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LIMONOATE DEHYDROGENASE FROM *ARTHROBACTER GLOBIFORMIS*: THE NATIVE ENZYME AND ITS N-TERMINAL SEQUENCE

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Key Word Index—*Arthrobacter globiformis*; citrus; bitterness; limonoate dehydrogenase; gene cloning; limonoids; transgenic plants.

Abstract—Bitter limonoids in citrus juice lower the quality and value of commercial juices. Limonoate dehydrogenase converts the precursor of bitter limonin, limonoate A-ring lactone, to nonbitter 17-dehydrolimonoate A-ring lactone. This enzyme was isolated from *Arthrobacter globiformis* cells by a combination of ammonium sulfate fractionation, Cibacron Blue affinity chromatography and DEAE ion exchange HPLC. Using this protocol a 428-fold purification of the enzyme was obtained. Gel filtration HPLC indicated a M_r of 118 000 for the native enzyme. SDS–PAGE indicated an individual subunit M_r of 31 000. N-Terminal sequencing of the protein provided a sequence of the first 16 amino acid residues. Since LDH activity in citrus is very low, cloning the gene for this bacterial enzyme into citrus trees should enhance the natural debittering mechanism in citrus fruit.

INTRODUCTION

The phenomenon of delayed bitterness is an important problem associated with commercial citrus juice production, particularly from navel oranges [1]. Delayed bitterness is caused primarily by limonin [1]. Citrus fruit tissues do not normally contain limonin, but a precursor of limonin, limonoate A-ring lactone (LARL). Shortly after juice extraction, LARL in the expressed juice is converted to limonin. This conversion occurs under acidic conditions and is accelerated by the enzyme, limonin D-ring lactone hydrolase (Scheme 1) [2]. Several approaches have been used to decrease the concentration of bitter limonoids in citrus juice including: passage of juice serum through a microbial bioreactor, addition of bitterness-suppressing agents to juice, and postharvest treatment of fruit with ethylene prior to processing [1]. Currently, commercial processing plants use an adsorption chromatography step to remove bitter limonoids from juice. All of the available methods for debittering juice have drawbacks because they alter or diminish the quality or flavor components of orange juice. The enhancement of naturally occurring debittering pathways in citrus by genetic engineering may provide the best solution to the bitterness problem.

Limonoid metabolizing soil bacteria are a readily available source of enzymes involved in limonin metabolism. Five different metabolic pathways have been identified, the 17-dehydrolimonoid pathway being the major pathway in bacteria [3]. Citrus also possesses this metabolic pathway, although it is a minor pathway [4, 5]. Limonoate dehydrogenase catalyses the conversion of LARL to 17-dehydrolimonoate A-ring lactone (Scheme 1) [3]. Formation of a keto group at the C-17 position inhibits closure of the D-ring thus preventing the conversion of the 17-dehydrolimonoate A-ring lactone back to limonin. This enzyme, isolated from *Arthrobacter globiformis*, has been partially characterized [6].

Our objectives for this work were (1) to purify the limonoate dehydrogenase to homogeneity and determine the molecular mass of the native enzyme and its subunits, and (2) to determine the N-terminal protein sequence to facilitate the construction of an oligonucleotide probe for the gene encoding this enzyme.

RESULTS AND DISCUSSION

Purification of limonoate dehydrogenase

Limonoate dehydrogenase was purified from *Arthrobacter globiformis* cells grown on a Na-limonoate mineral salt liquid medium as a carbon source. A summary of the purification scheme for limonoate dehydrogenase is given in Table 1. Limonoate dehydrogenase activity was recovered in the 30–80% ammonium sulphate fraction. The largest increase in purification was obtained by biospecific elution of the dehydrogenase from the Cibacron Blue dye ligand column with NAD [7]. This step provided a 217-fold increase in purity over the crude protein fraction. The fraction obtained from the dye ligand

