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Journal of Chromatography A, 760 (1997) 173–178

JOURNAL OF CHROMATOGRAPHY A

High-performance liquid chromatography quantitation of limonin D-ring lactone hydrolase and limonoate dehydrogenase activities

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Received 9 July 1996; revised 9 September 1996; accepted 10 September 1996

Abstract

Ion-pairing reversed-phase HPLC has been used to separate and determine different derivatives of limonin, i.e. disodium limonoate, 17-dehydrolimonoate, limonoate A-ring lactone and 17-dehydrolimonoate A-ring lactone. Consequently, it has been possible to measure, by this chromatographic technique, the activities of the two enzymes that sequentially catalize the first two steps in limonin degradation: limonin D-ring lactone hydrolase and limonoate dehydrogenase. In both cases linear responses were found when using increasing enzyme amounts. The determination of limonoate dehydrogenase by HPLC measurements of limonoid products was compared with that obtained by the spectrophotometric method, based on pyridine nucleotides reduction. A good correlation was detected by using both procedures, which allows the proposition of the chromatographic method as an alternative to pyridine nucleotides determination. Besides, the chromatographic method appears to be a better assay than thin-layer chromatography or radioactive procedures, the methods traditionally used to measure limonin D-ring lactone hydrolase activity.

Keywords: Limonoids; Limonoate dehydrogenase; Limonin D-ring lactone hydrolase; Enzymes

1. Introduction

Limonoids are a family of substances chemically related with terpenoids [1]. Their industrial and physiological importance is based on the bitterness they produce in many citrus juices [2] and their proposed anti-carcinogenic and insect feeding inhibitory activities [3,4]. Approaches used to quantitate limonin, the most intense bitter limonoid, have included thin-layer chromatography (TLC) [5–7], gas chromatography [8], spectrophotometric determination [9–11] and immunochemical quantitation [12,13].

Limonoids determination has been improved with the establishment of HPLC techniques [14] which allow the separation of limonoid aglycones [15,16], as well as their corresponding glucoside derivatives [17]. However, until very recently, the precise and direct separation of different limonin derivatives [i.e. disodium limonoate, 17-dehydrolimonoate, limonoate A-ring lactone (LARL), and 17-dehydrolimonoate A-ring lactone (17-dehydro-LARL)] has not been possible to perform by reversed-phase HPLC methods. Thus, LARL has been indirectly determined, after its conversion into limonin, by acid treatment [18,19]. The separation of these derivatives has been accomplished by ion-pair reversed-phase HPLC [20] but, with the exception of limonin, none

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PII S0021-9673(96)00772-8

of these compounds has been reliably quantitated until now. Consequently, neither limonin D-ring lactone hydrolase (LDRLase) nor limonoate dehydrogenase (LDase), the enzymes catalyzing the interconversions of these limonin derivatives, have been measured by HPLC procedures.

LDRLase activity has been previously determined by using TLC or radioactive methods to measure the LARL formed in the enzyme reaction which uses limonin as substrate [21]. Both methods are tedious and labor intensive. On the other hand, LDase activity has been quantitated spectrophotometrically by following NAD(P) reduction [22–24]. Although pyridine nucleotide measurement is a well established protocol, interferences can occur due to other dehydrogenases present in crude extracts or to sample turbidity. In the present paper, we describe the direct quantitation of LDRLase and LDase activities by ion-pair reversed-phase HPLC. For this purpose, we have performed and described for the first time the identification and determination of 17-dehydrolimonoate and 17-dehydrolimonoate A-ring lactone, products of LDase catalysis.

2. Experimental

2.1. Chemicals

NAD, Tris and triethanolamine were from Sigma (St. Louis, MO, USA). Tetrabutylammonium dihydrogenphosphate was from Aldrich (Steinheim, Germany). Acetonitrile and methanol were from Merck (Darmstadt, Germany). All other chemicals used were of analytical-reagent grade.

