molecular weight peptides (contrast Figures 5 and 6) suggest that condensation of sugar residues is a requisite for color formation. This conclusion is also strongly supported by our earlier observations (Clark and Tannenbaum, 1973) that dehydration of glucose at the hydroxymethyl carbon is strongly correlated with color formation.

Nutritional Significance. While the enzymes and model systems used are not those encountered *in vivo*, some comments can be made on the expected reduction in the nutritive value of foods through losses of amino acids other than lysine, arginine, and histidine. The models of the insulin pigments (Figures 5 and 6) demonstrate the incompleteness of the *in vitro* enzymatic digestion of the peptide near the bound carbohydrate. The low molecular weight pigment (Figure 6) apparently has undergone extensive cleavage of bonds B-6 to -7, B-3 to -4, and A-5 to -6. These bonds appear to be intact in the higher molecular weight pigment which contains a greater number of nearby sugar residues that can sterically interfere with hydrolysis.

Boctor and Harper (1968) demonstrated that the nutritional value of heat treated egg–albumin–glucose was lower than that which could be explained on the basis of the loss of fluorodinitrobenzene (FDNB) reactive lysine, and also that some FDNB-reactive lysine was excreted. Similarly, Dvorak (1968) also found that there could be FDNB-reactive lysine that was not nutritionally available. These observations agree with the results of Ford and Salter (1966) who found that the availability of several amino acids in heated cod fillets was lower than could be accounted for by direct losses. Thus, the observed difference in the susceptibility of the insulin pigments to enzymatic hydrolysis is a direct example of a nonspecific loss of amino acids (to undigested peptides) that contributes to deterioration of the protein-nutritive value of foods.

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Metabolism of Limonoids. Isolation and Characterization of Deoxylimonin Hydrolase from *Pseudomonas*

Shin Hasegawa,* V. P. Maier, Susan N. Border, and Raymond D. Bennett

A new limonoid-metabolizing enzyme, deoxylimonin hydrolase, was isolated from cell-free extracts of *Pseudomonas* 321-18 by $(NH_4)_2SO_4$ precipitation followed by three applications of DEAE-cellulose column chromatography. This enzyme catalyzes the hydrolysis of deoxylimonin to form deoxylimonic acid and apparently attacks only the closed D ring of deoxylimonin. The enzyme requires no cofactor and its activity is optimal at pH 8.0–8.5. The enzyme possesses sulfhydryl groups, which are involved in its catalytic action.

Limonin (I) is the intensely bitter triterpenoid dilactone present in citrus seeds (Arigoni *et al.*, 1960; Barton *et al.*, 1961) or formed during the delayed bittering of citrus juices (Maier and Beverly, 1968).

Because of a limonin bitterness problem in certain citrus juices and other processed products, the metabolic pathways of limonoids in plants and microorganisms have been intensively investigated (Nomura, 1966; Flavian and Levi, 1970; Chandler, 1971; Maier *et al.*, 1971; Hasegawa *et al.*, 1972a,b, 1974a,b). Limonoate A-ring lactone (II), the major limonoid in citrus fruit tissues, decreases in

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concentration with advancing maturity of the fruits (Higby, 1938; Scott, 1970) or during post-harvest treatments of navel oranges with ethylene or its analogs (Maier *et al.*, 1971), indicating that the fruit tissues possess limonoid-degrading systems. Recently, we have isolated the metabolite 17-dehydrolimonoate A-ring lactone (III) from various parts of citrus (Hsu *et al.*, 1973) and have also detected limonoate dehydrogenase activity in albedo tissues of navel oranges (Hasegawa *et al.*, 1974a).

In bacteria, limonoate and limonoate A-ring lactone have been shown to be metabolized through two pathways: one through 17-dehydrolimonoate or 17-dehydrolimonoate A-ring lactone, and the other through deoxylimonin (Hasegawa *et al.*, 1972a,b; 1974a,b). During the course of our investigation on bacterial limonoid metabolism, we have found and isolated a new enzyme, deoxyli-

monin hydrolase, from cell-free extracts of *Pseudomonas* 321-18, which catalyzes the hydrolysis of deoxylimonin to form deoxylimonic acid. This paper reports the isolation and characterization of this enzyme.

