stituents could contribute to their bitter taste.

Isolation, identification, and quantitation of oxidized fatty acid constituents of SPC's partially characterized are underway. Hopefully, the distinguishing properties of SPC-A and SPC-B will be characterized.

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Bitterness Prevention in Citrus Juices. Comparative Activities and Stabilities of the Limonoate Dehydrogenases from *Pseudomonas* and *Arthrobacter*

Linda C. Brewster,* Shin Hasegawa, and Vincent P. Maier

A comparison of the limonoate dehydrogenases of *Pseudomonas*-sp. 321-18 and *Arthrobacter globiformis* in orange juices and model systems has demonstrated the wide differences in activity and stability of the two enzymes. Both enzymes have been shown to function in freshly prepared navel orange juice. A comparison of the effectiveness of the two enzymes in reducing the eventual limonin content of freshly prepared navel orange juice revealed that 200 munits/ml of juice of the limonoate dehydrogenase of *Pseudomonas* reduced the eventual limonin content of a 21-ppm juice to 3 ppm, a level below the general bitterness threshold, whereas comparable activity levels of the enzyme from *Arthrobacter* caused substantially smaller decreases in eventual limonin content. This wide difference in activity at low pH is explained by the instability of the limonoate dehydrogenase of *Arthrobacter* at pH 3.5 and the relative stability of the *Pseudomonas* enzyme.

Limonin bitterness in citrus juices continues to be an important economic problem for the citrus industry, especially in California. As citrus production and processing increase, the problem becomes more acute. Recently, two methods were reported which reduced limonin-caused bitterness in citrus products. One is the metabolic debittering method of Maier et al. (1973) which reduces the amount of limonin precursor (Maier and Margileth, 1969) in the intact fruit. The other is an enzymatic juice treatment which converts the limonin precursor to a nonbitter product (Hasegawa et al., 1973). In the latter method, the enzyme limonoate dehydrogenase of *Arthrobacter globiformis* (LD-Ag), a limonoate:NAD^+ oxidoreductase, is used to convert the limonin precursor present in the juice, limonoate A-ring lactone (LARL), to nonbitter 17-dehydrolimonoate A-ring lactone (17-DLARL), and thereby prevent the formation of bitter limonin.

Further work to develop a more efficient enzyme system led to the isolation of another limonoate dehydrogenase, from *Pseudomonas*-sp. 321-18 (LD-Ps) (Hasegawa et al., 1974c). This enzyme, a limonate:$NAD(P)^+$ oxidoreductase, also catalyzes the conversion of LARL to 17-DLARL and

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requires Zn ions and sulfhydryl groups for its activity. It differs from LD-Ag in its pH optimum, 8.0 vs. 9.5, cofactor requirements, NAD^+ or $NADP^+$ vs. NAD^+ only, and lower affinity for DEAE-cellulose resins.

This paper reports some of the factors that affect the activity of LD-Ps in orange juice systems, the effectiveness of LD-Ps vs. LD-Ag in orange juice, and the relative stabilities of the two enzymes.

EXPERIMENTAL SECTION

Juice Preparation. Freshly prepared juice from early- to mid-season navel oranges was used in most of the experiments. A test was run to determine the solubility state of limonoids soon after juicing. Juice was prepared on a mechanical reamer and passed through a $^1/_{16}$ in. mesh screen. Immediately after juicing, one aliquot was filtered through Celite while another was left unfiltered. Both aliquots were then acidified and boiled to convert all LARL to limonin for analysis. The total limonin ("eventual limonin content"), determined by the method of Maier and Grant (1970), was 25.0 ppm in the filtered aliquot and 24.6 ppm in the unfiltered, whole juice. Thus, in juice prepared as above, all of the LARL was soluble in the serum immediately after juicing when the enzyme treatments would be initiated.

Due to the unavailability of fresh navel oranges during a major portion of the year, navel orange juice concentrate diluted to single strength was used for some of the experiments. When diluted concentrate was used, the D-ring of limonin was first opened, since the enzyme attacks only the open D-ring form as in LARL. To do this, the juice was adjusted to pH 8 with NaOH and either heated on a steam bath for 15 min or held for several hours at room temperature.

