Pergamon

Phytochemistry, Vol. 46, No. 1, pp. 33–37, 1997
Published by Elsevier Science Ltd
Printed in Great Britain

PII: S0031–9422(97)00216–1

PURIFICATION OF LIMONOID GLUCOSYLTRANSFERASE FROM NAVEL ORANGE ALBEDO TISSUES

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(*Received* 8 *January* 1997)

Key Word Index—*Citrus sinensis*; Rutaceae; limonoids; limonoid glucosides; UDP-D-glucose:limonoid glucosyltransferase.

Abstract—UDP-D-glucose:limonoid glucosyltransferase was purified from albedo tissues of navel orange (*Citrus sinensis*) cultivars, Frost and Newhall, by a combination of $(NH_4)_2SO_4$ fractionation, UDP-glucuronic acid affinity chromatography and DEAE ion exchange HPLC. This procedure resulted in a 452-fold increase in enzyme purification. This enzyme catalysed the glucosylation of both nomilin and limonin. SDS-PAGE showed a M_r of 56–58 k for the enzyme. The enzyme displayed a peak of activity between pH 6.5 and 9.0 with an optimum at 8.0. Mn^{2+} stimulated enzyme activity by 66% over basal activity observed with EDTA. Activity was lost when the purified enzyme was frozen and stored in Tris–HCl buffer at pH 8.0. Published by Elsevier Science Ltd

INTRODUCTION

Limonoids are a group of chemically related triterpenoids present in the Rutaceae and Meliaceae family plants, and they are one of two bitter principles in citrus juices. The bitterness due to limonoids is an important economic problem in commercial citrus juice production. Among 36 limonoids isolated from *Citrus* and its hybrids [1], limonin (**1**) is the major cause of limonoid bitterness in citrus juices. Nomilin (**2**) is also a bitter limonoid and present in grapefruit juice [2] and other citrus juices, but its concentration is generally very low, and its contribution to limonoid bitterness is very minor.

Limonoid bitterness is a problem of the early-season to mid-season fruit, but not in late-season fruit. As the fruit ripens, the concentration of limonoid aglycones, such as limonoate A-ring lactone (**3**), decreases. This natural debittering process was known for over a century, but the mechanism was not known until the discovery of limonoid glucosides in citrus tissues. In 1989, Hasegawa *et al.* [3] found that limonoid aglycones were converted to their respective nonbitter glucosides in fruit tissues and seeds during late stages of fruit growth and maturation. Seventeen limonoid glucosides have been isolated and identified from *Citrus*. [1]. Each possesses one D-glucose molecule attached to the C-17 position of each corresponding aglycone via a β-glucosidic linkage such as limonin 17-β-D-glucopyranoside (**4**). The limonoid glucosides are practically tasteless. The glucosylation of limonoid aglycones is catalysed by UDP-D-glucose:limonoid glucosyltransferase (limonoid GTase) [4]. The objective of this research was to purify the GTase from citrus fruits and characterize it.

RESULTS AND DISCUSSION

Limonoid GTase purification sequence

Limonoid GTase activity was recovered in the 40–80% $(NH_4)_2SO_4$ fraction from the crude protein extract obtained from Frost navel orange albedo tissues. Fractionation of this protein fraction on a UDP-glucuronic acid affinity column resulted in a 55-fold increase in enzyme purification over the $(NH_4)_2SO_4$ fraction (Table 1). The enzyme activity occurred in the M_r range of 40–60 k was determined by SDS-PAGE. Nucleotide sugar affinity chromatography has been used successfully in the purification scheme for a flavanone-specific 7-*O*-glucosyltransferase from grapefruit [5] and the purification of a UDP-glucosyltransferase from liver microsomes [6].