deoxylimonin   deoxylimonate

deoxylimonin hydrolase
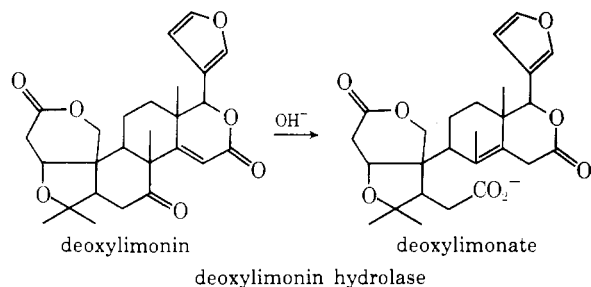
EXPERIMENTAL SECTION

Assay Methods. Protein concentration was measured by the procedure of Lowry *et al.* (1951). The molar extinction coefficient of deoxylimonin at 230 mμ is 8050, whereas that of deoxylimonic acid is 450. Thus, deoxylimonin hydrolase activity was assayed by following the decrease in absorbance at 230 mμ with a Varian Techtron 635. Activity was assayed in 1 ml of a reaction mixture consisting of 0.1 mM deoxylimonin, 0.1 M phosphate buffer at pH 8.0 and 10-25 munits of enzyme. The reaction was carried out at 23° in a standard silica cuvette with 1-cm light path. One unit of deoxylimonin hydrolase is defined as the amount which catalyzes the production of 1 μmol of deoxylimonic acid/min under the above conditions.

Growth of Cells. A 2-l. erlenmeyer flask containing 200 ml of 0.3% of sodium limonoate in a mineral salt medium was inoculated with 10 ml of a 48-hr culture of *Pseudomonas* 321-18. Incubation was carried out at room temperature on a shaker. After 48-72 hr of incubation, cells were collected by centrifugation at 20,000g for 10 min and washed with cold 0.1 M phosphate buffer at pH 7.0, and kept in a freezer until used.

Isolation of Deoxylimonin Hydrolase. The cells were suspended in approximately 15 vol of 0.1 M phosphate buffer at pH 7.0 containing 1 mM dithiothreitol and disrupted for 4 min in a 50-ml Rosset flask with a 20-kHz Branson Sonifier J-22 model. The resulting mixture was centrifuged at 20,000g for 10 min and the supernatant was used as the starting material for purification. All preparative procedures were carried out at 2-4°.

The supernatant was then brought to 90% saturation with $(NH_4)_2SO_4$ by addition of solid salt. The precipitate was collected by centrifugation at 20,000g for 15 min and dissolved in a minimum portion of 0.01 M phosphate buffer at pH 6.2 containing 0.5 mM dithiothreitol. The solution was then dialyzed against 0.01 M phosphate buffer at pH 6.2 containing 0.05 mM dithiothreitol for 2 hr.

The dialysate was loaded on a 2.4 × 25 cm jacketed column of DEAE-cellulose previously equilibrated with 0.01 M phosphate buffer at pH 6.2. The column was eluted with a linear gradient consisting of 150 ml of 0.01 M phosphate buffer at pH 6.2 and 150 ml of the same buffer containing 0.8 M NaCl. Fractions (5 ml) were collected. Fractions 43-55 containing enzyme activity were combined, dialyzed against 0.01 M phosphate buffer at pH 7.0, and freeze-dried.

The freeze-dried sample was dissolved in 3.4 ml of 0.01 M phosphate buffer at pH 7.0 and applied to the top of a 1.5 × 20 cm column of DEAE-cellulose which had been equilibrated with 0.01 M phosphate buffer at pH 7.0. The column was washed with a linear gradient using 150 ml of 0.01 M phosphate buffer at pH 7.0 and 150 ml of the same buffer containing 0.8 M NaCl. Fractions 27-32 containing enzyme activity were pooled, dialyzed against 0.01 M phosphate buffer at pH 7.5, and freeze-dried.

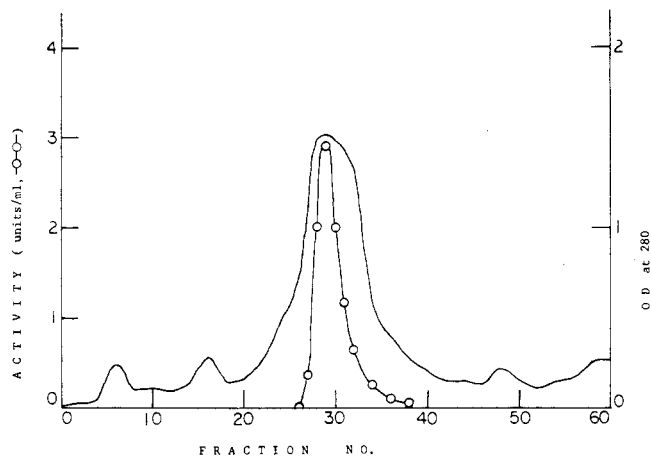The resulting sample was again fractionated on the above column. The column was eluted in a manner simi-

