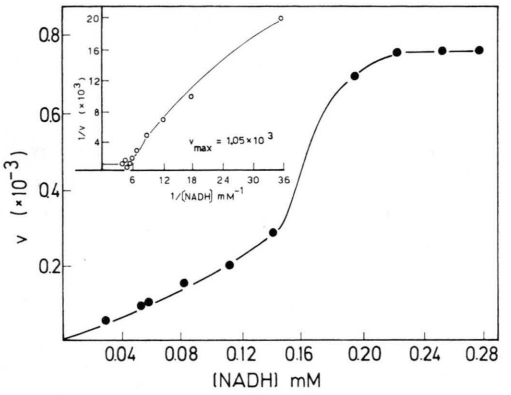

Fig. 4. The effect of NADH concentration on dehydrogenase activity.

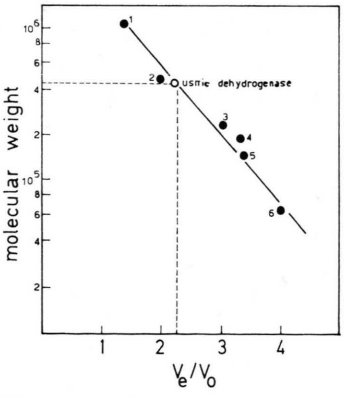

Fig. 5. Determination of the molecular weight of usnic acid dehydrogenase on Sephadex G-200. The void volume V_0 is determined from the elution volume of dextran blue. Molecular weights are estimated from standard plot of log molecular weights *versus* V_e/V_0. Molecular standards were: Glutamic acid dehydrogenase (1), urease (2), phosphorylase a (3), catalase (4), alcohol dehydrogenase (5) and glyceraldehyde-3-P dehydrogenase (6).

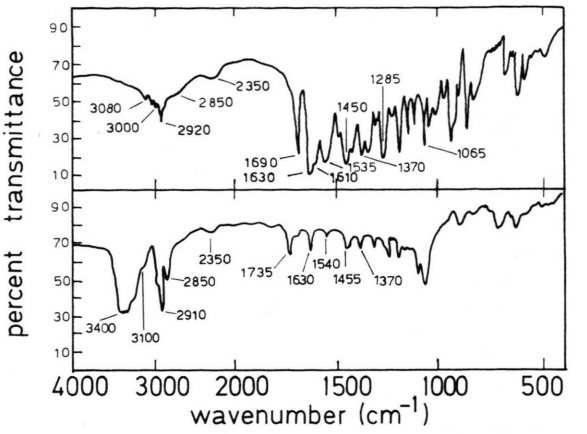

Fig. 6. Infra-red spectra of L-usnic acid (a) and enzymatic reduction product (b). Both substances were pressed in potassium bromide pellets.

Table II. Substrate and cofactor specificity of usnic acid dehydrogenase of *Evernia prunastri.*

Substrate	Cofactor	Specific acitivity (units)
L-usnic acid	NADH	0.23
D-usnic acid	NADH	0.09
L-usnic acid	NADPH	0.0
D-usnic acid	NADPH	0.0

Table III. Chromatographic behaviour of both L-usnic acid and its reduced form.

Compound	R_f	Fluorescence	
		254 nm	366 nm
L-usnic acid	0.96	yellow	Yellow
Product	0.82	violet	violet

1.42 (glutamate dehydrogenase), 2.05 (urease), 2.28 (usnic acid dehydrogenase), 3.11 (phosporylase a), 3.36 (catalase), 3.45 (alcohol dehydrogenase) and 4.02 (glyceraldehyde-3-P dehydrogenase).

Both substrate and product have been isolated from a reaction mixture, as described above, by thin layer chromatography on cellulose MN 300. L-usnic acid reveals by fluorescence, as a yellow spot of $R_f = 0.96$. Reaction product give a violet spot of $R_f = 0.82$ (Table III).

0.4 mg of pure L-usnic acid and, alternatively, 0.2 mg of the reaction product, have been used to made infra-red spectra. L-usnic acid spectrum (Fig. 6a) shows characteristical peaks at 955 and

Table IV. Spectral characteristics of both L-usnic acid and its reduced product.

