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Determination of Limonin D-Ring Lactone Hydrolase Activity by Solid Phase Extraction with Indirect Fluorescence Detection

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A method for the evaluation of limonin D-ring lactone hydrolase activity is described. The method utilizes solid phase extraction (SPE) for the isolation of limonin A-ring limonoate (LARL), which is subsequently converted to limonin and quantitated by fluorescence. The fluorescence method is capable of quantifying the formation of LARL in concentrations as low as 75 ng and is applicable to both purified and crude enzyme preparations. The coupling of SPE with fluorescence detection allows for the simple and rapid analysis of samples.

KEYWORDS: Limonoid; citrus; solid phase extraction; analysis; hydrolase activity

INTRODUCTION

Freeze damage or physical damage to citrus fruit, including juicing, initiates the formation of the bitter dilactone limonoids from nonbitter monolactone precursors. This phenomenon is referred to as delayed bitterness and is a major problem for both fresh fruit and juice producers worldwide. The presence of bitter limonoids in concentrations in excess of 6 ppm reduces the acceptability of citrus products to consumers and forces fresh fruit producers to discard their fruit; juice producers must rely on juice blending−dilution or removal of bitter limonoids to lower the bitter limonoid content in juice. The identification of commercial cultivars with reduced susceptibility to delayed bitterness is of considerable interest to citrus producers throughout the world. The major limonoid associated with delayed bitterness is limonin. The formation of limonin (**Figure 1**) from limonoate A-ring lactone (LARL) is catalyzed by the acidic pH of citrus juice and the enzyme limonin D-ring lactone hydrolase (LDLH) (*1*).

Several methods for the quantification of LDLH activity have been described including a thin-layer chromatography (TLC) method (*1*) and a radiochemical method (*2*). More recently, Merino et al. (*3*, *4*) described a high-performance liquid chromatography (HPLC)/UV method that utilizes ion pairing C18 chromatography to separate limonin and LARL. This method requires the generation of a LARL stock solution from a limonin solution chromatographically exposed to immobilized LDLH. The concentration of the LARL stock solution is calculated from the change in the limonin concentration. The method is not compatible with the testing of a large number of samples.

In this study, we describe a method (**Figure 2**) for the quantification of LDLH activity in purified and crude enzyme preparations, based on the isolation of the LARL reaction product by solid phase extraction (SPE) followed by it's stoichiometric conversion to limonin by the addition of acid and the quantification of the limonin product by fluorescence detection (*5*).

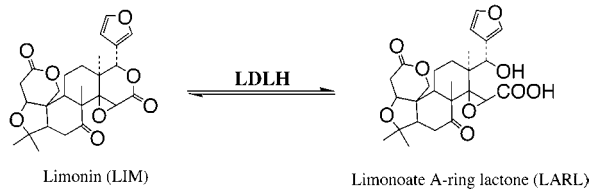

Figure 1. Reaction catalyzed by LDLH.

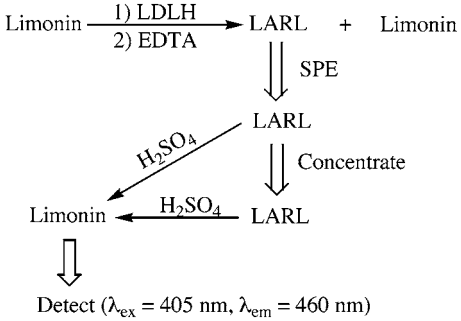

Figure 2. Principles of the method.

MATERIALS AND METHODS

Materials. Water was distilled and deionized. Solvents (Fisher, Pittsburgh, PA) were HPLC grade. Strata C18-E 500 mg, 3 mL columns were obtained from Phenomenex (Torrance, CA). Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad (Hercules, CA), and polyvinylpolypyrrolidone (PVPP) was purchased from Sigma (St. Louis, MO). All other reagents were reagent grade.

Preparation of Stock Solution and Standard Curve. The limonin, available in our laboratory, was used to prepare a limonin (1.045 mg/mL) stock solution in acetonitrile. The stock solution was diluted to

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give a 100 μg/mL limonin working solution in 30% acetonitrile(aq). The working solution was diluted with 30% acetonitrile to provide standard solutions with limonoid concentrations ranging from 5 to 80 μg/mL.