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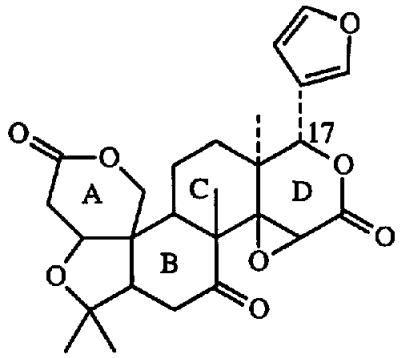
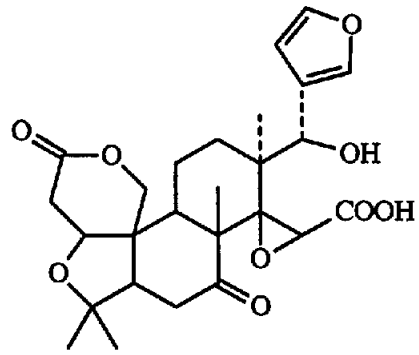
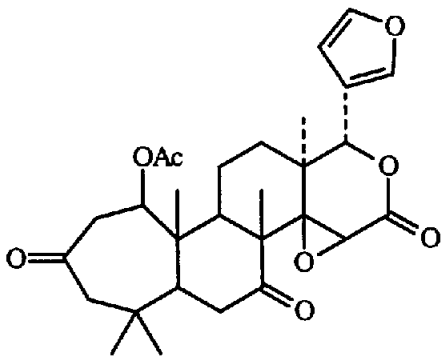


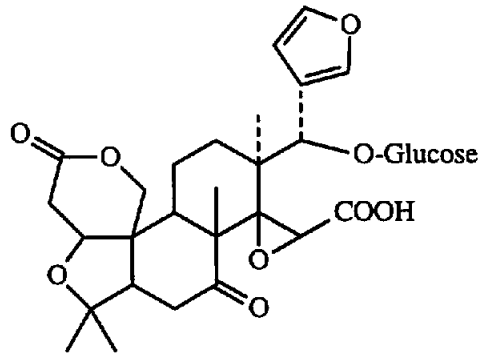
Limonin (1)



Limonoate A-ring lactone (3)



Nomilin (2)



Limonin 17-β-D-glucopyranoside (4)

Table 1. Purification of limonoid glucosyltransferase from Frost navel orange albedo tissues

Purification steps	Total protein (mg)	Total activity (pKat)	Specific activity (pKat/mg)	Purification	Recovery (%)
$(NH_4)_2SO_4$ (40–80%)	597.0	400.0	0.67	1	100
UDP-glucuronic acid affinity column	12.5	463.0	37.0	55	115
DEAE ion exchange HPLC at pH 7.0	—	—	—	—	—
DEAE ion exchange HPLC at 8.0 Fraction 12	0.078	23.5	301	449	5.9

The enzyme fraction obtained from the UDP-glucuronic acid affinity column was applied to a DEAE ion exchange HPLC and run at pH 7. Enzyme activity was eluted between 220 and 240 mM salt (two fractions) on a NaCl linear gradient system from 0 to 0.4 M. The two active fractions were pooled and applied to the same ion exchange HPLC column equilibrated at pH 8. The active fraction eluted at 240 mM NaCl when the column was washed with a NaCl linear gradient of 0 to 0.4 M. This final step provided a 452-fold increase in the purification of the GTase. These same purification procedures were successfully used for purifying limonoid GTase from Newhall navel orange.

Although extensive limonoid substrate specificities were not tested, this enzyme catalysed the glucosylation of both nomilin and limonin to form the respective 17-β-D-glucopyranoside derivatives. As seen with limonin D-ring lactone hydrolase [7], this GTase most likely takes all the citrus limonoid agly-

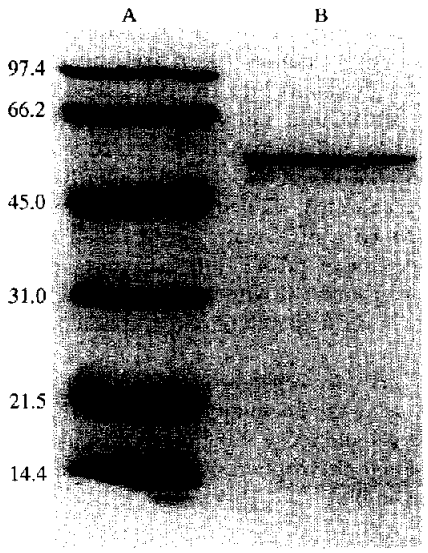


Fig. 1. SDS-PAGE of the purified limonoid glucosyltransferase. Lane A contains M_r standards and lane B contains the purified limonoid glucosyltransferase.

cones as substrate and converts them to their respective glucosides.

