Phytochemistry, 1969, Vol. 8, pp. 405 to 407. Pergamon Press. Printed in England

SHORT COMMUNICATION

LIMONIN D-RING-LACTONE HYDROLASE. A NEW ENZYME FROM *CITRUS* SEEDS

V. P. MAIER, S. HASEGAWA and ELVIRA HERA

Fruit and Vegetable Chemistry Laboratory,* Pasadena, California, U.S.A.

(*Received* 29 *August* 1968)

Abstract—An enzyme that catalyzes the reversible lactonization-hydrolysis of the open and closed D-ring lactones of: limonoate ⇄ limonoate D-ring lactone and limonoate A-ring lactone ⇄ limonin has been extracted from orange seeds, *Citrus sinensis*, and purified about 200-fold.

Citrus seeds are known to accumulate relatively large amounts of limonin[1,2] an intensely bitter tetracyclic triterpenoid dilactone.[3,4] However, recent work in our laboratory has shown that the *structural tissues* of *Citrus* fruits contain primarily a nonbitter form of limonin, limonoate A-ring lactone.[5,6] Only small amounts of this compound are detected in the seeds. During this work indications of enzymic conversion of limonoate A-ring lactone into limonin in extracts of *Citrus* fruits and seeds were noted.[6,7] These observations led us to undertake a detailed study of the enzyme activity of orange seeds [*Citrus sinensis* (L.) Osbeck] relative to limonin and its derivatives. We report here the isolation of a new enzyme for which we propose the name limonin D-ring-lactone hydrolase.

Products of the action of the purified orange seed enzyme on several limonoid substrates show (Table 1) that it catalyzes the reversible lactonization-hydrolysis of the open and closed D-rings of: limonoate (I) ⇄ limonoate D-ring lactone (II) and limonoate A-ring lactone (III) ⇄ limonin (IV) (Fig. 1). The enzyme does not catalyze the reversible lactonization-hydrolysis of the open and closed A-ring lactone of these compounds. In contrast to the enzyme-catalyzed reaction, acid catalysis leads to the simultaneous formation of both the A- and D-ring lactones and base catalysis leads to the simultaneous hydrolysis of both lactone groups. Since limonin D-ring-lactone hydrolase acts only on the open and closed D-ring

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

[1] Cf. J. F. KEFFORD, in *Advances in Food Research* (edited by C. O. CHICHESTER, E. M. MRAK and G. F. STEWART), Vol. 9, p. 285, Academic Press, New York (1959).

[2] D. DREYER, in *Progress in the Chemistry of Organic Natural Products* (edited by L. ZECHMEISTER), Springer-Verlag, New York, in press.

[3] D. ARIGONI, D. H. R. BARTON, E. J. COREY, O. JEGER, L. CAGLIOTA, SUKH DEV, P. G. FERRINI, E. R. GLAZIER, A. MELERA, S. K. PRADHAN, K. SCHAFFNER, S. STERNHELL, J. F. TEMPLETON and S. TOBINAGA, *Experientia* **16**, 41 (1960).

[4] D. H. R. BARTON, S. K. PRADHAN, S. STERNHELL and J. F. TEMPLETON, *J. Chem. Soc.* 255 (1961).

[5] V. P. MAIER and G. D. BEVERLY, *J. Food Sci.*, in press.

[6] V. P. MAIER and D. A. MARGILETH, *Phytochem.* **8**, 243 (1968).

[7] V. P. MAIER, *Proc. International Citrus Conference*, University of California, Riverside, 17–26 March 1968, Publications Department, University of California, Riverside, California, in press.

TABLE 1. IDENTIFICATION OF ENZYME REACTION PRODUCTS BY THIN-LAYER CHROMATOGRAPHY (TLC) AND PAPER ELECTROPHORESIS (PE)

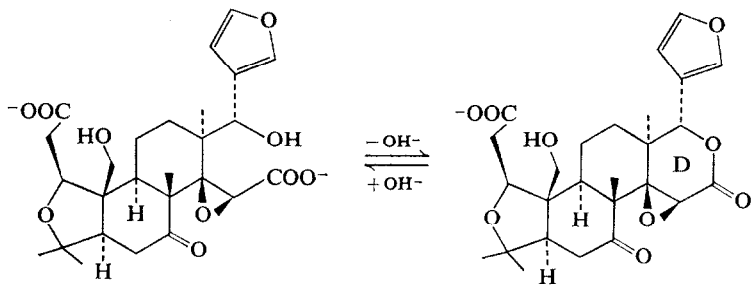
Substrate	pH of reaction mixture	Reaction product* TLC, R_f‡ SG–EtOH	SG–BzEWA	MCC–IAW	PE migration distance, cm pH 5·7	pH 3·1	Identity of reaction product†
Limonoate	6·0	0·30	0·18	0·75	6·5	0·0	Limonoate D-ring lactone
Limonoate A-ring lactone§	6·0	0·70	0·48	0·88	0·0	0·0	Limonin
Limonin	8·0	0·40	0·18	0·51	6·5	8·0	Limonoate A-ring lactone
Limonoate D-ring lactone	8·0	0·15	0·0	0·21	14·5	7·8	Limonoate

* The intensity of the product spot paralleled the decrease in intensity of the substrate spot. In each case at least 40 per cent of the substrate was converted into product and only one product was detected.

† Authentic compounds gave identical TLC and PE values when run adjacent to and superimposed on the reaction mixture.

