Limonoate Dehydrogenase from *Arthrobacter globiformis*

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The bacterium, *Arthrobacter globiformis*, isolated from soil produces intracellularly limonoate dehydrogenase when grown on a Na–limonoate medium. This dehydrogenase was purified by $(NH_4)_2SO_4$ precipitation, followed by two columns of DEAE cellulose. It reversibly catalyzes the conversion of limonoate to 17-dehydrolimonoate in the presence of NAD. The enzyme has its optimum activity at pH 9.5, and requires Zn ions and sulfhydryl groups for its catalytic action. It attacks only limonoids which have the furan ring, epoxide, and open D-ring in their molecules.

Bitterness due to limonin in citrus juices has become an increasingly important economic problem. In dealing with this bitterness problem, one of the approaches taken at our laboratory is to investigate the possibility of converting limonin to nonbitter compounds with a limonin-metabolizing enzyme. Since microorganisms are known to be a potential source of many enzymes, we have been investigating that source for such an enzyme.

During the course of our study, we found that a species of *Arthrobacter* isolated from soil produces a limonoate dehydrogenase which catalyzes the conversion of limonoate to 17-dehydrolimonoate (Hasegawa *et al.*, 1971). In this paper we report detailed studies on identification of the bacterium, isolation and structure determination of the metabolite, and isolation and characterization of the enzyme.

EXPERIMENTAL

Assay Methods. Protein concentration was measured by the procedure of Lowry *et al.* (1951). Limonoate dehydrogenase activity was assayed by following the increase in absorbance due to the formation of NADH at 340 mμ. Activity was assayed in 1 ml of a reaction mixture consisting of 10^{-2} *M* Na–limonoate, 0.07 *M* Tris buffer at pH 9.5, 5×10^{-4} *M* NAD, and 1–4 munits of enzyme. The reaction was carried out at 23° in a standard silica cuvette with 1-cm light path. One unit of limonoate dehydrogenase is defined as the amount which catalyzes the production of 1 μmol of 17-dehydrolimonoate per minute under the above conditions.

Characterization of Bacterium. The bacterium was isolated from soil on the same Na–limonoate medium described previously (Hasegawa *et al.*, 1972a), and after purification the organism was classified as *Arthrobacter globiformis* by following standard procedures (Society of American Bacteriologists, 1957).

Growth of Cells. A 2-l. Erlenmeyer flask containing 400 ml of 1% Na–limonoate in a mineral salt medium was incubated with 10 ml of a 48-hr culture of *A. globiformis*. Incubation was carried out at room temperature on a shaker. After 72 hr, cells were collected by centrifugation at 10,000 × *g* for 20 min and washed twice with cold 0.1 *M* phosphate buffer at pH 7.0 and kept in a freezer until used.

Preparation of Limonoate Dehydrogenase. Limonoate dehydrogenase was isolated from cell-free extracts of *A. globiformis*. Frozen cells were suspended in approximately 10 vol of 0.1 *M* phosphate buffer at pH 7.5 containing 10^{-2} *M* dithiothreitol and disrupted by a French press. After centrifugation at 20,000 × *g* for 10 min, the supernatant was used as the starting material for purification of the enzyme.

The supernatant was brought to 90% saturation with $(NH_4)_2SO_4$ by the addition of solid salt with continuous stirring and was placed in an ice bath for 1 hr. The resulting precipitate was collected by centrifugation at 20,000 × *g* for 10 min, and dissolved in a minimum portion of 0.1 *M* Tris buffer at pH 7.5 containing 10^{-2} *M* dithiothreitol. The solution was then dialyzed for 2 hr against 0.05 *M* phosphate buffer at pH 7.5 containing 10^{-4} *M* dithiothreitol and also flushed with N_2 gas just prior to dialysis.

The dialysate was then applied to a 1.5 × 25 cm jacketed column of DEAE cellulose which had been equilibrated with 0.01 *M* Tris buffer at pH 8.0. The column, which was maintained at 4°, was eluted at a flow rate of 40 ml per hr with a linear gradient formed between 150 ml of 0.01 *M* potassium phosphate buffer at pH 8.0 and 150 ml of the same buffer solution containing 1.0 *M* NaCl. The effluent was collected in 4-ml fractions (Figure 1).