Figure 1. DEAE-cellulose column chromatography of deoxylimonin hydrolase (second column). The procedure used is described in the text.
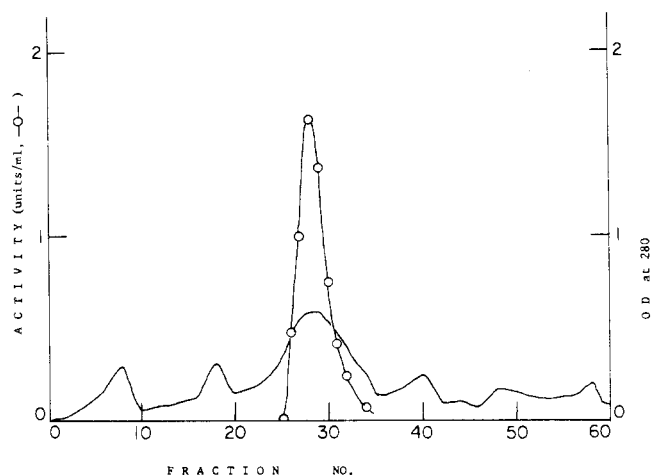

Figure 2. DEAE-cellulose column chromatography of deoxylimonin hydrolase (third column). The procedure used is described in the text.

lar to the above except that this time the column was eluted at pH 7.5. Fractions 26-31 were pooled, dialyzed against 0.01 M phosphate buffer at pH 7.0, and used for characterization of the enzyme.

Identification of Reaction Product. Silica gel G plates were used for thin-layer chromatography (tlc). Chromatograms were developed with (1) ethanol, (2) benzene-ethanol-water-acetic acid (200:47:15:1), (3) cyclohexane-ethyl acetate (30:70) and (4) dichloromethane-methanol (96:4), and were revealed by spraying with 50% H_2SO_4 and heating or by spraying with Ehrlich's reagent and exposing to HCl gas. Nmr spectra were run at 100 MHz using a Jeolco JNM-PS-100 spectrometer.

RESULTS

Purification of Enzyme. The deoxylimonin hydrolase was purified from cell-free extracts of *Pseudomonas* 321-18 by $(NH_4)_2SO_4$ precipitation followed by three applications of DEAE-cellulose column chromatography. Typical elution patterns from fractionation of the enzyme are shown in Figures 1 and 2. The purification steps are summarized in Table I. The overall purification resulted in a 326-fold increase in specific activity over the crude extract, and the recovery was 71%. The final preparation, fractions 26-30 in Figure 2, was used for the characterization of the enzyme.

Table I. Purification of Deoxylimonin Hydrolase

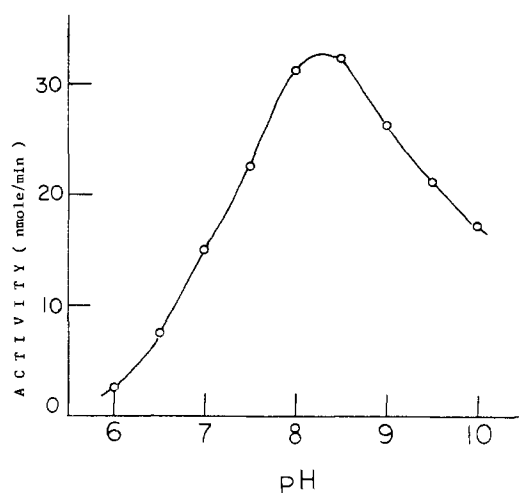| Treatments	Act., units	Protein, mg	Sp act., units/mg of protein	Purification	Recovery, %
Crude extracts	41.1	513.3	0.08	1	100
1st DEAE-cellulose column	38.8	60.52	0.64	8	94.3
2nd DEAE-cellulose column	37.3	2.35	15.8	198	90.6
3rd DEAE-cellulose column	29.3	1.12	26.1	326	71.1

Figure 3. Effects of pH on deoxylimonin hydrolase activity. The reaction mixture consisted of 0.1 mM deoxylimonin, 0.1 M phosphate–Tris buffer, and 0.032 unit of enzyme.