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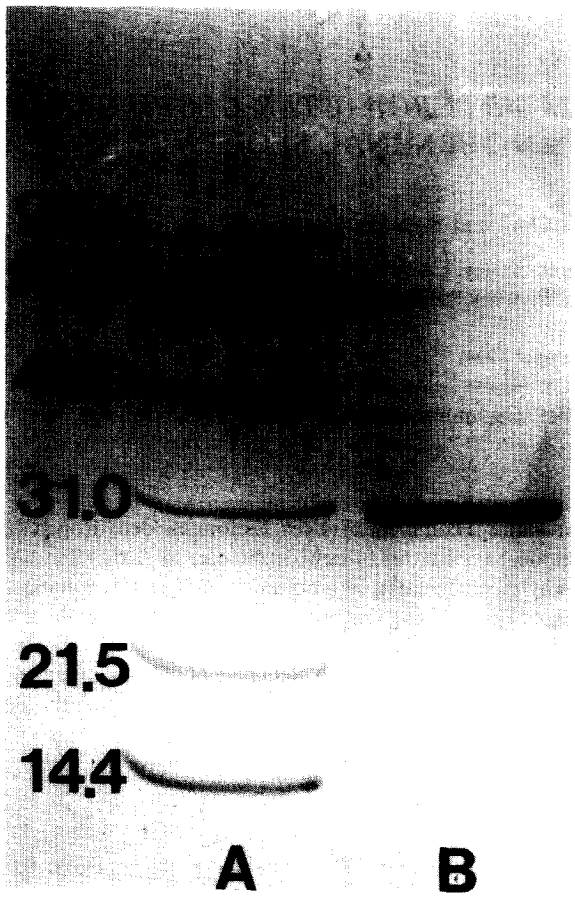


Fig. 1. SDS–PAGE of purified limonoate dehydrogenase. Lane A contains molecular weight standards, lane B contains the purified limonoate dehydrogenase.

chromatography step was applied to a HPLC DEAE ion exchange column and run at pH 8. Limonoate dehydrogenase activity eluted at 390 mM salt on the NaCl gradient. This step increased purity 271-fold over the crude protein preparation. The final step in the purification scheme, HPLC DEAE ion exchange at pH 7.0, provided a 428-fold increase in purity over the ammonium sulfate fraction. In this step limonoate dehydrogenase activity eluted at 360 mM salt on the NaCl gradient. Using this purification procedure a single protein band was obtained when the product was analysed by 1D SDS–PAGE (Fig. 1).

Physical characteristics of limonoate dehydrogenase

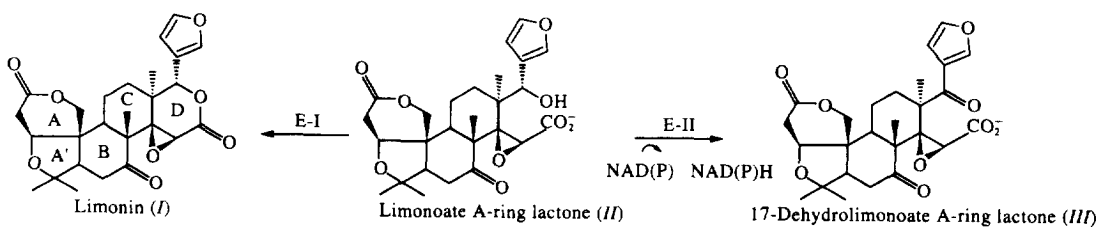
SDS–PAGE revealed a single protein band having a M_r of 31 000 (Fig. 1). Two-dimensional electrophoresis indicated a dominant acidic protein.

The M_r of native limonoate dehydrogenase was determined by HPLC gel filtration chromatography. The enzyme had an apparent M_r of 118 000 as compared to the BioRad gel chromatography standards (Table 2). The native limonoate dehydrogenase enzyme complex was apparently composed of four identical 31 kD subunits.

The 428-fold purification of limonoate dehydrogenase from *Arthrobacter globiformis*, yielding a single 31 kD protein band on SDS–PAGE gels completed the characterization of this enzyme. Previous work in our laboratory with a limonoate dehydrogenase preparation purified 68-fold allowed a partial characterization of the enzyme: a pH optimum of 9.5, NAD is the obligatory hydrogen acceptor, Zn^{2+} ions stimulated enzyme activity, and active site sulphydryls are required for activity [6]. For alcohol dehydrogenase, Zn^{2+} ions have a role in the structural integrity of the enzyme and are also part of the

Table 1. Purification scheme for NAD-dependent limonoate dehydrogenase from *Arthrobacter globiformis*

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Fold purification
Ammonium sulphate 30–80%	140.2	14.46	0.1031	1
Cibacron Blue	0.472	10.57	22.39	217
DEAE pH 8	0.178	4.97	27.94	271
DEAE pH 7	0.091	4.01	44.11	428



Scheme 1. Limonoate A-ring lactone (LARL) is converted to limonin under acidic conditions and this reaction is accelerated by limonin D-ring lactone hydrolase (E-I). LARL is converted to 17-dehydrolimonoate A-ring lactone by limonoate dehydrogenase (E-II).