Enzyme Preparation. *Pseudomonas*-sp. 321-18 (NRRL B-5777R) and *Arthrobacter globiformis* were grown on media containing limonoate as the sole carbon source. Limonoate dehydrogenase was partially purified from cell-free extracts of *Pseudomonas* or *Arthrobacter* by $(NH_4)_2SO_4$ precipitation followed by dialysis and separation on one DEAE-cellulose column by the method of Hasegawa (Hasegawa et al., 1972, 1974c). One unit of enzyme is defined as the amount which catalyzes the formation of 1 μmol of NADPH (LD-Ps) or NADH (LD-Ag) per min at the pH optimum of the enzyme (pH 8.0 for LD-Ps, pH 9.5 for LD-Ag).

Juice Treatment. All experiments on fresh juice were at its natural pH. Juice from concentrate was adjusted to the desired pH with HCl or NaOH after hydrolysis and just prior to enzyme addition. The enzyme and cofactors were added to 5- to 20-g aliquots of the juice soon after juicing. The juice was stirred and held loosely covered at 18 to 24°C during the enzyme treatment period. An aliquot of heat-inactivated enzyme was added to the control samples.

A preliminary study of the extent of reaction in raw juice as a function of time (60 munits of LD/ml of juice; 1 μmol/ml each of NAD^+ and $NADP^+$, 18°C) showed that after 15, 30, 60, and 120 min the enzyme-catalyzed decreases in eventual limonin content were 38, 67, 80, and 97%, respectively, of the total decrease observed after 24 and 48 hr. Most experiments were therefore run for 1–2 hr.

Limonoid Analyses. After incubation, the reaction mixtures were acidified to pH 2 with HCl and boiled for 10 min to convert any remaining LARL into limonin. The standard limonin procedure of Maier and Grant (1970) was followed, analyzing for total juice limonin. Since the residual LARL of the reaction mixtures and controls was converted to limonin before analysis, the limonin levels reported show the "eventual limonin content" of the juice after the "delayed bitterness" reaction is complete.

Table I. Effect of Enzyme Concentration on the Reduction of Eventual Limonin Content of Fresh Navel Orange Juice by Limonoate Dehydrogenase (LD) of *Pseudomonas*

Treatment[a]			
LD, munit/ml of juice	NAD^+ added, μmol/ml of juice	Length of incubation, hr	Eventual limonin content, ppm
0	0	0	21.3
0	1.0	1	21.0
50	5.0	1	12.1
100	5.0	1	7.4
200	5.0	1	3.3

[a] Incubated at 23° C. Natural pH of the juice was 4.0.

The reaction product (17-DLARL) was extracted from fresh navel orange juice treated with LD, and was identified by thin-layer chromatography (TLC); the product and its methyl ester were co-chromatographed with authentic compounds (Hasegawa et al., 1972).

Enzyme Stability Studies. Small aliquots of purified enzyme were incubated in 0.1 *M* buffers (citrate–phosphate buffer, pH 3.0–7.0; phosphate buffer, pH 6.0–8.0; Tris-HCl buffer, pH 7.5–9.0; and glycine–NaOH buffer, pH 9.0–10.5) in a constant temperature bath at 23°C for 5 min. Residual activity was assayed in 1 ml of a reaction mixture consisting of 0.01 *M* limonoate, 0.1 ml of LD (10 munits original activity), 0.15 *M* potassium phosphate buffer at pH 8.0 (LD-Ps) or Tris-HCl buffer at pH 9.5 (LD-Ag), and 0.002 *M* $NADP^+$ (LD-Ps) or NAD^+ (LD-Ag).

RESULTS AND DISCUSSION

Factors Affecting the Activity of *Pseudomonas* LD in Orange Juice. Table I shows the reduction in limonin content of freshly prepared navel orange juice treated with LD-Ps for 1 hr. The extent of reduction was directly related to the amount of enzyme added. With the addition of 100 munits of LD/ml of juice, the eventual limonin content of the juice was reduced from 21.3 to 7.4 ppm; with 200 munits of LD/ml of juice, limonin content was reduced to 3.3 ppm, a level which is below the general bitterness threshold range (Guadagni et al., 1973). The conversion of LARL to 17-DLARL was confirmed by TLC.

