Pergamon

0031-9422(94)0094-X

Phytochemistry, Vol. 39, No. 6, pp. 1305 1307, 1995
Elsevier Science Ltd
Printed in Great Britain
0031 9422/95 $9.50 + 0.00

LIMONOID GLUCOSIDE β-GLUCOSIDASE ACTIVITY IN LEMON SEEDS

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(*Received* 11 *October* 1994)

Key Word Index—*Citrus limon* seeds; β-glucosidase; nomilin 17-β-D-glucopyranoside β-glucosidase; limonoids.

Abstract—Radioactive tracer work demonstrated that dormant and germinated seeds of *Citrus limon* hydrolysed nomilin 17-β-D-glucopyranoside and liberated nomilin. The shoot portion of the germinated seeds also possessed this enzyme activity. These findings show the presence of limonoid glucoside β-glucosidase activity in lemon seeds. This is the first study to establish the presence of such an enzyme in plant tissues.

INTRODUCTION

Limonoids are triterpenoids present only in the Rutaceae and Meliaceae families, and are one of the two bitter principles in citrus juices. In 1989, Hasegawa *et al.* [1] discovered the presence of large quantities of water-soluble limonoid derivatives in both mature citrus fruit tissues and seeds. They were identified as 17-β-D-glucopyranoside derivatives of the limonoid aglycones. Limonoid glucosides are one of the major secondary metabolites in *Citrus* [2]. Commercial orange juices, for example, contain an average of 320 ppm of total limonoid glucosides [3], and in citrus seeds the glucosides approach 0.8% of the dry weight [4].

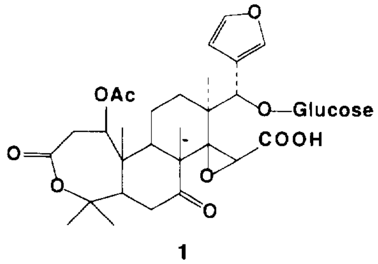
We have observed in many instances that while citrus seeds contain high concentrations of limonoid glucosides, their young seedlings contain practically no limonoid glucosides. This observation suggests that limonoid glucoside β-glucosidase activity is present in germinated seeds. In this study we have examined whether such an enzyme activity is present in germinated lemon seeds as well as in freshly harvested dormant seeds.

RESULTS AND DISCUSSION

Radioactive tracer work showed that both dormant and germinated lemon seeds hydrolysed [^{14}C] nomilin 17-β-D-glucopyranoside (**1**) and liberated labelled nomilin. When labelled nomilin glucoside (**1**) was fed to germinated seeds through the root, it was converted to several metabolites, mainly to component **B** (Fig.1). Each metabolite was isolated by TLC and identified by TLC using three different solvent systems. (1–3, see Experimental). Component **A** remained on the baseline with these solvent systems but it was identified as the unchanged substrate using the solvent EtOAc–MeCOEt–formic acid–water (5:3:1:1). Component **B**, the major metabolite, was identified as nomilin. Components **C** and **D** were likewise identified as limonin and obacunone, respectively. Previous tracer work has shown obacunone and limonin to be metabolites of nomilin in *Citrus* [5, 6].

When a germinated shoot without the attached seed portion was incubated for two days with either 150 000 or 300 000 cpm of labelled nomilin glucoside, 2.1–22% of the substrate was converted to nomilin (Table 1). A seed without the attached shoot portion also hydrolysed 9.6% of 400 000 cpm of labelled compound and liberated mainly nomilin and a small amount of obacunone and limonin. Freshly harvested dormant seeds also hydrolysed the labelled nomilin glucoside. When the seeds were incubated for two days in substrate solution containing 1.5×10^5 or 3.0×10^5 cpm, 4.6–13.8% of the radioactivity was converted to aglycones.

The decrease in substrate concentration, and the formation of nomilin plus traces of limonin and obacunone, show that both dormant and germinated lemon seeds possess nomilin 17-β-D-glucopyranoside β-glucosidase activity. The presence of this enzyme activity explains why there are no limonoid glucosides in citrus seedlings. The glucose moiety could be used by the plant



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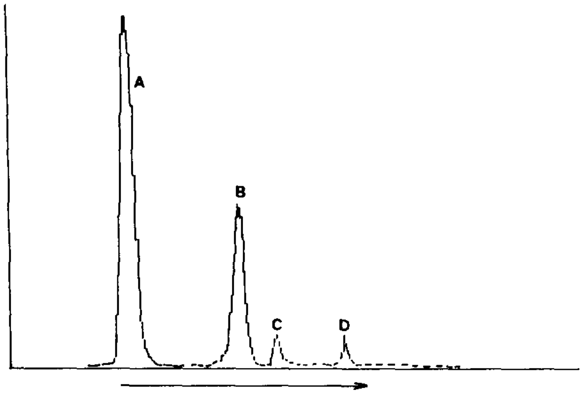

Fig. 1. Radiochromatogram of nomilin glucoside metabolites. The silica gel plate was developed with EtOAc–cyclohexane (3:2).