Purification of Limonin D-Ring Lactone Hydrolase from Valencia Seeds. Dried Valencia orange seeds (200 g, Sunkist Growers, Ontario, CA) were ground to a fine powder in the presence of dry ice using a blender. The resulting powder was suspended in 3 volumes of an aqueous mixture of 1% NaCl, 1% PVPP, and 0.5% SDS made up in water. After the mixture was incubated (12 h, 4 °C), the suspension was filtered through cheesecloth and clarified (20 000*g*, 10 min, 10 °C). The supernatant was transferred to a separatory funnel, and the aqueous layer was collected and filtered through sand. The resulting liquid was further clarified by centrifugation (26 000*g*, 10 min, 10 °C). The clarified liquid was cooled to 0 °C, and chilled acetone (−20 °C) was added to a final concentration of 25%. After the precipitate was allowed to form for 20 min, the slurry was subjected to centrifugation (12 000*g*, 10 min, 10 °C) and the supernatant was decanted. The precipitate was washed [2× acetone (−20 °C)] and dried under nitrogen. The solid was suspended in 20 mL of 10 mM phosphate, pH 7.2 (buffer A), and placed on a platform shaker for 20 min. The solution was clarified (26 000*g*, 10 min, 10 °C), the solution was collected, and the extraction was repeated.

The extracts were combined and desalted by fast protein liquid chromatography (FPLC, Amersham, Piscataway, NJ). The system was equipped with a conductivity monitor to monitor the salt gradient and a UV detector set at A_{280}. Desalting was conducted on a G-25M column (Amersham) (26 mm × 270 mm, flow rate = 20 mL/min). LDLH was purified to homogeneity from the desalted extract by FPLC according to the method of Maier et al. (*1*) with the substitution of DEAE-Sepharose (Amersham) (50 mm × 40 mm column) for DEAE-Sephadex. Three successive ion exchange chromatography (IEC) runs were conducted, and following each IEC run, active fractions were pooled together and desalted into the buffer of the next IEC step. This method yielded 1−2 mg of purified protein. The LDLH purity was estimated by SDS-polyacrylamide gel electrophoresis using a 5% stacking and a 12% separation gel. A Tris-glycine discontinuous buffer system was used in a mini-protein II slab gel apparatus (Bio-Rad). The protein was visualized by Coomassie staining.

Preparation of Crude Seed Extracts. Seeds were harvested from fruit obtained from the University of California at Davis Lindcove Research Center (Exeter, CA). Seeds [*Poncirus trifoliate*; *Citrus grandis*, cv. Chandler (pummelo); *Citrus paradisi*, cv. Duncan (grapefruit); *Citrus sinensis*, cv. Valencia (sweet orange); and *Citrus aurantium* (sour orange)] were peeled, diced with a razor blade, and suspended at a ratio of 1:2.5 mass to volume in a chilled aqueous solution of 1% NaCl and 1% PVPP. The suspension was homogenized (10 000 rpm, 30 s) using a Kinematica PT 3100 polytron (Brinkmann, Westbury, NY). The resulting homogenate was filtered through sand and further clarified by centrifugation using a tabletop centrifuge (13 000 rpm, 10 min, 4 °C). The supernatant was carefully separated from the lipid layer and clarified by centrifugation (13 000 rpm, 10 min, 4 °C). To remove endogenous limonoids in the extract, the supernatant (1 mL) was applied to a PD-10 column (Amersham) equilibrated in buffer B (10 mM phosphate, 150 mM NaCl, pH 7.5). The supernatant was followed by the equilibration buffer (2 mL), and the effluent was discarded. An additional 500 μL was added, and the effluent was collected and used for analysis.

Protein Concentration Determination. The protein concentration was determined using a Bio-Rad Protein Assay Kit (Richmond, CA) with bovine serum albumin (Sigma) as the standard. Protein standards were prepared in the same buffer as the sample that was under examination.

Enzyme Assay. The reaction solution consisted of the enzyme sample (120 μL), Tris-HCl (20 μL, 1 M, pH 8.0), and limonin stock solution (60 μL, 0.67 mM). The sample and Tris were combined, and the reaction was initiated with the addition of limonin. The reactions were terminated with the addition of ethylenediaminetetraacetic acid (EDTA, 50 μL, 0.25 M, pH 8.0). For time zero points, the EDTA solution preceded the addition of limonin. For blank reactions, the buffer was substituted for the sample and the blank was processed identical to the samples.

Strata C18-E columns were prepared by washing with MeOH (2 mL) followed by 30% CH_3CN(aq) (2 mL). To process the samples, an aliquot (200 μL) of the reaction was applied to the column and the effluent was collected. Additional eluant (1.3 mL) was added and collected. The effluents were combined, evaporated to dryness, and stored until they were to be quantified. The column was then prepared for the next sample by washing with MeOH (3 mL) and 30% CH_3CN (2 mL). If desired, the unreacted limonin can be collected in the MeOH wash.

Quantification of Samples. The samples were reconstituted in 30% CH_3CN(aq) (100 μL). An aliquot of each sample (25 μL) and standard (25 μL) was transferred to a well in a Costar 3631 assay plate (Corning Incorporated, Corning, NY) and combined with concentrated sulfuric acid (250 μL). The standards were plated in replicates of three. After incubation (37 °C, 1 h), the fluorescence was measured on a Molecular Devices Gemini-EM (Sunnyvale, CA) plate reader. The plate was top read (λ_{ex} = 405 nm and λ_{em} = 460 nm) with a cut off of 455 nm used. The software supplied with the instrument was used for data analysis.