2.2. Equipment and chromatographic conditions

HPLC determinations were carried out by using a Beckman liquid chromatograph equipped with a 126 solvent system fitted with an injection loop of 20 μl and a 128 diode array detector. Reversed-phase chromatography was performed in an ODS Spherisorb column (15×0.4 cm), with a particle size of 5 μm, fitted with a precolumn of the same material (1.5×0.4 cm). Elution was carried out at room temperature under isocratic conditions by using water–acetonitrile–methanol (58.5:28.5:13, v/v) and 20 m*M* tetrabutylammonium dihydrogenphosphate as ion-pairing agent. The eluent was adjusted at pH 3.7 with phosphoric acid and the flow rate was 1 ml/min. Detection of limonoids was performed at 214 nm. Chromatograms were analyzed with the Gold software version 8.4 from Beckman. High-purity water was obtained using a Milli-Q System (Millipore). Mobile phases were degassed in an ultrasound bath and filtered through 0.45 μm membranes before use.

2.3. Limonoids production and determination

Limonin was obtained from citrus seeds by essentially following the method described by Barton et al. [1]. For LARL production, a saturated solution of limonin was passed through a column with immobilized LDRLase [25]. Limonin concentration was measured by HPLC in aliquots of the loading and eluted solutions. The decrease in limonin concentration was quantitated as the amount of LARL formed. Calibration curves were obtained from the HPLC peak areas for different dilutions of the LARL solution. For preparation of disodium limonoate, one gram of limonin was added to 48 ml of 0.1 *M* NaOH. The mixture was refluxed until most of the starting limonin was transformed into limonoate and neutralized to pH 7.5 [26]. Differences in limonin mass at the beginning and the end of refluxing were used to obtain disodium limonoate concentration. Calibration curves for disodium limonoate were obtained by HPLC quantitation of the corresponding dilutions.

2.4. Biological materials and enzymes obtention

LDRLase was purified from peeled citrus seeds by essentially following the procedure previously described [27]. LDase was obtained from *Rhodococcus fascians* grown as described by Hasegawa and King [24], and purified as described elsewhere [28].

2.5. Enzyme assays

LDRLase activity was assayed in a reaction mixture that consisted of: 100 m*M* Tris–HCl pH 8.0, 0.48 m*M* limonin, 25% acetonitrile and an appropriate amount of enzyme in 300 μl total volume.

After 5 min at 37°C, an aliquot of 20 μl from the reaction mixture was injected into a C_{18} reversed-phase column to measure by HPLC the limonoate A-ring lactone formed. Spectrophotometric assay for LDase activity was performed at room temperature by following the production of NADH at 340 nm. Activity was assayed in 1 ml of reaction mixture containing 100 m*M* Tris–HCl pH 8.5, 0.5 m*M* NAD, 4 m*M* disodium limonoate and a convenient aliquot of the enzyme [24]. HPLC assay of LDase was carried out by injecting into the C_{18} column, 20 μl of the reaction mixture used for the spectrophotometric assay. One unit of activity is the amount of enzyme that catalyzes the formation of one μmol of product per minute. Activity values reported throughout the paper correspond to the average of 4–6 determinations.

3. Results and discussion

Disodium limonoate, chemically obtained from limonin, has been traditionally used as the substrate for LDase, which converts this limonoid into 17-dehydrolimonoate. However, the reported physiological substrate for LDase is LARL, which is also transformed into its 17-dehydro derivative (17-dehydro-LARL). When disodium limonoate and LARL are together in the presence of the enzyme, the two above cited reactions occur producing a mixture of both dehydro forms (Fig. 1).

Preliminary attempts to separate the above cited limonoid species by reversed-phase HPLC were unsuccessful. The addition of phosphate ions to the eluent allowed a better separation, but the different limonoid species were not accurately determined under these conditions. Fig. 2 shows the elution profile of limonoids subjected to ion-pair reversed-phase HPLC including the peaks corresponding to substrates and products of LDRLase and LDase reactions. Thus, Fig. 2A represents the chromatographic behaviour of limonin (b) and LARL (a), respectively substrate and product of LDRLase, after performing the enzyme reaction. On the other hand, Fig. 2B shows disodium limonoate (c), 17-dehydrolimonoate (d), LARL (a) and 17-dehydro-LARL (e). Both dehydro forms were obtained after incubation of a mixture of disodium limonoate and LARL with