Table 1. Nomilin glucoside β-glucosidase activity in *Citrus limon* seeds

	^{14}C NG. fed (cpm)	^{14}C-Aglycones (cpm)	Conversion (%)
Dormant			
	150 000	13 670	9.1
	150 000	20 640	13.8
	300 000	13 890	4.6
Germinated			
	150 000	52 500	35.0
Shoots without the attached seed			
	300 000	6330	2.1
	300 000	16 170	5.4
	150 000	32 960	22.0
	150 000	6920	4.6
Seeds without the attached shoot			
	400 000	38 440	9.6

NG: Nomilin 17-β-D-glucopyranoside.

as an energy source during germination. On the other hand, the limonoid moiety most likely remains in the tissue. Citrus seedlings contain large amounts of limonin and obacunone other than nomilin, despite the fact that the seedling tissue has been shown to synthesize mainly nomilin only from labelled acetate [7].

Radioactive tracer work has shown the absence of β-glucosidase activity in citrus fruit tissue [8]. Limonoid glucosides have been shown to be stable in citrus fruit tissues and the concentration increases as fruit maturation progresses. We have observed also that commercial enzymes such as β-glucosidase of almonds and naringinase of moulds do not attack limonoid glucosides. However, a species of bacterium isolated from soil by enrichment on limonin glucoside as a sole carbon source exhibits β-glucosidase activity on limonoid glucosides [1].

The β-glucosidase activity in lemon seeds was demonstrated with nomilin glucoside. It is uncertain at this time whether this single enzyme catalyses the hydrolysis of all other limonoid glucosides. However, it is certain that all other limonoid glucosides disappear during germination. Seventeen limonoid glucosides have been isolated from *Citrus* and its hybrids [9].

The presence of limonoid β-glucosidase in citrus seeds could be important to the citrus industry. Seeds crushed during juice processing may contribute glucosidase activity to juice, which may liberate limonin from limonin 17-β-D-glucopyranoside, the predominant limonoid glucopyranoside, present in citrus juices [3]. Limonin is the major cause of the limonoid bitterness in the juice.

EXPERIMENTAL

Materials. [^{14}C] Nomilin was prepared by the procedure described previously [7]. Up to 10% of [1-^{14}C] acetate was converted to nomilin. Labelled nomilin glucoside was biosynthesized from labelled nomilin by previously published procedures [8]. Labelled nomilin was fed to a $1 \times 2 \times 0.3$ cm^3 piece of mature navel orange albedo. The tissue was incubated at room temp. for 2 days under moist conditions in a closed container. The labelled nomilin glucoside was extracted with 70% MeOH. The MeOH extract was dried, redissolved in H_2O and loaded on a C-18 Sep-pak cartridge (Waters, Milford, MA). The nomilin glucoside was eluted first with 20% MeCN and nomilin was eluted with the MeOH wash.

Feeding experiment. Germinated (3–5 cm long) and freshly harvested lemon seeds were used. Preliminary tests showed that when a germinated seed was fed through the root, 35% of 1.5×10^5 cpm of nomilin glucoside was converted to, mainly, nomilin. Germinated shoots without the attached seed portion were fed with 1.5×10^5 or 3.0×10^5 cpm of nomilin glucoside in 0.1 M Tris buffer at pH 7.5 through the root. Detached seeds from the rest of the germinated seeds and dormant seeds were soaked in the substrate solution. After two days of incubation at room temp., the tissue was thoroughly rinsed with H_2O and the limonoids were extracted and prepared for TLC analysis according to procedures previously described [7].

Isolation and analysis of labelled limonoids. The extracts were spotted on silica gel TLC plates, which were developed with the following three solvent systems: (1) EtOAc–cyclohexane (3:2), (2) EtOAc–CH_2Cl_2 (2:3) and (3) CH_2Cl_2–MeOH (97:3). A Berthold Automatic TLC-linear Analyzer LB 2832 was used to scan radioactive TLC plates. Limonoids were revealed by spraying plates with Ehrlich's reagent followed by HCl gas exposure.

Isolation and identification of labelled limonoids. The extract was first analysed with solvent (1). Radioactive peaks were scraped off and the limonoids were extracted from the silica with EtOAc. Each extract was examined with two other solvent systems and limonoid standards.

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