Figure 4. Heat stability of deoxylimonin hydrolase at various hydrogen ion concentrations. Enzyme solutions in 0.05 M phosphate buffer at various pH values were treated at 41° for 5 min. The residual activity was assayed by the method described in the text.

Characterization of Reaction Product. Two milligrams of deoxylimonin were dissolved in 40 μl of acetonitrile and added slowly to 10 ml of enzyme–buffer solution (0.1 M phosphate buffer at pH 8.0 containing 0.05 unit of the enzyme) at a rate of 10 μl/5 min. The mixture was incubated at 30° overnight. Then, the reaction mixture was acidified to pH 2.0 with 1 N HCl and two 5-ml portions of chloroform. The chloroform fraction was extracted successively with 2 ml of 5% KHCO₃ and 2 ml of H₂O. The two aqueous extracts were combined, acidified to pH 2.0 with 1 N HCl, and extracted with two 2-ml portions of ethyl acetate. An nmr spectrum of the product in deuterioacetone was identical with that of an authentic sample of deoxylimonic acid. The spectrum showed identifiable signals at 7.55 (α-furans), 6.50 (β-furan), 5.31 (H-17), 4.71 (H-1), 4.41 and 4.25 (H-19), 3.46 (H-15), 1.89, 1.13, and 1.04 (C-methyls).

The product was also examined by tlc. It was positive to Ehrlich's reagent and had the same mobility as authentic deoxylimonic acid in three tlc systems (Hasegawa et al., 1972a). It was also examined in the form of its methyl ester, which corresponded chromatographically to methyl deoxylimonoate in two tlc systems (Hasegawa et al., 1972a).

Effects of pH and Temperature. A study of deoxylimonin hydrolase activity at various hydrogen ion concentrations showed that the optimum was in the range of 8.0–8.5 (Figure 3).

The results of heat-stability studies showed that a solution of the enzyme containing 0.05 unit/ml of 0.05 M phosphate buffer at pH 7.0 was stable for 5 min at temperatures below 35°, but was almost completely inactivated by heating at 55° for 5 min. Approximately 50% of the
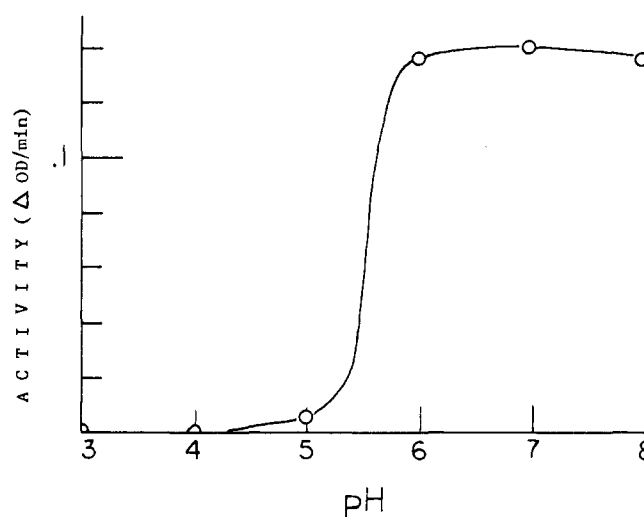
activity was lost after 5 min at 47°. The results of heat treatments of the enzyme at various pH values are shown in Figure 4. When the pH was 6 or higher the enzyme was stable after 5 min at 41°. There was a sharp decrease in stability between pH 5 and 6, and the activity was lost at pH values below 5.

Effects of Inhibitors. EDTA and other metal-binding agents were ineffective as inhibitors (Table II). As expected, metal ions such as CaCl₂, MgCl₂, ZnCl₂, and FeCl₂ also had no effect on enzyme activity.

The inhibition of deoxylimonin hydrolase by reagents that react with sulfhydryl groups was examined. As shown in Table II, the enzyme was completely inhibited by 0.1 mM p-chloromercuribenzoate and 0.1 mM HgCl₂.

Limonin was found to be an effective inhibitor. Addition of 0.05 mM of limonin to the reaction mixture inhibited activity 36% and the inhibition increased to 52% when 0.1 mM limonin was added (Table III). On the other hand, limonoate had no effect on the rate of the deoxylimonin hydrolase reaction.