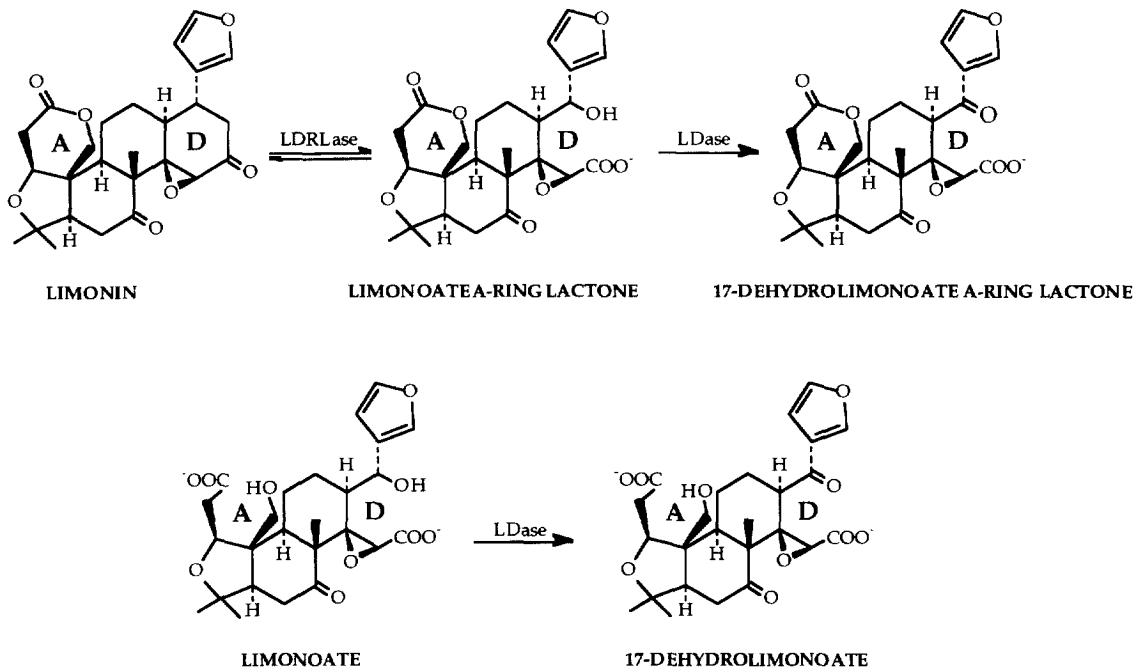

Fig. 1. Reactions of limonin D-ring lactone hydrolase (LDRLase) and limonoate dehydrogenase (LDase).

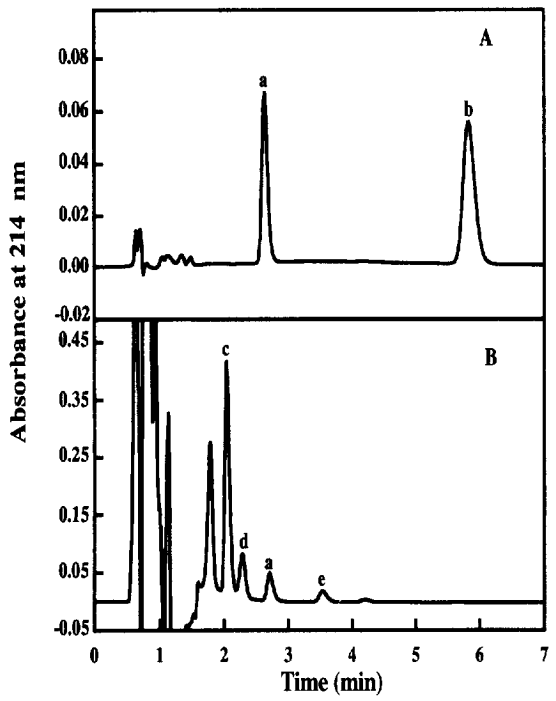

Fig. 2. HPLC elution pattern of limonoid species involved in LDRLase and LDase reactions. Chromatographic conditions were as described in Section 2, with an injection volume of 20 μl in (A) 100 m*M* Tris–HCl pH 8.0 and (B) 100 m*M* Tris–HCl pH 8.5. A: LARL (a, 1.23 μg), limonin (b, 1.55 μg). B: LARL (a, 0.88 μg), disodium limonoate (c, 1.51 μg), 17-dehydrolimonoate (d, 0.23 μg), 17-dehydro-LARL (e, 0.44 μg).