Determination of Optimal SPE Elution Condition for LARL. For these experiments, columns were initially washed with MeOH (2 mL) per the manufacturer's directions prior to preconditioning. Enzyme reactions using purified LDLH were incubated (8 h, 37 °C) prior to their application to the SPE column to ensure sufficient production of LARL. Sample volumes (100−500 μL) were applied to columns, and preconditioning and elution conditions utilizing 100% water to solutions up to 50% aqueous acetonitrile were examined. The column effluent was collected, and the elution profile of LARL was followed in a semiquantitative fashion by reverse phase electrospray ionization (ESI) LC-MS. The analysis was conducted on a Micromass LCZ single quadrapole mass spectrometer equipped with an ESI probe and coupled to a Waters Alliance 2690 solvent/sample delivery system. The mass spectrometer was operated in the single ion monitoring mode with a capillary temperature of 500 °C, a capillary voltage of 3.85 kV, and a cone voltage of 41 V. The detector was set to the negative mode for the first 1.2 min and then switched to the positive mode for the following 1.3 min of each analysis. LARL was detected in the negative mode at *m*/*z* 487.2, whereas limonin was detected in the positive mode at *m*/*z* 471.4. HPLC on an ODS C-18 (4.0 mm × 2.0 mm i.d.) security guard column (Phenomenex) was accomplished with a 4 mM formic acid:MeOH (65:35) isocratic mobile phase, a flow rate of 0.4 mL/min, and a column temperature of 30 °C. The sample injection volume was 3 μL.

RESULTS AND DISCUSSION

The choice of fluorescence for the quantification was based on the report of Fisher (*5*) in which limonin samples were dissolved in sulfuric acid and the fluorescence was measured. In that report, standards were prepared in chloroform and evaporated to dryness prior to combination with sulfuric acid (18.4 g). Because our method was to utilize a plate reader with smaller sample volumes (300 μL total) and we desired the convenience of using solution standards without the necessity of evaporating them, the ratio of sulfuric acid to standard solution that would yield the greatest sensitivity was investigated. The combination of 25 μL of standard with 250 μL of sulfuric acid was found to provide the greatest sensitivity, and the volume was sufficient enough to minimize sample to sample variance due to pipetting. Under these conditions, standards were incubated at 37 °C for 1 h and the fluorescence (λ_{ex} = 405 nm and λ_{em} = 460 nm) was measured. The fluorescence response was linear up to 100 μg/mL with correlation coefficients consistently greater than 0.98 (**Figure 3**), and the quantification limit was found to be 3 μg/mL using a 25 μL standard (75 ng of limonin). The difference in the calibration curve slope from plate to plate was less than 10%. The samples were treated identical to standards for quantification. The samples out of

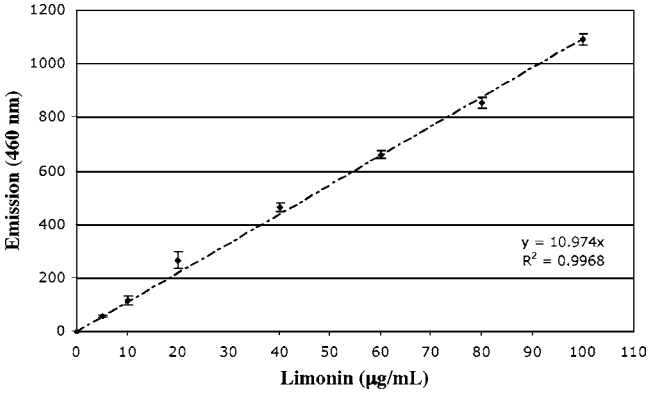

Figure 3. Calibration curve. Experimental details are described under the Materials and Methods.

the range of the calibration curve were diluted with 30% CH_3CN(aq). The response to dilution was linear across the range of the calibration curve.

The sulfuric acid used in fluorescence detection also conveniently functions as the catalysis for the conversion of LARL to limonin. The acid-catalyzed conversion of LARL to limonin was employed in the quantification of LARL in juice and vegetative tissue samples (*6*). However, in those examples, LARL was not directly isolated and quantified but calculated from the difference in limonin isolated before and after the addition of acid. That approach effectively requires two isolation and quantification steps to be completed. Having optimized the fluorescence detection, we sought a method to separate LARL from limonin. Maier et al. (*1*) and Merino et al. (*3*) had demonstrated that LARL can be chromatographically separated from limonin by TLC and ion pairing HPLC, respectively. In this study, C18 chromatography with a water:acetonitrile mobile phase was investigated. SPE was chosen to simplify the isolation process and capitalize on the compatibility of SPE for handling large numbers of samples. Solvent compositions [0−50% CH_3CN(aq)] and the volume required to isolate LARL by SPE were examined by LC-MS analysis. A composition of 30% CH_3CN(aq) was chosen because this concentration effectively separated LARL from limonin using a single solution and resulted in a greater than 99% recovery in a minimal elution volume (1.5 mL total). In addition, the effluent could be directly combined with sulfuric acid and the limonin concentration quantified because the CH_3CN concentration was the same as the standards.