DISCUSSION

Deoxylimonin hydrolase was isolated from cell-free extracts of Pseudomonas 321-18. This organism grows well on a limonoate–mineral salt medium and produces deoxylimonin and deoxylimonic acid in the growth medium (Hasegawa et al., 1974b). The enzyme responsible for the formation of deoxylimonin has not been isolated. The isolation of deoxylimonin hydrolase, however, confirmed the previous findings that limonoate is metabolized through a

Table II. Effects of Inhibitors on Deoxylimonin Hydrolase Activity

Inhibitors	Concn, mM	Act., ΔOD/min	Inhibition, %
Expt. 1			
PCMB	0.05	0.062	36
	0.1	0	100
HgCl₂	0.1	0	100
Control		0.097	
Expt. 2			
EDTA	1	0.183	0
	5	0.183	0
NaN₃	1	0.180	1.6
	5	0.180	1.6
KCN	5	0.182	0.6
Control		0.183	

Table III. Effects of Limonin and Limonoate on Deoxylimonin Hydrolase

Limonoids	Concn, mM	Act., ΔOD/min	Inhibition, %
Limonin	0.05	0.100	36
	0.1	0.074	52.6
Limonoate	0.05	0.154	1.2
	0.1	0.158	−1.2
Control		0.156	

deoxylimonin–deoxylimonic acid pathway (Hasegawa et al., 1974b).

The enzyme catalyzes the addition of one molecule of water to deoxylimonin, but the reaction is not a simple hydrolysis, as a carbon–carbon bond is cleaved and a double bond shifted. The reaction can also be carried out by heating deoxylimonin with strong base and a mechanism, involving attack by hydroxide ion on the 7-keto group, has been proposed (Barton et al., 1961). The conditions required for this chemical reaction, however, are much more vigorous than those under which the enzyme is active. A control experiment showed that no deoxylimonic acid was produced by treatment of deoxylimonin under the conditions used for the enzyme reaction. It is quite interesting that sulfhydryl groups are involved in the catalytic action of the enzyme, which is not the case with other common hydrolases.

The question of whether the enzyme would be capable of attacking deoxylimonin if the D ring were open could not be answered directly, because attempts to open the D ring by treatment with base caused the formation of deoxylimonic acid. Limonin D-ring lactone hydrolase was also inactive toward deoxylimonin (Maier et al., 1969). Therefore, an indirect approach was taken, in which limonin and limonoate were used as inhibitors. Enzyme activity was inhibited as much as 52% when equimolar amounts of deoxylimonin and limonin, in which the D ring is closed, were present in the reaction mixture. This indicates that limonin has an affinity for the enzyme. On the other hand, no inhibition was observed with limonoate, in which both the A and D rings are open. Since the enzyme attacks methyl deoxyisoobacunoate (IV), it would appear that an open A ring should not prevent inhibition. It

seems likely, therefore, that the open D ring of limonoate is responsible for its lack of inhibitory activity (affinity). This suggests that the enzyme may not attack an open D ring of deoxylimonin.

limonin (I)

limonoate A-ring lactone (II)

17-dehydrolimonate A-ring lactone (III)

methyl deoxyisoobacunoate (IV)
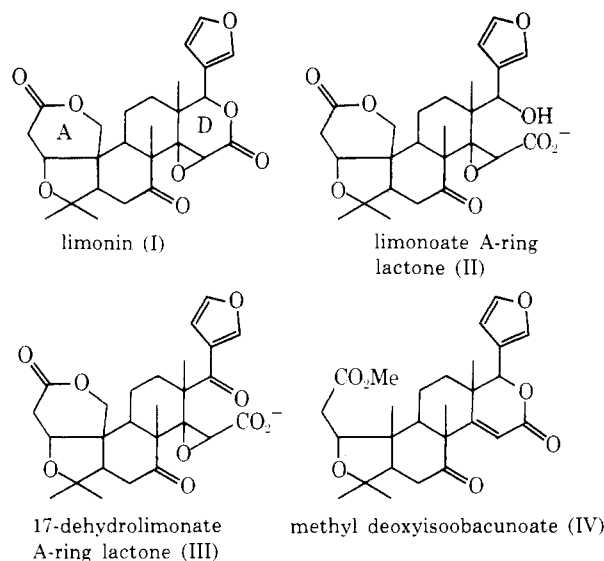
Albedo tissues of navel oranges possess a limonoate dehydrogenase which converts limonoate A-ring lactone, the major limonoid in the fruit tissues, to 17-dehydrolimonoate A-ring lactone (Hasegawa et al., 1974a). Recent experiments in our laboratory have indicated that there may be also deoxylimonin hydrolase activity in citrus. Based on knowledge obtained in this study, our investigation on this matter is underway.

ACKNOWLEDGMENT

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