Protein electrophoresis

SDS-polyacrylamide gel electrophoresis revealed a single protein band having an M_r of 58 k for the enzyme isolated from Frost navel orange and an M_r of 56 k for the enzyme isolated from Newhall navel orange (Fig. 1). These small differences in M_r fall within the error of the SDS-PAGE technique, thus we can only predict an average M_r of 56–58 k for the limonoid GTase from navel orange.

Glucosyltransferase from plant sources that utilize flavonoids and other small molecules as substrates are generally single subunit enzymes, are acidic proteins and fall in the M_r range of 40 60 k [5, 8–18]. The limonoid GTase isolated from navel oranges in this report has an M_r close to that of two other transferases isolated from *Citrus* sp. The flavanone glucosyltransferase from pummelo leaves has a reported M_r of 52 k [15] and a flavanone glucosyltransferase from grapefruit seeds has a reported M_r of 54.9 k [5].

Factors influencing limonoid GTase activity

An open D-ring is necessary for the glucosylation of limonoid aglycones to proceed via the GTase reaction. Limonoid aglycones are present as the open D-ring form in citrus fruit tissues, leaves and stems [1]. In seeds, limonoid aglycones are present in both open and closed D-ring forms [1]. Alkaline conditions open the D-ring, whereas acidic conditions close it [7]. Studies on the pH dependence of the GTase, using ^{14}C nomilin and UDP-D-glucose as substrates, showed that the enzyme displays a peak of activity between pH 6.5 and 9.0 with an optimum at 8.0. Activity below pH 6.5 is inhibited by a closed D-ring.

Most mammalian GTases and some plant GTases require metal ions for maximal activity. The Mn^{2+} dependence of the limonoid GTase determined at pH 7.3 showed that optimal stimulation of GTases occurs at 50 μM Mn^{2+}, this metal ion concentration providing a 66% stimulation of activity over the basal activity observed with 100 μM EDTA. Concentrations above 500 μM either had no effect or were inhibitory.

In our study we initially encountered problems with enzyme storage during the purification procedure. When partially purified enzyme preparation was placed into 50 mM Tris–HCl buffer, pH 8.0 and stored at $-80°$, all activity was lost. Replacing Tris buffer with 10 mM Mes–KOH, pH 7, allowed frozen storage of the enzyme with full recovery of activity. We postulate that this loss of enzyme activity in Tris–HCl buffer may be due to sensitivity of the enzymes to high Cl^- concentration. A GTase from strawberry has been shown to be inhibited by phosphate ions [17].

Possible practical application

Navel orange is one of the major commercial crops which are subject to limonoid bitterness. In navel orange grown in California, the natural glucosidation of limonoid aglycones begins in September and continues until the fruit is harvested [4]. The harvesting season of navel oranges begins in November. During the period of maturation which may be as short as two months, glucosidation of the aglycones is incomplete. An enhancement of the limonoid GTase activity through genetic engineering could reduce aglycone concentration.

Mandarin orange, pummelo and citron are ancient citrus species and believed to be three of the original species of *Citrus* [19]. Sweet oranges, hybrids of Mandarin orange, in general have relatively high levels of limonoid UDP-D-glucose transferase activity. In contrast, pummelo and its hybrids such as grapefruit, sour oranges and Oroblonco possess low levels of this enzyme. Consequently, Pummelo juices, for instance, extracted from fruits harvested at very late season have a severe limonoid bitterness. For instance, the juices from 16 cultivars of well matured pummelo fruits grown in California contained an average 18 ppm of limonin and only 29 ppm of total limonoid glucosides [20]. This limonin concentration is well above the 6 ppm, bitterness threshold of limonin in orange juice [21]. However, the insertion of a gene encoding for GTase into commercial cultivars through genetic engineering could create transgenic citrus varieties producing fruits potentially free of limonoid bitterness.