Direct addition of LD to the raw juice would be the simplest and most convenient use of the enzyme in preventing limonin bitterness in navel orange juice. For this, the enzyme must have sufficient stability and catalytic activity at pH 3–4 to convert most of the LARL to 17-DLARL in the presence of the competing acid- and enzyme-catalyzed conversion of LARL to limonin (the "delayed bitterness" reaction). Table I shows that juice of this high eventual limonin content requires an enzyme activity of about 200 munits/ml of juice to produce a nonbitter juice in 1 hr. Higher levels of enzyme activity would appear to cause further conversion of LARL to 17-DLARL since all of the LARL present in whole juice was shown to be soluble and available as substrate for the enzyme (see Experimental Section). For juices of lower eventual limonin content than that of this experiment, lower levels of enzyme activity would achieve adequate conversion.

Table II shows the effect of cofactors on the LD-Ps-catalyzed reaction in 1 hr. Added cofactors with denatured LD had no effect on the eventual limonin content of the juice. Addition of enzyme without added cofactors decreased the eventual limonin content of the juice by 31%.

Table II. Effect of Cofactor Addition on the Action of Limonoate Dehydrogenase (LD-Ps) in Navel Orange Juice

Treatment[a]			Eventual limonin content, ppm	Decrease in limonin content, %
LD, munit/ml of juice	Cofactor added, μmol/ml of juice	Length of incubation, hr		
0	0	0	21.3	0
0	1.0 NAD^+	1	21.0	0
0	1.0 $NADP^+$	1	22.7	0
100	0	1	14.6	31
100	1.0 NAD^+	1	10.1	52
100	5.0 NAD^+	1	7.4	63
100	1.0 $NADP^+$	1	6.0	72

[a] Incubated at 23°C. Natural pH of the juice was 4.0.

Table III. Effect of Diaphorase on Limonoate Dehydrogenase (LD-Ps) Treatment of Fresh Navel Orange Juice

Treatment[a]				
LD, munit/ml of juice	Cofactor added,[b] μmol/ml of juice	Diaphorase, unit/ml of juice	Eventual limonin content, ppm	Decrease in limonin content, %
0	0	0	12.8	0
25	0	0	10.7	16
25	1.0	0	6.7	48
25	0	0.1	9.9	23
25	0	1.0	7.7	40

[a] Incubated for 90 min at 24°C. Natural pH of the juice was 3.8. [b] Equal quantities of both NAD^+ and $NADP^+$ were added.

In the presence of 100 munits of enzyme/ml of juice, the addition of 1 and 5 μmol of NAD^+/ml reduced the eventual limonin content by 52 and 63%, respectively. These results indicate that the raw juice contained enough cofactor(s) to support substantial conversion of LARL to 17-DLARL, but not enough for optimum activity. Added cofactor, therefore, allowed more efficient use of the enzyme.

Table II also shows that addition of $NADP^+$ (1 μmol/ml of juice) caused a much greater reduction in the eventual limonin content than did addition of a like molar amount of NAD^+. This result agreed with that of the enzyme in a model system; with $NADP^+$, the reaction rate was twice that with NAD^+ (Hasegawa et al., 1974c).

Since added cofactors substantially increased LD activity in the juice, a method was sought to recycle the native cofactors for increased efficiency. Diaphorase is one enzyme that could be used for this purpose. It oxidizes NADH and NADPH to NAD^+ and $NADP^+$ in the presence of any of a variety of electron acceptors. Table III shows that the effect from 1 unit of diaphorase (Worthington) per ml of juice was almost the same as that from 1 μmol of both NAD^+ and $NADP^+$ per ml of juice, and 2.5 times that from no added cofactor in juice. Thus, recycling the reduced cofactors of the LD-catalyzed reaction appears to be a feasible alternative to direct addition of NAD^+ or $NADP^+$.