Fractions containing enzyme activity were combined, dialyzed, and fractionated again on a 1.5 × 25 cm DEAE cellulose column. The column was eluted in a manner similar to the first column except that this time the elution was at pH 7.0 (Figure 2).

Identification of Metabolite. Silica gel G plates were used for thin-layer chromatography (tlc). Chromatograms were revealed by spraying with 50% H_2SO_4 and heating or by spraying with Ehrlich's reagent and exposing to HCl gas (Dreyer, 1965). Nmr spectra were run at 100 MHz in $CDCl_3$ using a Jeolco JNM-PS-100 spectrometer.

The supernatant from a culture of *A. globiformis* which had been incubated with 4 g of Na–limonoate in 400 ml of the mineral salt medium for 3 days was acidified with HCl to pH 2 and allowed to stand overnight. It was then extracted twice with $CHCl_3$, and the extracts were combined and evaporated to give 1.63 g. Tlc with benzene–ethanol–water–acetic acid (200:47:15:1) showed two major components, one of which was limonin. The second compound remained at the origin upon tlc with nonacidic systems, which suggested that it was an acid. The extract was therefore dissolved in 50 ml of ethyl acetate and extracted with two 20-ml portions of 5% $KHCO_3$ and 20 ml of H_2O. The extracts were washed with 10 ml of ethyl acetate, combined, acidified with HCl, and extracted with two 30-ml portions of CH_2Cl_2. These extracts were washed with 10 ml of H_2O, combined, and evaporated to give an acidic fraction weighing 870 mg. Tlc showed that this consisted mainly of one compound, but attempts to crystallize it were unsuccessful. The acidic fraction was therefore methylated with CH_2N_2, and crystallization from methanol then gave 600 mg of the major compo-

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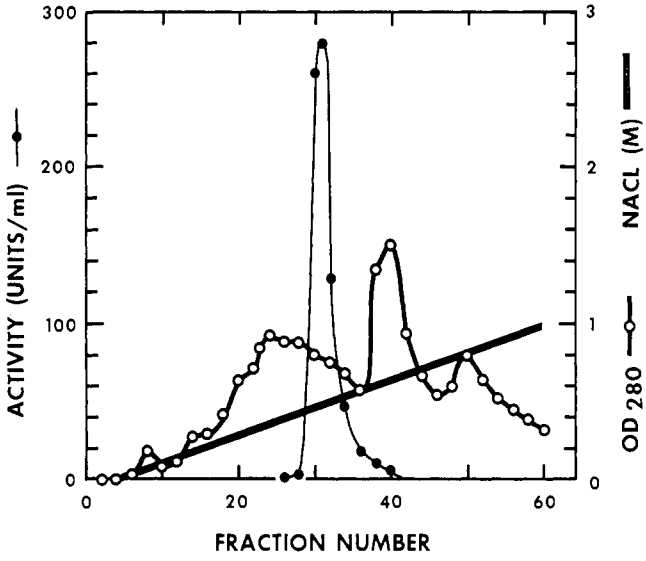


Figure 1. Chromatography of limonoate dehydrogenase on DEAE cellulose. Column (1.5 × 25 cm) was developed with linear gradient of NaCl at pH 8.0 as described in Experimental Section

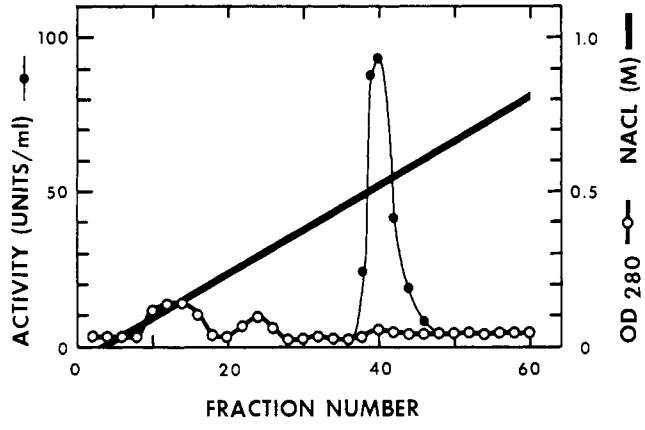


Figure 2. Chromatography of limonoate dehydrogenase on DEAE cellulose. Chromatography was performed under conditions similar to those used for Figure 1 except that the column was eluted at pH 7.0

nent. After two more crystallizations from methanol, the metabolite ester was pure enough for structure determination.