Although LDLH has not been reported to be a metalloenzyme, we examined the addition of EDTA (5−500 mM, 50 μL) as a means to terminate the assay. The addition of 250 mM EDTA effectively terminated the assay and did not interfere with the SPE or quantification steps. After the addition of EDTA, samples could be processed within the next hour without a detectable change in the LARL concentration relative to a blank.

The method was used to measure the LDLH activity of a variety of samples including the purified enzyme from Valencia seeds and crude seed extracts. LDLH was purified from whole Valencia seeds and assayed as described in the Materials and Methods. An example of a kinetic experiment is shown in **Figure 4**. Data points represent the mean ($n = 3$), and error bars represent the standard deviation (SD). The specific activity of purified LDLH was determined to be 157 μmol LARL mg^{-1} enzyme h^{-1} ($n = 5$, SD = 24). Previous reports of LDLH activity have not included specific activity values or sufficient experimental details to calculate a value; therefore, a comparison is not possible. Fold enrichments were reported but are not in the scope of this report.

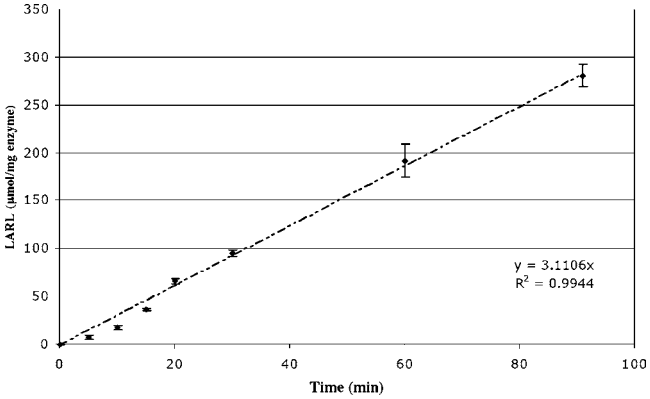

Figure 4. Time course of the LDLH-catalyzed formation of LARL. LDLH was purified from Valencia seeds, and additional experimental details are described under the Materials and Methods.

Table 1. LDLH Activity in Crude Seed Extracts

sample	activity (nmol/mg h)[a]	simplified parentage
Poncirus trifoliata	8.0 ± 0.5	Poncirus trifoliata
Pummelo (Chandler)	5.6 ± 0.6	Pummelo
grapefruit (Duncan)	12.0 ± 1.3	Pummelo × sweet orange
sweet orange (Valencia)	24.8 ± 2.4	Pummelo × Mandarin
sour orange	34.2 ± 0.5	Pummelo × Mandarin

[a] Assays were conducted at 30 °C and carried out for 1 h for the Valencia and sour orange extracts and 3 h for the remainder. Units are nmol of LARL generated per mg of protein per hour. Additional experimental details are described under the Materials and Methods.

The results for the crude seed extracts are shown in **Table 1**. Activities ranged from 5.6 to 34.2 nmol LARL mg^{-1} protein h^{-1}, with the lowest and highest activities exhibited by Pummelo and sour oranges, respectively. We had anticipated that the LDLH activity for *Poncirus* would be minimal or undetectable since the limonin and total limonoid content of *Poncirus*' seeds have been reported to be quite low as compared to other citrus seeds (*7*). A possible explanation for this observation is that LDLH participates throughout the limonoid biosynthetic pathway rather than only at the terminus by controlling the solubility of limonoids through their interconversion between water soluble A-ring monolactones and hydrophobic dilactones. This hypothesis is supported by Hasegawa's reports that LDLH catalyzes the hydrolysis of a broad range of substrates including obacuonone, nomilin, and inchangin (*2*) and that nomlinate A-ring lactone (monolactone form of nomilin) is the limonoid that is translocated from stems to other tissues where it is converted into other limonoids (*8*). Further investigation of the limonoid biosynthetic pathway is warranted to address the differences in LDLH activity.

The method that we have described is ideally suited for the task of rapidly quantifying LDLH activity in both purified and crude samples. As compared to other procedures, this method is more sensitive than the TLC procedure, does not require radioactive materials, and is more efficient than the ion pairing HPLC method. In addition, we have included in this report an improved purification method for LDLH and determined that LDLH activity is inhibited by the addition of EDTA.

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