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Partial Purification and Characterization of Limonoate Dehydrogenase from *Rhodococcus fascians* for the Degradation of Limonin

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Abstract An extracellular limonoate dehydrogenase was purified 10-fold from a cell-free extract of *Rhodococcus fascians* by ammonium sulfate precipitation, dialysis, and ultrafiltration. This purified dehydrogenase catalyzed the conversion of limonoate to 17-dehydrolimonoate. The enzyme showed optimum activity at pH 8.0 and 40°C, with K_m value of 0.9 μM, and requires Zn ions and sulfhydryl groups for catalytic action. The enzyme activity was inhibited by Hg^{2+} and NaN_3 ions. The degradation of limonin (66%) in Kinnow mandarin juice was successfully demonstrated with partially purified limonoate dehydrogenase. With scale-up preparation of limonoate dehydrogenase, a successful debittering operation of fruit juices appears feasible.

Key words: Purification, limonoate dehydrogenase, limonin, debittering, *R. fascians*, Kinnow mandarin juice

"Delayed bitterness" in citrus juices is a problem of considerable industrial importance [8, 14]. Citrus tissues possess limonin-A-ring lactone (LARL), a non-bitter limonoid, which is a natural precursor of limonin (a highly oxygenated triterpene derivative), the compound responsible for the bitter taste [12]. An intact citrus fruit barely contains limonin; however, after its juice is extracted, the acidic pH conditions facilitate the conversion of LARL to limonin, a process known as delayed bitterness [5]. Limonin contributes a more intense bitterness per unit weight compared to naringin (another component responsible for bitterness) which shows a relatively mild bitterness even at high concentration. Several approaches have been used to avoid limonin formation or to eliminate limonoid from citrus juices [6, 7, 9, 16, 23]. Limonoate dehydrogenase (LDase), an enzyme detected in various microorganisms, can prevent limonin production by catalyzing the oxidation of LARL to the corresponding 17-dehydrolimonoate, a non-bitter derivative. This enzyme has been isolated from *Arthrobacter globiformis* and *Pseudomonas* sp. strain 321-18 [6, 7]. Partial purification and characterization of LDase has been described and the enzyme has been used for the degradation of limonin from citrus fruit juices [for review see 4, 16].

Debittering enzymes of microbial origin have some inherent advantages such as: a) single step hydrolysis, b) operation under mild conditions, (c) cost effectiveness and energy saving, and d) preservation of natural qualities of juice such as flavor, color, vitamins, and organoleptic compounds. These advantages have prompted many workers to isolate and purify enzyme(s) for the debittering of citrus fruit juices [5, 18, 22].

Kinnow mandarin, a hybrid of *Citrus deliciosa* and *Citrus nobilis*, is widely grown in Northern India [10, 17, 21]. The processing of Kinnow fruit to juice has faced several problems due to "bitterness" and "delayed bitterness" [15]. As limonin is a consistent factor in non-acceptance of processed Kinnow juice, we have attempted to optimize the use of a partially purified LDase from *Rhodococcus fascians* for the degradation of limonin in Kinnow mandarin juice.

MATERIALS AND METHODS

Organism and Growth Conditions

Rhodococcus fascians NRRL-B-15096 was procured from the Department of Agriculture Research Service, Peoria, U.S.A. and grown in liquid medium as described earlier [14]. A volume of 50 ml medium in a 250-ml Erlenmeyer flask was adjusted to pH 7.0 with 3 M NaOH and inoculated with 1% of 48 to 72 h old culture. Incubation was carried out at 25°C on an orbital shaker (150 rpm). Fructose (0.4%)

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was used as the sole carbon source, and bacterial growth was monitored by measuring the increase in absorbance of the culture at 600 nm. Cells were collected after 72 h by centrifugation at 15,000 ×g for 10 min, washed with 0.1 M phosphate buffer (pH 7.0), and stored frozen at -20°C. The supernatant was assayed for enzyme activity and 1,000 ml supernatant was used in purification steps.