‡ SG–EtOH = silica gel developed with ethanol; SG–BzEWA = silica gel developed with benzene–ethanol–water–acetic acid, upper (200:47:15:1); MCC–IAW = microcrystalline cellulose developed with isopropanol–ammonium hydroxide–water (9:1:1).

§ Compound available only as a mixture with limonoate D-ring lactone; the limonoate D-ring lactone was unchanged after the enzyme reaction whereas the limonoate A-ring lactone had decreased substantially.



(I) Limonoate (II) Limonoate D-ring lactone

(III) Limonoate A-ring lactone (IV) Limonin

FIG. 1

lactone group it exhibits a very high degree of intramolecular specificity. The net reaction catalyzed by the hydrolase depends on the pH of the reaction mixture. At pH 6 the lactonization reaction is predominant whereas at pH 8 the hydrolytic reaction is predominant. Further studies of the properties and biosynthetic significance of this reversible limonin D-ring-lactone hydrolase are in progress.

EXPERIMENTAL

Extraction and Purification

Peeled seeds [*Citrus sinensis* (L.) Osbeck] were macerated for 3 min in the presence of 5 vols. of 3·0 per cent NaCl solution containing 1·0 per cent polyvinylpyrrolidone. The slurry was held at 5° overnight and then centrifuged at 12,800 *g* for 10 min at 0°. The supernatant was dialyzed against distilled water for 3 hr and centrifuged to remove the small amount of precipitate. The enzyme was precipitated from the supernatant by addition of 2·5 vols. of −20° acetone, collected by centrifugation, and dissolved in a minimum amount of water. The crude enzyme extract was run successively on three 1·5 × 20 cm DEAE-cellulose columns equilibrated at pH's 7·2 and 6·0 with potassium phosphate buffers, and at pH 8·0 with *tris* buffer. The enzyme was eluted with NaCl solution (0·20, 0·11, and 0·22 M, respectively) in a linear gradient system (0 to 0·7 M) containing 0·01 M buffer. After each column run the active fraction was dialyzed at 1° against distilled water for 3 hr and reintroduced onto the next column. Following purification, which resulted in a 214-fold increase in specific activity, the enzyme fraction was dialyzed for 3 hr and lyophilized.

Test of Enzyme Purity by Disc Electrophoresis

Electrophoresis of the enzyme was carried out in 0·5 × 6·5 cm columns of polyacrylamide gel buffered with 0·2 M glycine-*tris*, pH 8·3, and using 2 mA/column for 30 min. After staining with aniline-black (and destaining) the purified enzyme preparation showed one major and two very minor protein bands (the crude acetone-precipitated enzyme showed seven major and numerous minor bands). The comparable major band from unstained companion columns of the purified enzyme gave a positive test for lactonizing activity with disodium limonoate and a positive test for hydrolyzing activity with limonin (see reaction systems below).

Lactonizing Activity

The reaction mixtures used to test for lactonizing activity consisted of: (1) $2{\cdot}5 \times 10^{-3}$ M disodium limonoate, 0·1 M citrate-phosphate buffer, pH 6·0, and an appropriate amount of enzyme (1–3 μg protein) in 100 μl; and, (2) 5×10^{-4} M limonoate monolactones (approximately 40 per cent limonoate A-ring lactone), 0·1 M citrate-phosphate buffer, pH 6·0, and an appropriate amount of enzyme in 500 μl. After incubation at 29·2° for 30–60 min, 10 and 40 μl of the reaction mixture was spotted on a thin-layer chromatography (TLC) plate and on filter paper for paper electrophoresis (PE) along with known amounts of authentic limonoid compounds (Table 1). PE was run on Whatman No. 1 filter paper at 113 V/cm for 40 min. The buffers used were: 0·05 M citrate, pH 5·7, and 5 per cent acetic acid adjusted to pH 3·1 with NH_4OH.[6] Following TLC and PE, the plates and papers were dried and sprayed with Ehrlich's reagent and fumed with HCl. Limonoid compounds were detectable at levels as low as 0·5 μg and appeared as orange–brown spots.[8] The sizes and intensities of the spots as compared with those of standards were used as a measure of the amount of product formed. Specific activity was determined using reaction system (1) above. The amount of product formed in 30 min at 29·2° was determined by TLC.

Hydrolyzing Activity

The reaction mixtures used to test hydrolyzing activity consisted of: (1) $8{\cdot}2 \times 10^{-4}$ M limonin, 0·16 M phosphate-citrate buffer, pH 8·0, 33·3 per cent methanol and an appropriate amount of enzyme in 300 μl; and, (2) $1{\cdot}32 \times 10^{-3}$ M limonoate D-ring lactone, 0·22 M phosphate-citrate buffer, pH 8·0, and an appropriate amount of enzyme in 90 μl. Identification of the reaction products was carried out as before, Table 1.

Nonenzymic Reactions

Experiments in which heat inactivated enzyme or buffer alone (or with methanol) were used showed that no significant nonenzymic lactonization or hydrolysis occurred under the test conditions used.

Acknowledgements—We wish to thank Messrs. Dale R. Genung and H. Peterson, Sunkist Growers, Inc., Ontario, California, and Dr. G. L. K. Hunter, Minute Maid Corporation, Plymouth, Florida, for supplying the orange seeds used in this work.

[8] D. L. DREYER, *J. Org. Chem.* **30**, 749 (1965).