Table IV shows the relationship between the pH of the juice and decrease in eventual limonin content. This study was made with hydrolyzed, reconstituted, navel orange juice concentrate. The effect of pH was substantial. Under equivalent conditions the decreases in eventual limonin content at pH 7.5, 5.0, and 3.5 were 80, 37, and 15%, respectively. Thus, the enzyme did function at the low natural juice pH, but far from optimally. However, the extent of the reaction at pH 3.5 could be increased by

Table IV. Effect of pH on Limonoate Dehydrogenase (LD-Ps) Treatment of Reconstituted Navel Orange Juice Concentrate

Treatment[a]				
pH	LD, munit/ml of juice	$NADP^+$ added, μmol/ml of juice	Eventual limonin content, ppm	Decrease in limonin content, %
7.5	0	2.0	17.5	0
7.5	11.8	2.0	3.5	80
7.5	11.8	0	5.4	69
5.0	11.8	2.0	11.0	37
5.0	11.8	0	16.0	9
3.5	11.8	2.0	14.9	15
3.5	11.8	0	16.9	3
3.5	0	10.0	15.6	0
3.5	21.6	10.0	10.5	33

[a] Incubated for 20 hr at 24°C.

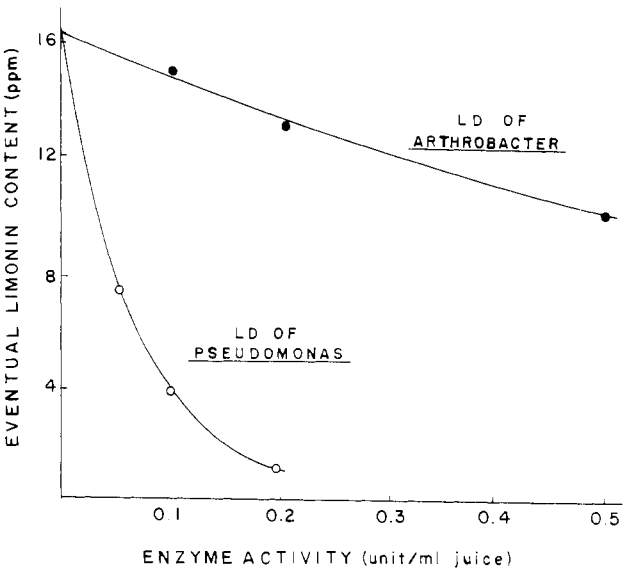

Figure 1. Comparison of effectiveness of limonoate dehydrogenase (LD) enzymes in navel orange juice. Juice treated at pH 3.5 with LD and 5 μmol of NAD^+/ml of juice for 2 hr at 24°C.

increasing the enzyme concentration. Thus, 21.6 munits of enzyme/ml of juice was about twice as effective as 11.8 munits/ml.

Comparative Activities and Stabilities of the LD's from *Arthrobacter* and *Pseudomonas*. After independently studying the limonate dehydrogenases of *Pseudomonas*-sp. 321-18 and *Arthrobacter globiformis* (Hasegawa et al., 1972, 1974c), we undertook comparative studies to investigate the differences in the two enzymes as they relate to the debittering treatment of citrus juice systems.

The reported lower pH optimum and greater activity at low pH of LD-Ps as compared with LD-Ag (Hasegawa et al., 1974c) suggested that the former would have greater activity in orange juice. The data of Figure 1 show that this is indeed the case. At all levels of enzyme activity tested (measured at the pH optimum of each enzyme) LD-Ps reduced the eventual limonin content of the juice substantially, whereas LD-Ag had only a slight effect. In 2 hr, at the activity level of 0.1 unit/ml of juice, the decrease in eventual limonin content was 76% for LD-Ps and 7.5% for LD-Ag; at 0.2 unit/ml of juice the decreases were 92 and 23%, respectively. Doubling the units of activity of LD-Ag about doubled the extent of the reaction. With LD-Ps, however, the extent of the reaction, which was great, was limited not by enzyme but by substrate con-