Preparation and Analysis of Kinnow Juice

Kinnows procured from the local fruit and vegetable markets were washed, peeled, and the juice extracted using a juicer. The juice was filtered through cheese cloth. The limonin [23] and naringin [3] contents in the juice were estimated spectrophotometrically. Vitamin C and total acidity were determined as per the standard method of AOAAC (Association of Official Analytical Chemists, Washington, DC) [1]. Reducing sugars were estimated by the method of Miller [13] using glucose as the standard.

Extraction of Limonin

Limonin was extracted from the juice according to Rouseff and Fischer [20] and the extract was preserved at 4°C.

Enzyme Assay and Protein Determination

The LDase activity was measured according to the method of Hasegawa *et al.* [7] with minor modifications. To 1 ml of limonin (100 µg ml^{-1}), 100 µl of enzyme and 1 ml of the limonin reagent were added. The contents were incubated at 35°C for 90 min. The time course of limonin degradation was measured at absorbance 500 nm and expressed as percent degradation of limonin. One unit of activity is defined as the amount of enzyme that catalyzes degradation of 1 µmol of limonin per min. The protein content in the soluble fraction was determined according to Lowry *et al.* [11] with bovine serum albumin as a standard.

Purification and Characterization of Enzyme

The fermented broth (1 liter) was subjected to 20, 40, 60, 80–100% ammonium sulfate saturation at 4°C. The resultant precipitates were collected, centrifuged (10,000 ×g for 15 min), and redissolved in sodium acetate buffer (0.1 M, pH 7.0). The resultant fractions were then dialyzed to remove the salts and concentrated by ultrafiltration. The activity of the partially purified enzyme was estimated at different period of times, temperatures, and pHs, and kinetic parameters were determined. All assays were performed in triplicate. The reaction mixture consisted of substrate solution (1 ml) and the partially purified LDase (100 µl), incubated at 35°C for 90 min. The resultant color change was estimated spectrophotometrically at 500 nm. The percent degradation of limonin was monitored as a function of debittering duration.

Table 1. Chemical composition of Kinnow mandarin juice.

Parameter	Value
pH	3.9
Total suspended solids (TSS) expressed as Brix (°)	12.1
Acidity (%)	0.88
Reducing sugars (%)	5.76
Limonin[a]	0.35–0.53
Naringin[a]	250
Vitamin C[a]	123
Water (%)	80

[a]Expressed as mg/100 ml.

RESULTS AND DISCUSSION

The chemical characteristics of the fresh juice are summarized in Table 1. It contained 0.35 mg of limonin and 25 mg of naringin per 100 ml, which are the two components responsible for bitterness in citrus fruit juices [2, 14]. Subsequent analysis of pasteurized juice (preserved at 4°C) revealed an increase in limonin content from 0.35 to 0.53 mg, probably due to the conversion of its precursors released during juice extraction [2].

Purification of LDase

By ammonium sulfate precipitation (60–80% saturation), overnight dialysis at 4°C, and ultrafiltration (30 kDa cut off, Millipore, U.S.A.; phosphate buffer pH 8.0, 0.1 M), a 10-fold purification of enzyme could be achieved (Table 2).

Degradation of Limonin as a Function of Incubation and pH

Incubation of purified enzyme with standard limonin for 140 min at 35°C (pH 8.0) resulted in 64% degradation (Table 3) followed by a steady state. This was pH dependent, showing 61% degradation at pH 8.0 (Fig. 1);

Table 2. Purification of limonoate dehydrogenase from *R. fascians*.

Steps	Total protein (mg)	Total activity (IU)	Specific activity (IU mg^{-1})	Purification (fold)	Yield (%)
1. Crude extract	5,152	11,850	2.3	1.0	100
2. $(NH_4)SO_4$ 80%	1,104	10,640	10.0	4.0	88
3. Dialysis (24 h)	689	8,985	13.0	5.6	75
4. Ultrafiltration	320	7,225	22.5	9.8	60

Table 3. Degradation of limonin as a function of incubation in processed Kinnow mandarin juice.

