STUDIES ON LICHEN ENZYMES
PART I. PREPARATION AND PROPERTIES OF A DEPSIDE HYDROLYSING ESTERASE AND OF ORSELLINIC ACID DECARBOXYLASE

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Although there may be as many as 15.000 different species of lichens representing symbiotic partnership of fungi (mycobiont) and algae (phycobiont), to the authors' knowledge no report has appeared in the literature on investigations of enzymes isolated from these organisms with the exception of a paper by Koller and Pfeiffer (1933). They prepared powdered lichen thalli capable of breaking down depsides to phenols. In the study of symbiotic organisms it is of great interest to establish the role of each component in anabolic as well as catabolic processes. In the synthesis of depsides and depsidones in lichens, these structures may be the result of a collaborative effort of both symbionts. For example, in the case of the lichen acid gyrophoric acid, a tridepside consisting of three orsellinic acid molecules the phycobiont may play a role in coupling the phenolcarboxylic acids or their activated forms into depsides. The mono-cyclic building unit orsellinic acid however, is most likely derived from the mycobiont since it has been found as a metabolite in some fungi. Both aromatic substances have been shown to be "acetate-polymalonate"-derived (Mosbach, 1964). It is also conceivable that the phycobiont is participating in the catabolism of such depside structures. In order to study this latter question, the enzymic decomposition of various depsides and their phenolcarboxylic acids by a cell-free, partially purified lichen extract from Umbilicaria pustulata was compared with that of a cell-free extract from a corresponding phycobiont (Trebouxia).

EXPERIMENTAL

Isolation of the enzyme system from Umbilicaria pustulata: Dry lichen thalli were ground in a meat-grinder to give a fine powder which was

washed several times with acetone to remove gyrophoric acid and other acetone-soluble material. All subsequent procedures described below were carried out at 4°. The dry acetone-treated powder was further homogenized by grinding in a mortar for 20 min with sand and sufficient 0.1 M phosphate-buffer (pH 6.8) to form a thin paste. It was allowed to stand for additional extraction another 40 min. After passing through cheese-cloth, the filtrate was centrifuged at 5.000 x g for 15 min. The precipitate was discarded and the supernatant fraction further centrifuged at 35.000 x g for 45 min. The active supernatant fraction contained about 10 mg of protein per ml as determined by the method of Kalckar and was used in the assays described below.

Fractional heat denaturation of the enzyme system was achieved by heating the preparation on a waterbath to 75° for 15 min.

The values given in Table II on the specific activity of the decarboxylase have been obtained from preparations further purified as follows: To the above supernatant, enough acetone was added to make a 45 % solution. This was centrifuged at 5.000 x g for 10 min, the precipitate discarded and more acetone was added to make a 65 % solution. After centrifuging at 5.000 x g for 10 min, the precipitate was dissolved in 15 times its volume of 0.02 M phosphate-buffer (pH 6.2) and subsequently dialyzed against the same buffer for 10 h. In the next step, the enzyme solution was purified by negative adsorption on a CMC column treated with 0.02 M phosphate buffer (pH 6.2). The enzyme fraction obtained contained about 1 mg of protein per ml as determined by the method of Kalckar and was used in the decarboxylase assay described below. The over-all purification was approximately 10-fold.

Isolation of the enzyme system from Trebouxia: The alga used in these studies is a Trebouxia sp. isolated from Umbilicaria papulosa. The same enzyme systems studied in U. pustulata were found in this lichen. The algae were grown in 2.8 l Fernbach flasks containing 500 ml of Trebouxia medium (970 ml of Bristol's solution, 10 g of peptone, 20 g of glucose per liter of medium (Ahmadjian)). After two weeks of growth as stationary cultures at 23° in day-light, the algae were harvested and centrifuged at 5.000 x g for 5 min. The cells were washed with distilled water and recentrifuged. The extraction of the enzyme system from the algae was performed as already described for the lichen (filtration through cheese-cloth was omitted). The extract obtained was centrifuged at 5.000 x g

for 15 min followed by centrifugation of the supernatant fraction at 35.000 x g for 45 min. The active supernatant solution contained about 6.5 mg of protein per ml as determined by the method of Kalckar and was used in the assays as described below.

Esterase assay: To 0.5 ml of enzyme solution (pH adjusted to 6.2) together with 1.8 ml of 0.02 M phosphate buffer (pH 6.2) were added 6 μmoles of the substrate in 0.2 ml of acetone. After 2 h of incubation at 32°, the reaction was stopped by acidifying with 1 M HCl. This was followed by immediate extraction with ether. The phenolcarboxylic acids obtained from the hydrolytic action of the esterase as well as the controls were submitted to paper-chromatography using different solvent systems and identified by R_F-values and staining reactions (Reio, 1958).

Decarboxylase assay: Orsellinic acid decarboxylase was assayed manometrically by measurement of CO_2 evolution at 32°. 6 μmoles of substrate in 0.2 ml of acetone were placed together with 1.8 ml of 0.02 M phosphate-buffer (pH 6.2) in the main-chamber of the Warburg vessels. 0.5 ml of enzyme solution adjusted to pH 6.2 were placed in the side-arm. Readings were made for a total of 5 min and enzyme activity calculated as μl CO_2 evolved per minute.