LDase under assay conditions. While in the case of LDase, both substrates and products possess a similar degree of polarity and, consequently, their peaks showed retention times between 2 and 3.5 min, limonin, due to its non-polar character was clearly separated from the other limonoids under our chromatographic conditions. The mobile phase contained tetrabutylammonium dihydrogenphosphate as ion-pairing agent. The phosphate salt was used due to its low absorbance in the ultraviolet region as compared with other commonly used anions. Separation of ionized limonoids required the presence of 20 m*M* tetrabutylammonium as previously described [20]. Lower and higher concentrations of the ion-pair former elicited a less precise resolution of peaks. We have improved the separation of ionized forms by including in the eluent 13% (v/v) methanol, which in turn promotes the resolution of 17-dehydrolimonoate and 17-dehydro-LARL. Both dehydro species appeared as a single peak when the mobile phase was devoided of methanol. Resolution of limonoids was better at an eluent pH range of 3.5–4.0 than at 4.5–5.0. Enzyme activity measurements have been carried out by injecting directly aliquots of the reaction mixtures (pH 8.0–8.5). If samples were acidified before injection, lactonization of disodium limonoate and LARL was promoted, thus modifying the equilibrium of limonoids to be determined.

Linear relationships were obtained when the peaks areas at 214 nm were represented against the amounts of limonoids injected into the column. Homogeneous preparations of limonin, disodium limonoate and LARL were obtained as described in Section 2. Quantitation of both 17-dehydro derivatives, products of LDase activity, was carried out on the basis of the decrease in concentration of the corresponding substrates after the enzyme reactions. As far as we know, this is the first time that these limonoids have been separated and directly estimated by HPLC techniques. Consequently, these results open up the possibility of measuring the activities of LDase and LDRLase by HPLC.

The time-course of LDRLase activity was determined by using, as substrate, limonin dissolved in acetonitrile. The amount of acetonitrile used in the enzyme assay was a compromise between substrate solubility and enzyme stability. Linearity (r=0.97) was maintained for at least 8 min (the time chosen for routine assays was 5 min). On the other hand, a perfect linearity was observed in the range of 10 to 80 ml of enzyme preparation (r=0.98) under the assay conditions described in Section 2.

Studies on LDRL hydrolase have been difficult to perform due to several reasons. Separation of limonoids involved in the enzyme reaction used to be made by TLC and their quantitation was carried out on the basis of the spots sizes and intensities after staining [27] or by radioactive methods [21]. The use of reversed-phase HPLC with an ion-pair former has allowed a better resolution and faster quantitation of reaction products and enzyme activity.

Disodium limonoate has been the substrate generally used for the in vitro spectrophotometric assays of LDase. HPLC determination of 17-dehydro-

limonoate showed a good correlation (r=0.98) between the amounts of the formed product and the enzyme concentration. In addition, the time course of the reaction indicated a constant rate for at least 20 min. It has been widely reported that LARL is the physiological substrate of the enzyme. However, the difficulty in obtaining homogeneous preparations of LARL by chemical procedures has hampered its use in LDase reaction. Our group has obtained pure LARL solutions by using a reactor of immobilized LDRLase [25]. This allowed the comparison of the affinity of LDase for both substrates. K_m values, determined by both spectrophotometric and HPLC methods, were 2–3 times higher for disodium limonoate, which reinforces the proposed physiological role of LARL as substrate for the dehydrogenase. Consequently, the characteristics of LDase HPLC assay with LARL as substrate have been studied. The effect of the reaction time on the rate of 17-dehydro-LARL formation is shown in Fig. 3. A good linearity was observed during the first 5–10 min of reaction.