Incubation period (min)	Degradation (%)	
	Limonin	in Kinnow juice
20	26.0	18.0
40	36.2	26.0
60	46.9	32.0
80	55.2	37.0
100	58.6	48.0
120	60.3	54.0
140	64.1	62.0
160	62.1	62.0
180	55.2	62.0

Reaction conditions: Partially purified LDase (100 µl) was individually added to the substrate solution (1 ml) and incubated under the assay conditions *pH* 8.0, 35°C.

however, at lower pH (3–4), relatively small degradation of limonin was obtained. In the case of *Pseudomonas* 321-18, a similar pH-dependent degradation of limonin has been reported [8].

Enzymes involved in limonin degradation usually show an alkaline pH optimum, therefore, the application at acidic pH of fruit juices to degrade limonin has been difficult. An immobilized cell system may be an option to protect enzymes from external pH to improve degradation of substrate, entrapment of the enzyme, or binding to some natural supports [19].

Degradation of Limonin as a Function of Temperature

Incubation of limonin (pH 8.0) at different temperatures (25–45°C) showed maximum degradation (66%) at 40°C (Fig. 2). A similar optimum condition has been reported for *Corynebacterium fascians* [7].

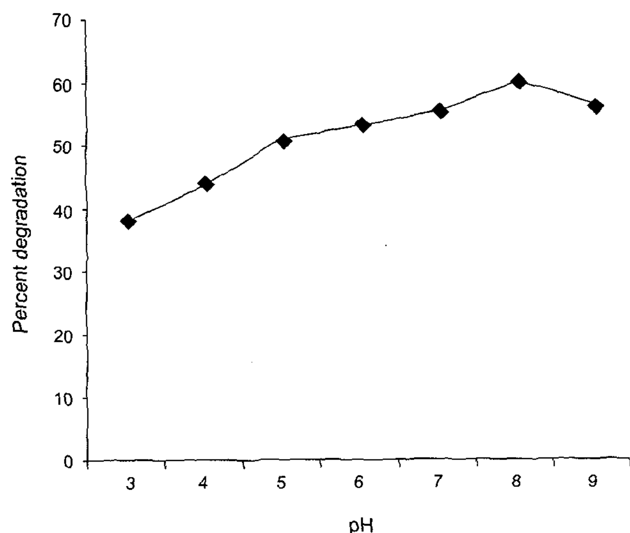

Fig. 1. Degradation of limonin as a function of pH.

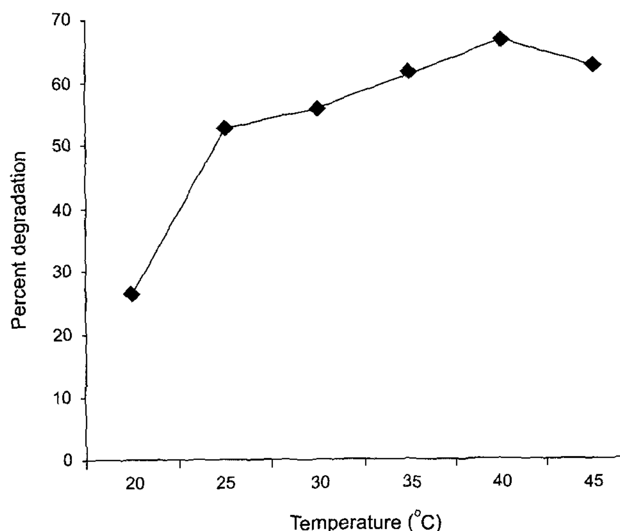

Fig. 2. Degradation of limonin as a function of temperature.

Degradation of Limonin as a Function of Agitation

The percent degradation of limonin remained the same (to stationary phase) regardless of agitation at different rates (150 and 200 rpm). This condition favors economization of the process, as agitation on industrial scale involves additional costs.