RESULTS

Identification of the Bacterium. The bacterium *Arthrobacter globiformis* was isolated from soil. The bacterium changed from gram-negative rods to gram-positive rods and eventually to gram-positive cocci. These characteristics are those of the genus *Arthrobacter*. Branching and mycelial development characteristics of *Nocardia* were not observed (Cann and Dimmick, 1947; Breed *et al.*, 1957; Skerman, 1967). The descriptive tests indicated that this bacterium is *Arthrobacter globiformis*, as they closely match the species description (Cann and Dimmick, 1947). The exception from the description of the species is that we did not observe starch hydrolysis, whereas *A. globiformis* is reported to hydrolyze starch.

When *A. globiformis* was grown on a mineral salt medium containing Na–limonoate as a single carbon source, a typical correlation between the bacterial growth and the decrease in substrate concentration was observed. This showed that *A. globiformis* is capable of metabolizing Na–limonoate and utilizing it as an energy source for growth.

Identification of the Metabolite. When incubated on Na–limonoate, the bacterium produced predominantly one metabolite which was acidic and negative to Ehrlich's reagent. This compound was isolated and crystallized as the methyl ester. The mp was 287–291° (dec). *Anal.* Calcd for $C_{27}H_{33}O_9$: C, 64.8; H, 6.44. Found: C, 64.7; H, 6.50.

The negative Ehrlich reaction initially suggested that the furan ring had been metabolized, but the nmr spectrum showed three aromatic protons at 8.60, 7.41, and 6.92 ppm. The three furan resonances in limonin appear at 7.42, 7.42, and 6.33 ppm, so two of these protons in the metabolite ester have moved far downfield. Such downfield shifts have previously been observed for furans attached to carbonyl groups (Gronowitz *et al.*, 1962). A structure of this type would explain the negative Ehrlich reaction, as the presence of an electronegative group prevents the color formation (Kubota, 1969). Other spectral data are also consistent with a carbonylfuran structure. The infrared spectrum in $CHCl_3$ shows a strong band at 1652 cm^{-1} (conjugated carbonyl), and a peak at 265 mμ, characteristic of furylketones (Scott, 1964), was observed in the ultraviolet spectrum in methanol.

Thus, the evidence suggests that the 17-hydroxyl group in Na–limonoate has been oxidized to a ketone by the organism. This would also explain the fact that the metabolite is an acid, since in the absence of the 17-hydroxyl, the D-ring carboxyl group could no longer form a lactone. Further evidence for the absence of the D-ring lactone in the metabolite ester is given by nmr and circular dichroism (CD). The H-17 resonance characteristically seen at about 5.5 ppm in limonoids does not appear in the nmr spectrum of the metabolite ester. The CD curve of limonin shows negative Cotton effects at 235 (D-ring lactone) and 293 mμ (7-ketone). For the metabolite ester, however, only the Cotton effect of the 7-ketone is observed at 308 mμ.

Other significant features of the nmr spectrum of the metabolite ester include a pair of AB doublets, centered at 4.43 and 4.33 ppm (J = 13 Hz), a broadened singlet at 4.23 ppm, a methyl ester signal at 3.82 ppm, a sharp singlet at 3.40 ppm, and four tertiary methyl signals at 1.29, 1.11, 0.98, and 0.96 ppm. The chemical shifts and coupling constant of the AB system are characteristic of the two H-19 protons in the A-ring lactone of limonoids. The broadened singlet may be ascribed to H-1 of the A-ring lactone, and the sharp singlet to the H-15 epoxy proton. The signal of the latter appears at higher field than is normal in limonoids, probably because opening of the D-ring changes its spatial position relative to the deshielding zone of the 7-keto group.