RESULTS

On investigating the substrate specificity of the esterase from the cell-free preparation of the lichen Umbilicaria pustulata and that of the phycobiont Trebouxia, the same picture was obtained for both systems (Table 1). It is also of interest that under the assay conditions given no hydrolysis of the depside m-digallic acid, the natural substrate of the enzyme tannase found in molds, was observed. Furthermore, phenylbenzoate was not attacked, a substrate of both liver and mold carboxylesterase. This indicates a rather high substrate specificity of the esterase studied, preferentially hydrolysing gyrophoric acid, a naturally occurring metabolite in this lichen, and closely related lichen depsides. However, further studies should be made with a variety of assay conditions and substrates to determine the degree of specificity of this esterase.

The partially purified lichen preparation also showed decarboxylase activity towards phenolcarboxylic acids. This activity was completely lost on partial denaturation of the cell-free lichen enzyme system by heating under the conditions described, whereas the hydrolytic activity on depside linkages was retained. In order to investigate the spe-

Table I

Specificity of the depside hydrolysing esterase

substrate	product in moles/mole of substrate hydrolyzed
gyrophoric acid	3 orsellinic acid
umbilicaric acid[x]	2 orsellinic, 1 isoeverninic acid
evernic acid	1 orsellinic, 1 everninic acid
methyl gyrophorate tetramethyl ether	no reaction
homoorsellinic acid ethyl ester	no reaction
m-digallic acid	no reaction
phenyl benzoate	no reaction

[x]In some preparations the hydrolytic products formed were orsellinic acid and an unidentified aromatic compound whose R_F-values and staining reactions indicate umbilicarinic acid. On longer incubation and using larger amounts of enzyme solution the hydrolytic products listed were obtained.

Table II

Specificity of orsellinic acid decarboxylase

substrate	specific activity U/mg[x]
orsellinic acid	1.35
homoorsellinic acid	1.30
everninic acid	1.25
isoeverninic acid	no reaction
6-methylsalicylic acid	no reaction
β-resorcylic acid	no reaction
gentisic acid	no reaction
gallic acid	no reaction
benzoic acid	no reaction

[x]One unit (U) of enzyme is defined as that amount which catalyzes the decarboxylation of 1 micromole of substrate per minute under the specified assay conditions. The specific activity is expressed as units per mg of protein.

cificity of the decarboxylase, its activity towards a series of aromatic carboxylic acids was determined (Table II). It was found that only orsellinic acid (2-methyl-4,6-dihydroxy-benzoic acid) and some of its derivatives were decarboxylated. Structurally similar compounds like 6-methylsalicylic acid and β-resorcylic acid were not attacked. Thus the enzyme responsible, which we call orsellinic acid decarboxylase, has as requirement for activity the orsellinic acid structure in which the methyl group can be substituted by an ethyl group (homoorsellinic acid) and the hydroxyl group at position 4 by a methoxyl group (everninic acid) without loss of activity. Although the over-all purification of the cell-free lichen extract was not great, the specific activity of the decarboxylase (1.35 units per mg of protein per minute) is high. No orsellinic acid decarboxylase activity was found in the cell-free enzyme system from Trebouxia.

The following conclusions can be drawn. The enzymic breakdown of a lichen depside such as evernic acid by the lichen enzymes studied, proceeds in two steps (Fig. 1): a.) hydrolysis of the depside to phenolic carboxylic acids by the esterase activity of the phycobiont (a possible participation of the mycobiont as well cannot be excluded), b.) decarboxylation of the phenolic carboxylic acids to their corresponding phenols by the orsellinic acid decarboxylase activity of the mycobiont.

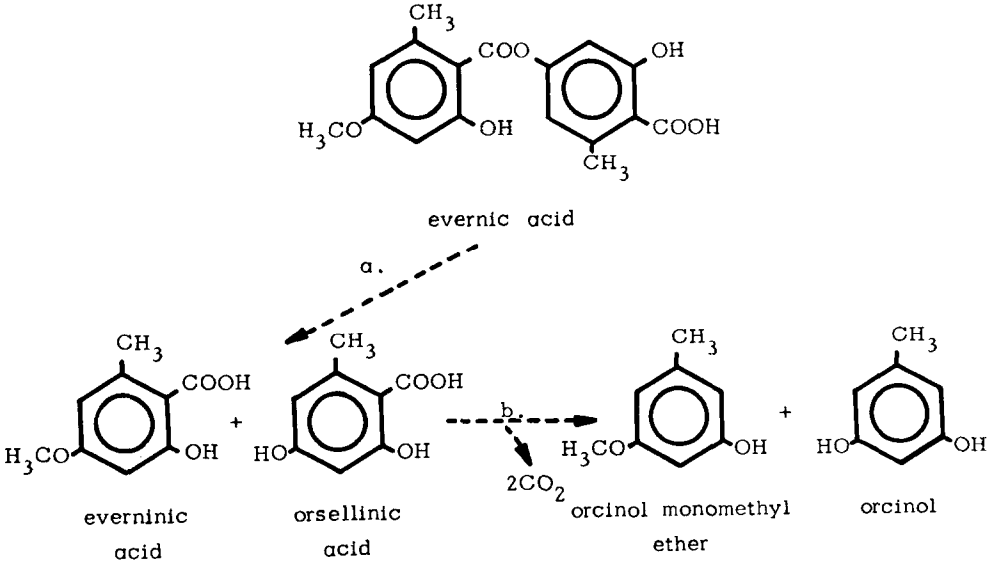

Fig. 1

In the lichen investigated, normally no orcinol is found. The conditions under which these enzymes become active *in vivo* and what function they may play in the general metabolism of the lichen remains to be shown.

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