Kinetic Characterization

The effects of different limonin concentrations (25, 50, 75, 100, and 125 µg ml^{-1}) on LDase activity were investigated in triplicate and the results were plotted by the method of Lineweaver and Burk. Michaelis constant (K_m) and V_{max} for LDase activity were 0.89 µM and 9.01 µmole/min/mg, respectively (Fig. 3).

Effect of Metal Ions

The effect of divalent metal ions (10^{-3} M) on limonoate dehydrogenase was determined. Ca^{2+}, Mg^{2+}, and Mn^{2+} had no effect on the enzyme activity, but Zn^{2+} increased the relative activity (44%) significantly, suggesting that the enzyme requires Zn^{2+} ions for catalytic action. Addition of 10^{-3} M EDTA to the reaction mixture did not reduce the activity, whereas $HgCl_2$ and NaN_3 inhibited 66% and 32% of enzyme activity, respectively. $HgCl_2$ and NaN_3 were found to be potent inhibitors of the enzyme activity, indicating the possibility that the enzyme requires sulfhydryl group(s) for its activity.

Based on the above studies, a laboratory scale protocol for the degradation of limonin has been established. It was, therefore, considered worthwhile to apply this protocol to degrade limonin in Kinnow mandarin juice [14].

Debittering of Kinnow Fruit Juice

Incubation of Kinnow mandarin juice (50 ml each in 250-ml Erlenmeyer flasks) with LDase (2.5 ml) at optimum

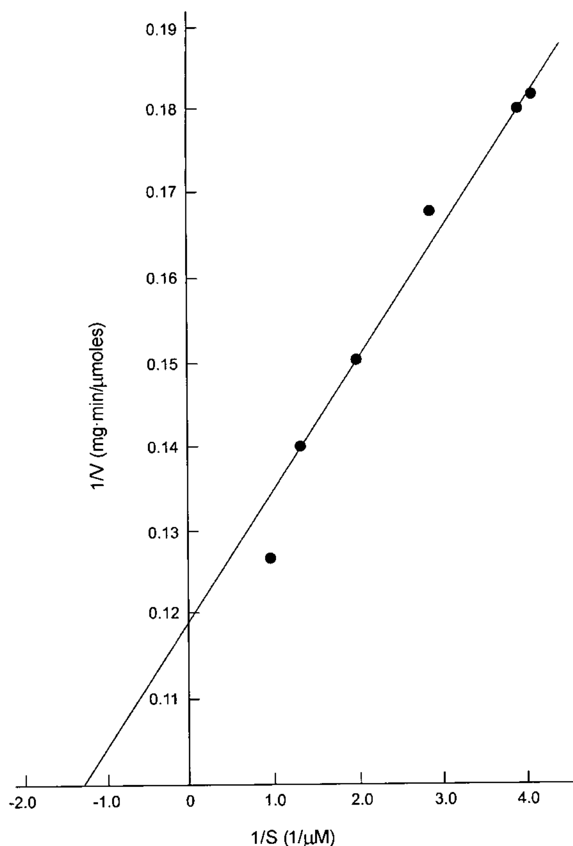

Fig. 3. Lineweaver-Burk plot for determining the K_m of partially purified LDase.

conditions resulted in the maximum degradation (62%) of limonin in 140 min (Table 3), and thereafter a steady state was attained.

CONCLUSION

The observation that the partially purified enzyme successfully degrades limonin (66%) in Kinnow fruit juice was encouraging. The enzyme may be immobilized in natural polymer to protect it from the acidity of the juice, thus increasing limonin degradation. For commercial purposes, it may not be necessary to have highly purified limonoate dehydrogenase preparations. The result of this study marks one step closer to finding a solution to debittering abuse.