By using the standard conditions described for the HPLC assay, a linear response was observed in a range of 20–80 μl of enzyme for 17-dehydro-LARL production (Fig. 4). Moreover, a high level of correlation between the appearance of NADH, determined spectrophotometrically, and that of 17-dehydro-LARL, measured by HPLC, was observed in the assay of LDase.

These results allowed the establishment of the HPLC assay as an alternative to the spectrophotometric method previously used to determine the reduction of pyridine nucleotide [22–24,29]. The advantages of this chromatographic method are various. It enables discrimination between the different species of substrates and products, is particularly reliable for turbid enzyme samples and it avoids interferences produced by other dehydrogenases present in crude extracts.

Acknowledgments

This work was supported by CICYT (Spain) grant No. Bio 94-0378 and Junta de Andalucia No. 1240. L. H. was recipient of a fellowship from the Universidad de Córdoba and M.T. M. from OTRI, Spain.

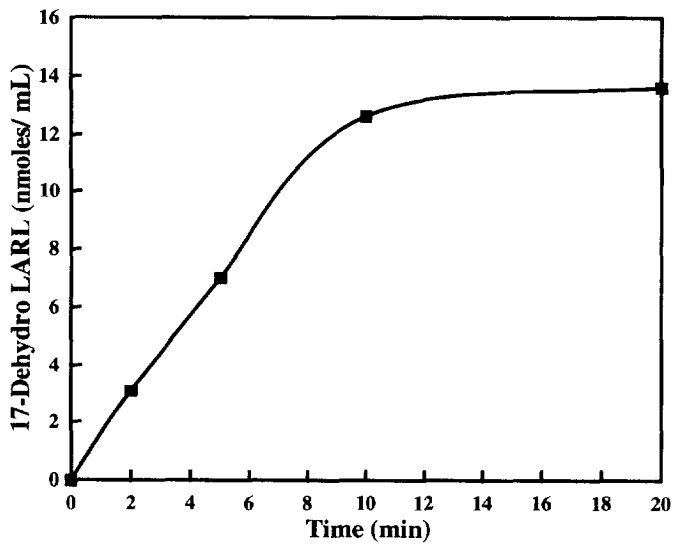

Fig. 3. Time-course of LDase assay with LARL as substrate. 60 μl aliquots of partially purified LDase from *R. fascians* were incubated for the indicated times in the standard assay mixtures. Volume injected 20 μl, sample solvent 100 m*M* Tris–HCl pH 8.5.

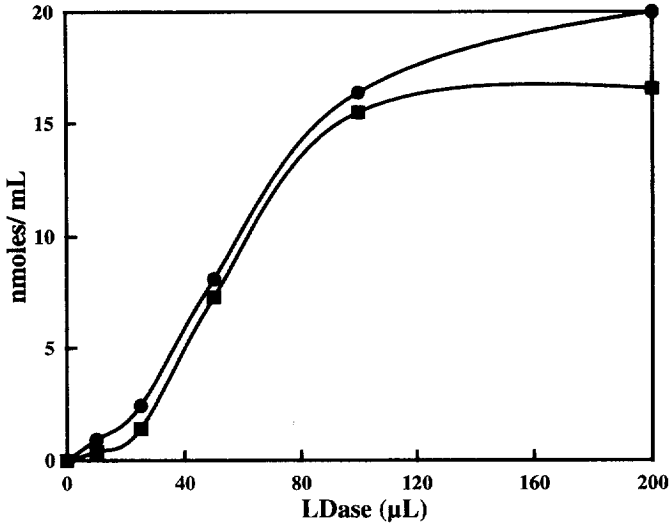

Fig. 4. Correlation between spectrophotometric and HPLC assay of LDase with LARL as substrate. The reaction mixtures contained the indicated amounts of *R. fascians* enzyme. The assay conditions used were the same as in the spectrophotometric assay, but LARL was used instead of disodium limonoate. NADH measured at 340 nm (●). 17-Dehydro-LARL determined by HPLC (■).

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