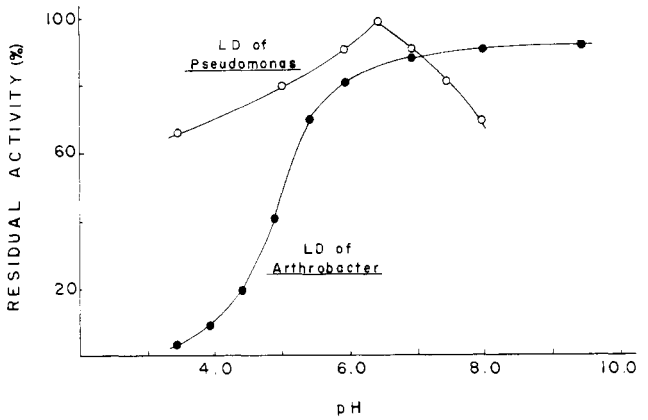

Figure 2. pH stability of limonoate dehydrogenase (LD) enzymes. Enzyme incubated 5 min at 23° C.

centration. At substrate concentrations that were not limiting, the activity of LD-Ps was roughly 15 times that of LD-Ag.

Figure 2 shows that the stabilities of the two enzymes over the pH range 3.5–9.0 differed widely. The differences between the stabilities of the two enzymes at pH values lower than 5.5 could explain the wide difference in their activities in orange juice. At 23°C, the stability of LD-Ps was maximum at pH 6.5 and decreased above and below that pH. LD-Ps retained substantial activity after 5 min at pH 3.5, 23°C. LD-Ag was stable at pH 7.0–9.5 but its stability fell off sharply below pH 6. Very little activity remained after 5 min at pH 3.5.

The limonoate dehydrogenases of *Arthrobacter* and of *Pseudomonas* functioned both in freshly prepared navel orange juice and in hydrolyzed reconstituted navel orange juice concentrate. The LD of *Pseudomonas* was the more stable at low pH and therefore the most effective in reducing the eventual limonin content of the juice. Its effectiveness increased with increasing pH (up to pH 6.5), and with cofactor and enzyme concentration.

With regard to the potential use of limonoate dehydrogenase in citrus juice systems, it should be noted that both a limonoate dehydrogenase enzyme (Hasegawa et al., 1974b) and its reaction product 17-dehydrolimonoate A-ring lactone (Hsu et al., 1973) occur naturally in citrus fruits.

Work is underway to further explore the potential utility of the limonoate dehydrogenase of *Pseudomonas*-sp. 321-18. We are now investigating the growth of the organism and its production of limonoate dehydrogenase (Hasegawa et al., 1974a). Of special interest is whether a sufficiently high yield of enzyme per unit volume of growth media can be attained to make feasible commercial production of the enzyme. The search for new limonoid-degrading microorganisms and new enzymes is continuing. Several new microorganisms which metabolize limonoids have recently been isolated and are being studied.

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Metabolism of Limonoids. Limonin D-Ring Lactone Hydrolase Activity in *Pseudomonas*

Shin Hasegawa

Pseudomonas sp. 321-18 possesses a limonin D-ring lactone hydrolase similar to that isolated from grapefruit seeds. The two enzymes have similar pH optima for both hydrolysis and lactonization, and also have similar substrate specificities. However, they differ greatly in heat resistance.

Limonin (I) is the intensely bitter triterpenoid dilactone present in citrus seeds (Arigoni et al., 1960; Barton et al., 1961). Because of bitterness in certain citrus juices and other processed products, the metabolic pathways of limonoids in citrus and microorganisms have been intensively investigated (Chandler, 1971; Flavian and Levi, 1970; Hasegawa et al., 1972a,b, 1974a–c; Hsu et al., 1973; Nomura, 1966).

We have found that there are at least two limonoid metabolic pathways in bacteria: one through 17-dehydrolimonoate A-ring lactone (IV) and the other through deoxylimonin (III) (Hasegawa et al., 1972b, 1974b). In plants, the 17-dehydrolimonoate pathway has been shown to be present in navel orange tissues (Hasegawa et al., 1974a), and a metabolite of that pathway, 17-

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