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REFERENCES

1. AOAC. Official Methods of Analysis. 14th ed. Association of Official Analytical Chemists, Washington, DC, 1985.
2. Bhalerao, S. D. and G. V. Mulmuley. 1992. Studies of bitterness in orange cultivars. *Indian Food Packers* **12:** 23–29.
3. Davis, W. B. 1947. Determination of flavonones in citrus fruits. *Anal. Chem.* **31:** 426–428.
4. Fellers, P. J. 1989. A review of limonin in grapefruit juice, its relationship to flavor and efforts to reduce it. *J. Sci. Food Agric.* **40:** 389–404.
5. Hasegawa, S., R. D. Bennett, V. P. Maier, and A. D. King. 1972. Limonoate dehydrogenase from *Arthrobacter globiformis*. *J. Agric. Food Chem.* **20:** 1031–1034.
6. Hasegawa, S., L. C. Brewster, and V. P. Maier. 1973. Use of limonoate dehydrogenase of *Arthrobacter globiformis* for the prevention or removal of limonin bitterness in citrus products. *J. Food Sci.* **38:** 1153–1155.
7. Hasegawa, S. and A. D. King. 1983. A species of bacterium producing constitutive enzymes for limonoid metabolism. *J. Agric. Food Chem.* **31:** 807–809.
8. Hasegawa, S. and V. P. Maier. 1990. Biochemistry of limonid citrus juice bitter principles and biochemical debittering processes, pp. 293–308. *In* R. L. Rouseff (ed.), *Bitterness in Foods and Beverages*. Elsevier, New York, NY, U.S.A.
9. Iborra, J. L., A. Manjon, M. Canovas, P. Lazano, and C. Martinez. 1994. Continuous limonin degradation by immobilized *Rhodococcus fascians* cells in k-carragenaan. *Appl. Microbiol. Biotechnol.* **41:** 487–493.
10. Jawanda, J. S. and K. Singh. 1973. Kinnow holds out promise for Punjab. *Punjab Horticulture J.* **13:** 89–93.
11. Lowry, O. H., A. L. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
12. Maier, V. P., S. Hasegawa, and E. Hera. 1969. Limonin-D-ring lactone hydrolase. A new enzyme from Citrus seeds. *Phytochemistry* **8:** 405–407.
13. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* **31:** 426–428.
14. Marwaha, S. S., M. Puri, M. Bhullar, and R. M. Kothari. 1994. Optimization of parameters for hydrolysis of limonin for debittering of Kinnow mandarin juice by *Rhodococcus fascians*. *Enz. Microb. Technol.* **16:** 723–725.
15. Puri, M. 1993. Immobilized enzyme technology for the clarification and debittering of citrus fruit juices with special reference to Kinnows. PhD thesis, Punjabi University, Patiala, India.
16. Puri, M., S. S. Marwaha, R. M. Kothari, and J. F. Kennedy. 1996a. Biochemical basis of bitterness in citrus fruit juices and biotech approaches for debittering. *CRC Rev. Biotechnol.* **16:** 145–155.
17. Puri, M., S. S. Marwaha, and R. M. Kothari. 1996b. Studies on the applicability of alginate entrapped naringinase for the

debittering of Kinnow juice. *Enz. Microbiol. Technol.* **18:** 281–285.
18. Puri, M. and U. C. Banerjee. 2000. Production, purification and characterization of the debittering enzyme naringinase. *Biotechnol. Adv.* **18:** 207–217.
19. Puri, M., M. Seth, S. S. Marwaha, and R. M. Kothari. 2001. Debittering of kinnow mandarin juice by covalently bound naringinase on hen egg white. *Food Biotechnol.* **15:** 13–23.
20. Rouseff, R. L. and J. F. Fischer. 1980. Determination of limonin in citrus juices by high pressure liquid chromatography. *Anal. Chem.* **52:** 1228–1233.
21. Sethi, V., J. C. Anand, and S. K. Saxena. 1980. Kinnow orange in juice and beverage making. *Indian Farming* **Oct-Dec:** 13-16.
22. Ting, S. V. 1958. Enzymatic hydrolysis of naringin in grapefruit. *J. Agric. Food Chem.* **6:** 546–549.
23. Vaks, B. and A. Lifshitz. 1981. Debittering of orange juice by bacteria which degrade limonin. *J. Agric. Food Chem.* **29:** 1258–1261.