L-usnic acid		Reduced Product	
υ [cm^{-1}]	Function	υ [cm^{-1}]	Function
–		3400	new chelated hydroxy group
3000–3080	chelated 5-hydroxy group	3100	chelated 5-hydroxy group
2920	chelated 7-hydroxy group	2910	chelated 7-hydroxy group
2850	aromatic C-acetyl group	2850	aromatic C-acetyl group
2320–2350	very strongly hydrogen bounded enolic hydroxy group	2350	very strongly hydrogen bounded enolic hydroxy group
–		1735	reflection of 3400 cm^{-1} band
1690	displacement of the ether band in relation to furanic nature of the heterocycle	–	
1610–1630	enol ether double bond which coalesces with aromatic C-acetyl group	1630	aromatic C-acetyl group
1535	conjugated chelated 4-carbonyl group	1540	conjugated chelated 4-carbonyl group
1065–1285	ether bond	–	

990 cm^{-1}, typical of conjugated bonds, and a single, well-defined, peak at 1535 cm^{-1} which reveals conjugated chelated 4-carbonyl group in B ring. Ether function shows bands at 1065 and 1285 cm^{-1} and a main peak at 1690 cm^{-1} by displacement in relation to the furanic nature of the heterocycle. The enol ether double bond shows a band near 1610 cm^{-1} which coalesces with the aromatic acetyl group band at 1630 cm^{-1}. Methyl groups show peaks at 1370 and 1450 cm^{-1}. A net peak near 2900 cm^{-1} can be adscribed to the chelated 7-hydroxy group. The band near 3000 cm^{-1}, as well as 3080 cm^{-1}, must be attributed to the 5-hydroxy group. These data are in agree with those described by Chan and Hassall [13] and Forsen *et al.* [14].

Spectrum of the reaction product (Fig. 6 b) shows very significant differences in relation to that unmodified acid (Table IV):

i) disappearance of the peaks at 1065, 1285 and 1690 cm^{-1}, typical of the ether bond.

ii) disappearance of the peak at 1535 cm^{-1}, typical of the 4-carbonyl group.

iii) appearance of a broad band at 3400 cm^{-1} which reveals the presence of new chelated hydroxy groups. The small band at 1735 cm^{-1} is a reflection of the strong hydrogen bonded hydroxy group at 3400 cm^{-1}.

Being constant the rest of the spectral characteristics, it can be proposed the structure shown in Fig. 7 for the product of L-usnic acid reduction.

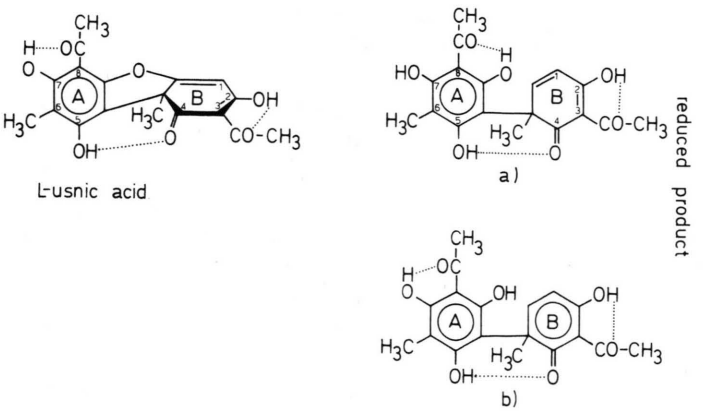

Fig. 7. Structural formulae of L-usnic acid and its reduced form. Structures a) and b) indicate that 7-hydroxy group and that formed by reductive cleavage of the ether bond appear as chelate.

Discussion

Catabolism of usnic acids reported for *E. prunastri* in starvation conditions [9] can be started by a reduction reaction which implies NADH oxidation and a highly specific dehydrogenase. Tentatively, it can be affirmed that the mobilization of usnic acids and the evolution of dehydrogenase activity are two rhythmically simultaneous facts in the lichen thallus (unpublished results). The purified enzyme is a termolabile, allosteric and heavy protein showing a very restricted specificity for both substrates. Only one reaction product has been described, arising from a reductive cleavage on the ether bond in L-usnic acid molecule. It could be expected that C-C-bond in heterocycle was reduced by enzyme action because its formation from two molecules of methylphloroacetophenone involves a dehydrogenase-catalyzed oxidation [15]. In addition, the biosynthetic enzyme system exhibits a rhythmic behaviour of activity [16]. However, the integrity of the linkage between both cycles can be assured because of the persistence of the 840 cm^{-1} peak in the infra-red spectrum of the product. Although the attempts to prepare the biosynthetic enzyme gave no successful results [17], it seems probable that both catabolic and biosynthetic dehydrogenases must to be two distinct proteins since the oxidative coupling of methylphloroacetophenone in lichens should be stereospecific [18] whereas both D- and L-forms of usnic acid appear to be reduced by the purified dehydrogenase described here.

Acknowledgements

We wish to thank Prof. F. Bustinza for a generous supply of both D- and L-usnics acids and Mr. C. Macías for his expert technical assistance.

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