Table 2. HPLC gel filtration determination of limonoate dehydrogenase molecular mass

Standards	Molecular mass (daltons)	Elution time (min)
Gamma globulin (bovine)	158 000	9.51
Ovalbumin (chick)	44 000	11.04
Myoglobin (horse)	17 000	12.86
Vitamin B-12	1350	14.99
Limonoate dehydrogenase	118 000	9.99

catalytic site [8]. Zinc-dependent alcohol dehydrogenases from bacteria and yeasts are tetrameric, whereas the mammalian and higher plant alcohol dehydrogenases are dimeric [9]. Zinc stimulation of limonoate dehydrogenase activity indicates that zinc is involved in the structure and/or function of this enzyme much like Zn-dependent alcohol dehydrogenases. We have shown the native limonoate hydrogenase from *Arthrobacter* to be tetrameric, consisting of four 31 kD subunits. Thus it is similar to the tetrameric Zn-dependent alcohol dehydrogenases found in other bacteria. Another bacterial enzyme, NAD(P)-dependent L-sorbosone dehydrogenase, a cytosolic enzyme from *Gluconobacter melanogenus*, is a tetrameric enzyme composed of identical 50 kD subunits with an apparent native molecular mass of 190 kD [10].

N-Terminal sequence of limonoate dehydrogenase

Protein sequencing of the subunit's N-terminus indicated the following amino acid sequence:

^{1}Met-Pro-Phe-Asn-Arg-^{6}Leu-Glu-Asn-Glu-Val-Ala-^{12}Ile-Val-Val-Gly-Ala

We are currently in the process of cloning the gene for limonoate dehydrogenase from *Arthrobacter* and intend to use the *Agrobacterium*-mediated transformation and regeneration system developed for citrus [11] to create transgenic navel orange trees. This natural debittering mechanism should be enhanced in transformed navel orange plants expressing the limonoate dehydrogenase gene.

EXPERIMENTAL

Culture of bacteria. Arthrobacter globiformis cells were grown in 400 ml of a pH 7.4 mineral salt medium [6] containing 0.1% Na-limonoate as the sole carbon source. The cells were grown in a 2.8 l culture flask at 22° for 72 hr and then harvested. The bacteria were collected by centrifugation at 10 000 *g* and the pellet washed with 0.1 M phosphate buffer, pH 7. The cells were frozen and stored at −85°.

Enzyme and protein assays. Limonoate dehydrogenase activity was measured in 100 mM Tris–HCl, pH 9.0 at 24°. The reaction volume was 1.0 ml and contained 0.5 mM NAD, 1.0 mM Na-limonoate, and a small aliquot of enzyme. The reaction rate was measured by following the formation of NADH at 340 nm. One unit of enzyme activity was defined as μmol NADH formed min^{-1} at 24° under standard assay conditions. Protein was determined using the dye binding protein assay [12].

Isolation of limonoate dehydrogenase. Approximately 5 g of frozen cells were resuspended in ice-cold homogenizing buffer in a sonication flask. The homogenizing buffer consisted of 0.2 M Tris/HCl containing 5 mM EDTA, 1 mM PMSF, 15 mM 2-mercaptoethanol, 5 mM DTT, pH 8. The sonication flask containing the buffer and bacteria was placed in an ice water bath and the cells were disrupted by sonication using five 2-min pulses with cool down periods of 2 min between each sonication. The sonicate was centrifuged at 20 000 *g* for 20 min to pellet cell debris.