Although the combined spectral data evidence is convincing that the metabolite is 17-dehydrolimonoic acid A-ring lactone, attempts were made to confirm this by synthesis of the compound. This proved to be unexpectedly difficult and synthesis was not achieved. To produce a 17-keto group by oxidation, the D-ring must be open, and this can be achieved by treatment of limonin with limonin D-ring lactone hydrolase (Maier *et al.*, 1969). However, the oxidation reaction must be run in aqueous alkaline solution, which limits the choice of oxidizing agents. Under these conditions $KMnO_4$, RuO_4, and silver(II) picolinate all attacked the furan ring, while with sodium chromate no reaction occurred. Another possible approach involved esterifying the carboxylate anion of the open D-ring. The product could then presumably be oxidized in an organic solvent with CrO_3. However, reaction with dimethyl sulfate caused ring closure to produce limonin, and *p*-bromophenacyl bromide did not react with the carboxylate ion, possibly because of steric hindrance. Attempts to reduce the metabolite back to limonin with sodium boro-

Table I. Purification of Limonoate Dehydrogenase

Treatments	Activity, units	Protein, mg	Specific activity, units/mg protein	Purification	Recovery, %
Cell extracts	3159	75.6	41.8	1.0	100.0
$(NH_4)_2SO_4$ precipitate after dialysis	2704	54.4	49.7	1.2	85.6
DEAE cellulose, 1st column	2372	7.92	300.0	7.2	75.1
DEAE cellulose, 2nd column	1753	0.612	2846.0	68.1	55.5

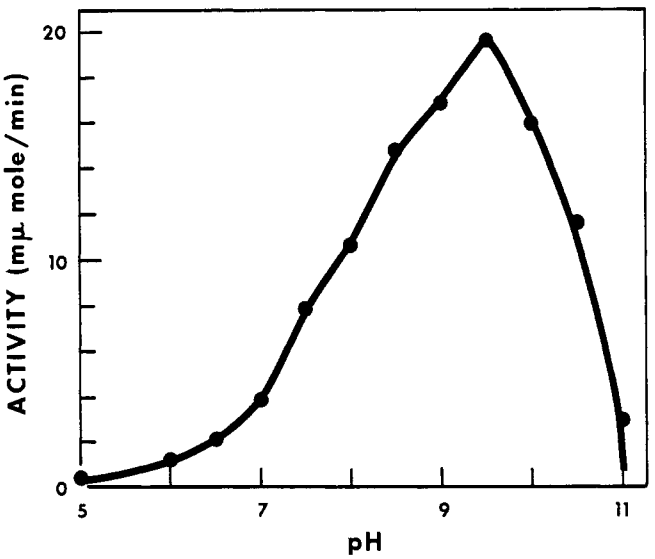


Figure 3. Effect of pH on limonoate dehydrogenase activity. Reaction mixture consisted of 10^{-2} *M* Na-limonoate, 0.2 *M* phosphate-Tris buffer, and 20 mμ units in 1.0 ml

hydride or aluminum isopropoxide were unsuccessful, as the furyl ketone was not affected by these reagents. In spite of our inability to correlate the metabolite with limonin by synthesis, its structure is well established. In addition to the spectral data discussed previously, the elemental analysis agrees closely with the calculated values. Furthermore, limonoate dehydrogenase attacks only limonoids which have an open D-ring, which confirms that change at only one position of the molecule, C-17, is involved.

Isolation of Limonoate Dehydrogenase. Since we observed large amounts of 17-dehydrolimonoate in the bacterial growing medium, it was reasonable to believe that this compound is the first metabolite of Na-limonoate produced by *A. globiformis*. Thus, attempts were made to isolate an enzyme responsible for the conversion of limonoate to 17-dehydrolimonoate. No activity was found in the medium but we did find activity in cell-free extracts of the bacterium. The enzyme was partially purified by $(NH_4)_2SO_4$ precipitation followed by two columns of DEAE cellulose.