EXPERIMENTAL

Collection of fruit and isolation of albedo tissue. Mature Frost and Newhall navel orange fruit were

collected from the University of California Riverside Citrus collection in March 1993 and 1994. Fruit albedo tissue served as the source of the glucosyltransferase. Fruit were peeled, and the albedo was cut from the peel and was placed into 0° infiltration buffer consisting of 0.2 M Tris–HCl, pH 8, 1 mM PMSF, 5 mM DTT, 15 mM 2-mercaptoethanol. Following vacuum infiltration, the tissue was removed from the buffer solution, placed into plastic bags, frozen on dry ice and stored at −80°.

Enzyme and protein assays. GTase activity was routinely assayed between pH 7.0 and 8.0 in a 50–100 μl vol. The assay soln contained 100 μM UDP-glucose, 50 μM Mn^{2+}, and 39 μM ^{14}C-nomilin (with an open D-ring). Tubes were incubated at 34° for 15 or 30 min. The reaction was stopped by adding 3 μl of 1 M HCl; this also closed to D-ring of unreacted nomilin. Protein was determined using the dye binding protein assay [22] purchased from BioRad.

TLC identification of nomilin glucoside. Aliquots of the enzyme reaction mixture were spotted onto silica gel plates. The plates were developed in EtOAc–MeEtCO–H_2O–HCOOH (5:3:1:1). In this solvent system the R_f value for the aglycone of nomilin is 0.88, and the value for nomilin glucoside is 0.42. The developed plates were scanned for radioactivity with a Berthold Automatic TLC-Linear Analyser, Model LB 2832.

Isolation of the limonoid glucosyltransferase. Frozen albedo tissue was thawed and placed into homogenizing buffer consisting of 50 mM Tris–HCl, pH 8, 5 mM DTT, 15 mM 2-mercaptoethanol, 0.5 mM PMSF in DMSO, 100 mM KCl, 0.5% PVP (w/v). The ratio of homogenizing buffer to tissue was 4 (v/w). The tissue was homogenized with a Polytron at low-medium speed for 4 min. The resulting slurry was centrifuged at 8000 *g* for 20 min to pellet cell debris and PVP.

The supernatant was brought to 40% satn with $(NH_4)_2SO_4$ and stirred in a 0° 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 $(NH_4)_2SO_4$ and stirred in a 0° bath for 1 hr and the ppt. was collected by centrifugation as before. The supernatant was discarded and the centrifuge tubes containing the protein pellets were sealed and stored at −85°.

The $(NH_4)_2SO_4$ precipitated protein pellets were redissolved in 10 mM Mes/KOH buffer, pH 7 containing 15 mM 2-mercaptoethanol and 50 μM $MnCl_2$ (buffer A). The dissolved protein was desalted on a PD-10 gel filtration column (Pharmacia), equilibrated with buffer A. The desalted, buffer exchanged sample was loaded at 2 ml min^{-1} onto a uridine 5′-diphosphoglucuronic acid affinity column (Sigma), having a 75 ml bed vol. that was pre-equilibrated with buffer A. The column was washed with buffer A until the UV absorbance of the eluate returned to baseline. The column was eluted with 10 mM UDP-glucose dissolved in buffer A. The active frs were pooled and loaded onto an HPLC Bio-Gel TSK-IEX DEAE 5PW (75 × 7.5 mm) column (BioRad) which was equilibrated with 50 mM Tris–HCl, pH 7 with 2 mM DTT (buffer B). A linear salt gradient, 0–400 mM NaCl, was used to elute the column over a 20 min period at a flow rate of 1.0 ml min^{-1} using buffer B. The active frs were collected, diluted 10-fold into buffer C (50 mM Tris–HCl, pH 8 with 2 mM DTT) and loaded onto the above DEAE ion exchange column and eluted with buffer C with a linear NaCl gradient, 0 400 mM, over a 20 min period at a flow rate of 1 ml min^{-1}.

SDS gel electrophoresis. SDS-PAGE was performed using the Laemmli buffer system [23]. A 12% running gel and a 5% stacking gel was used for one-dimensional electrophoresis. Gels were stained with Coomassie Blue.

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