The supernatant was brought to 30% satn with $(NH_4)_2SO_4$ and stirred in an ice bath for 1 hr. The ppt. was removed by centrifugation at 20 000 *g* for 30 min. The supernatant was taken to 80% satn with $(NH_4)_2SO_4$, stirred in an ice bath for 1 hr and the ppt. removed by centrifugation as before. The protein pellet was resuspended in a minimal volume of buffer (50 mM Tris–HCl, 1 mM EDTA, 5 mM DTT, pH 8.0) and desalted on a PD-10 gel filtration column (Pharmacia). The desalted protein fraction was loaded onto a Cibacron Blue dye chromatography column at 1.0 ml min^{-1} (Sigma Chemical Co., St Louis, MO) having a 2.5 ml bed volume and that was equilibrated with 50 mM Tris–HCl, 1 mM EDTA, 2 mM DTT, pH 8 buffer (buffer A). The column was washed with buffer A until the UV absorbance of the eluate returned to baseline. Biospecific elution was carried out by washing the column with buffer A containing 10 mM NAD. The active fractions were pooled and loaded onto an HPLC Bio-Gel TSK-IEX DEAE 5PW (75 × 7.5 mm) column (BioRad Laboratories, Hercules, CA) equilibrated with buffer A. A linear salt gradient, 0–600 mM NaCl, was used to elute the column over a 20 min period at a flow rate of 1.0 ml min^{-1}. The active fractions were desalted and buffer exchanged into 50 mM Tris–HCl, 1 mM EDTA, 2 mM DTT, pH 7 on a PD-10 column. The protein fraction was rechromatographed on the above DEAE ion exchange column at pH 7 and eluted with a linear salt gradient, 0–600 mM NaCl, over a 20 min period at a flow rate of 1.0 ml min^{-1}.

The M_r of the native enzyme was determined by HPLC on a Bio-Sil TSK-250 (300 mm × 7.5 mm) gel filtration column (Bio-Rad Laboratories, Hercules, CA). The column was equilibrated and eluted with 0.05 M Na_2SO_4 and 0.02 M NaH_2PO_4, pH 6.8 buffer. BioRad gel filtration standards were chromatographed under the same conditions. Protein was detected by UV absorbance at 280 nm.

SDS gel electrophoresis. SDS–PAGE was performed using the Laemmli buffer system [13]. A 12% running gel and 5% stacking gel was used for 1-D electrophoresis. Gels were stained with Coomassie Blue.

N-Terminal protein sequencing. The purified enzyme was run on a 17% SDS–PAGE gel. Protein from a developed gel was electroblotted onto PVDF membrane and the band of interest visualized by Coomassie Blue

staining. The 31 kD band was cut out with a razor blade and analysed using a peptide analyser (Beckman LF3400). Protein microsequencing was conducted according to the method of ref. [14].

REFERENCES

1. Hasegawa, S. and Maier, V. P. (1983) *Food Technol.* June, 73.
2. Maier, V. P. and Beverly, G. D. (1968) *J. Food Sci.* **33**, 488.
3. Hasegawa, S. (1989) in *Quality Factors of Fruits and Vegetables*, ACS Symposium Series 405 (Jen, J. J., ed.), p. 84. American Chemical Society, Washington, DC.
4. Hsu, A. S., Hasegawa, S., Maier, V. P. and Bennett, R. D. (1973) *Phytochemistry* **12**, 563.
5. Hasegawa, S., Maier, V. P. and Bennett, R. D. (1974) *Phytochemistry* **13**, 103.
6. Hasegawa, S., Bennett, R. D., Maier, V. P. and King, A. D. (1972) *J. Agric. Food Chem.* **20**, 1031.
7. Scopes, R. K. (1992) in *Methods in Molecular Biology*, Vol. 11: *Practical Protein Chromatography* (Kenney, A. and Fowell, S., eds), p. 209. Humana Press, Totowa, NJ.
8. Vallee, B. L. and Auld, D. S. (1990) *Biochemistry* **29**, 5647.
9. Ammendola, S., Raia, C. A., Caruso, C., Camardella, L., D'Auria, S., De Rosa, M. and Rossi, M. (1992) *Biochemistry* **31**, 12514.
10. Hoshino, T., Sugisawa, T. and Fujiwara, A. (1991) *Agric. Biol. Chem.* **55**, 665.
11. Hidaka, T., Omura, M., Ugaki, M., Tomiyama, M., Kato, A., Ohshima, M. and Motoyoshi, F. (1990) *Japan. J. Breed.* **40**, 199.
12. Bradford, M. (1976) *Analyt. Biochem.* **72**, 248.
13. Laemmli, U. K. (1970) *Nature* **227**, 680.
14. Hirano, H. (1989) *J. Protein Chem.* **8**, 115.