Typical elution patterns of DEAE cellulose columns are shown in Figures 1 and 2. The overall purification during the preparative procedures gave a 68-fold increase in activity over the crude extract and approximately 55% of the original activity was recovered (Table I). With this preparation we investigated the properties of the dehydrogenase.

Properties of Limonoate Dehydrogenase. Tests showed that NAD is the obligatory hydrogen acceptor. Substitution of NADP for NAD in the reaction mixture resulted in no activity of limonoate dehydrogenase.

The activity of limonoate dehydrogenase at various hydrogen ion concentrations is shown in Figure 3. The enzyme has its optimum activity at pH 9.5. We observed also that at pH 7.0 the reverse reaction occurred. However, it was only 5% of that of the forward reaction.

Table II. Effects of Divalent Cations on Limonoate Dehydrogenase Activity

Ions	Concentrations	Relative activity
Control	...	100
$CaCl_2$	10^{-3} *M*	105
$MgCl_2$	10^{-3} *M*	100
$MnCl_2$	10^{-3} *M*	95
$ZnCl_2$	10^{-3} *M*	144
EDTA	10^{-3} *M*	100

Table III. Effects of Inhibitors on Limonoate Dehydrogenase Activity

Inhibitors	Concentrations	Inhibition, %
$HgCl_2$	10^{-4} *M*	68
	10^{-3} *M*	100
PCMB	10^{-3} *M*	59
NaN_3	10^{-3} *M*	44

The effect of divalent cations on activity of limonoate dehydrogenase is summarized in Table II. Ca, Mg, and Mn had no effect on the activity, but Zn ions increased the activity significantly, suggesting that the enzyme required Zn ions for its catalytic action. The direct addition of 10^{-3} *M* of EDTA to the reaction mixture did not reduce the activity, but dialysis against 10^{-4} *M* EDTA at pH 7.0 for 3 hr inhibited the activity completely, while the control which was dialyzed against water only lost 20% of its activity. This indicates that EDTA did inhibit limonoate dehydrogenase activity apparently through its ability to form a complex with the intrinsic Zn ions of the enzyme. In addition to EDTA, the metal-binding agent NaN_3 also reduced the activity significantly (Table III).

$HgCl_2$ and *p*-chloromercuribenzoate were found to be potent inhibitors of the enzyme (Table III). This indicates that sulfhydryl groups are required for its activity.

The results of substrate specificity studies are shown in

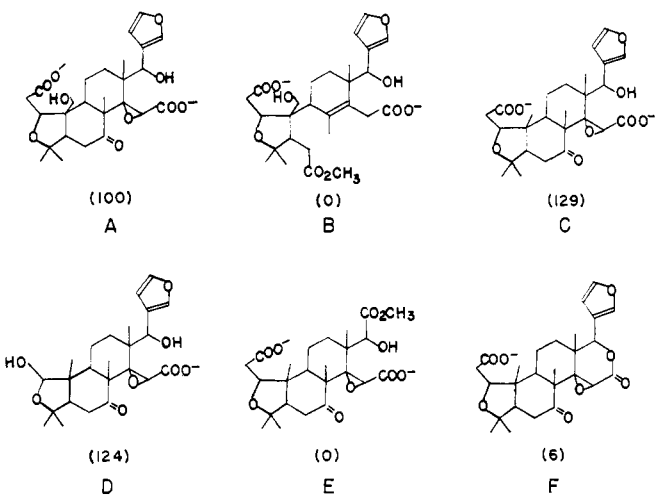
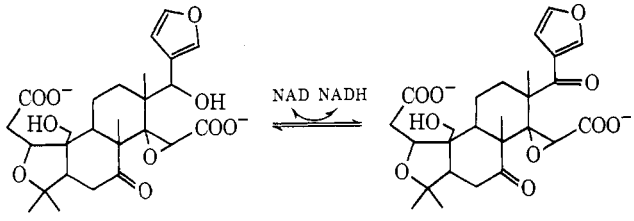


Figure 4. Substrate specificity. Figures in parentheses indicate activities relative to that of Na-limonoate

Figure 4. The enzyme was completely inactive toward deoxylimonic acid (B) and etioisoobacunoic acid (E), but attacked isoobacunoic acid (C) and the compound (D) at rates faster than that of limonoate (A). Changes in the structure around the A-ring of limonoid molecules gave compounds which are attacked more rapidly by the enzyme. On the other hand, the removal of epoxide (B) or furan ring (E) resulted in compounds which are completely resistant to attack. In the case of the D-ring closed isoobacunoic acid (F), the enzyme attacked it, but the rate was less than 5% of that of the open D-ring isoobacunoic acid (C), suggesting that in order to be a substrate, the limonoid compound must have an open D-ring.

DISCUSSION

Limonoate dehydrogenase was isolated from cell-free extracts of *Arthrobacter globiformis*. This enzyme catalyzes reversibly the conversion of Na-limonoate to 17-dehydrolimonoate in the presence of NAD.



Like alcohol dehydrogenases (Theorell *et al.*, 1955; Vallee and Hoch, 1955, 1957; Witter, 1960; Yonetani and Theorell, 1962), the enzyme requires Zn ions and sulfhydryl groups for its catalytic action.

Recently, we showed that several species of bacteria obtained from soil are capable of metabolizing limonin and limonoate (Hasegawa *et al.*, 1972a). From a cultural medium of these organisms, two metabolites were isolated and identified as deoxylimonin and deoxylimonic acid. Judging from those results coupled with the results obtained in this study, it appears that there are at least two different metabolic pathways of limonoids in bacteria: one is *via* 17-dehydrolimonoate and the other is through deoxylimonin. Enzymes involved in the latter pathway, however, have not been isolated.

In earlier work, Nomura (1966) reported also that several species of *Aspergillus* and *Penicillium* are capable of producing enzymes metabolizing limonin and nomilin, but their metabolites have not been identified. Recently, we observed also the growth of certain molds on a limonoid medium. The rate of their growth is, however, much slower than that of bacteria.

Since limonin is a bitter principle of citrus juices (Higby, 1938; Emerson, 1948; Kefford, 1959), considerable research has been done on limonoid chemistry. The biochemistry of limonoids, however, is just beginning to be understood. It has been shown that the naturally occurring form of limonin in citrus fruit tissues is the monolactone, limonoate A-ring lactone (Maier and Margileth, 1969). In addition, a limonin hydrolase, which specifically catalyzes the hydrolysis of the D-ring lactone of limonin and other limonoids, has been isolated from citrus seeds and has been shown to be present in the fruit tissues (Maier *et al.*, 1969). This enzyme appears to be responsible for the presence of the limonoate A-ring lactone form of limonin in the fruit tissues. The pre- and postharvest metabolism of limonoate A-ring lactone has been demonstrated with intact citrus fruits (Scott, 1970; Maier *et al.*, 1971) but neither metabolites nor enzymes have been isolated. Hypothetical pathways of limonoid biosynthesis have been proposed based upon the structures of citrus limonoids (Dreyer, 1968; Bennett, 1971), but enzymes of the proposed pathways have yet to be isolated.

It is possible that limonoid metabolism in citrus fruits is similar, at least in some respects, to that of *Arthrobacter globiformis*. Based upon that premise, recent work in this laboratory has resulted in the isolation of 17-dehydrolimonoate A-ring lactone from citrus fruit tissues (Hsu *et al.*, 1972).

Since 17-dehydrolimonoate and 17-dehydrolimonoate A-ring lactone are both nonbitter, attempts were made to remove limonin from orange juices with limonoate dehydrogenase. Preliminary tests showed that in reconstituted navel orange juice adjusted to the pH optimum of the enzyme, essentially all of the limonin was converted to 17-dehydrolimonoate (Hasegawa *et al.*, 1972b). Activity was also good in juice at pH 6.5, provided that the D-ring of limonin was open. Although upward adjustment of the juice pH was necessary, this work shows the feasibility of this overall approach to developing a debittering process for citrus juices.

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