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Food Chemistry 76 (2002) 431–436

Food Chemistry

www.elsevier.com/locate/foodchem

Isolation and characterization of limonoid glucosyltransferase from pummelo albedo tissue

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Received 13 March 2001; received in revised form 17 July 2001; accepted 17 July 2001

Abstract

An enzyme that catalyses the transfer of a glucose unit from UDPG to limonoids was isolated from the albedo tissue of pummelo fruit (*Citrus grandis* Osbeck) in electrophoretically homogeneous form. The enzyme was purified to 180-fold by a combination of $(NH_4)_2SO_4$ fractionation, ion exchange chromatography on DEAE-cellulose and DEAE-Toyopearl. SDS-PAGE showed a molecular weight of 55 kDa for the enzyme. The purified enzyme, limonoid glucosyltransferase, displayed an optimum activity at pH 7.8 and 37 °C with apparent K_m values of 65 and 200 μM for limonin and UDPG, respectively. Mn^{2+} and Co^{2+} stimulated the enzyme activity by 33 and 30%, respectively, while EDTA completely inhibited it. Cu^{2+}, Hg^{2+} and diethyl pyrocarbonate also inhibited the enzyme, indicating a possible role of histidine in catalysis. The enzyme was stable at 4 °C for 6 months in Tris–HCl buffer, pH 7.5.

Keywords: Limonoid glucosyltransferase; Pummelo albedo; Bitterness in citrus juice; Enzyme purification; Juice debittering

1. Introduction

Citrus fruits are popular and have established nutritional and medicinal values. Juices from most of the citrus varieties are facing commercial problems due to bitterness, caused by limonoids, a group of chemically related, highly oxygenated, tetracyclic triterpenoids. Among different citrus limonoids, limonin and nomilin are the primary causes of bitterness (Hashinaga, Ejima, Nagahama, & Itoo, 1977). The limonoid content in citrus fruits reportedly decreases during the process of ripening (Hashinaga & Itoo, 1981). This decrease is considered to be due to conversion of limonoid to a corresponding glucoside. In citrus juice, the glucoside levels are much higher than the free limonoids (Fong, Hasegawa, Herman, & Ou, 1990). The free limonoids cause a bitterness problem after juice preparation.

A number of postharvest methods have been developed to minimize the limonoid bitterness problem. These methods include the use of cyclodextrin polymer, affinity columns and immobilized bacterial cells or enzymes, either to remove the bitter component or to convert it to non-bitter ones (Hasegawa & Pelton, 1983; Shaw, 1990). Some reports have suggested that limonoids and their glucosides have many health benefits, including prevention of cancer (Lam, & Hasegawa, 1989; Lam, Li, & Hasegawa, 1989; Miller, Gonzales-Sanders, Courillon, Wright, Hasegawa, & Lam 1992). For this reason, researchers are putting emphasis on alternative ways to remove bitterness without affecting the levels of limonoids or their metabolites.

Enhanced activity of limonoid glucosyltransferase (LGTase) can increase the glucoside level, which in turn reduces the bitterness problem; this can be achieved by regulation of enzyme activity at molecular level. Another possible way of reducing bitterness in juice may be the introduction of the LGTase gene to bacterial cells and the genetically engineered bacteria can be used in cell-affinity column to convert the free limonoids in juice into glucoside. The enzyme itself might also be immobilized on different carriers and possibly be used in a column to reduce bitterness in juice. Therefore, it is important to study the properties of LGTase and its gene to explore ways to reduce bitterness.

Although pummelo is reported to have very low levels of limonoid glucoside in its juice (Hasegawa, Berhow, & Fong, 1996), we found a high activity of LGTase in its

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0308-8146/02/$ - see front matter
PII: S0308-8146(01)00300-4

albedo tissue (Karim & Hashinaga, 2001). The present study reports the purification and characterization of LGTase from pummelo albedo tissue.

2. Materials and methods

2.1. Plant materials and chemicals

Pummelo fruits were obtained from the wholesale market and stored at 7–10 °C. Tris, dimethyl sulfoxide (DMSO), phenyl methyl sulfonyl fluoride (PMSF), and polyvinyl pyrrolidone (PVP) were from Wako Pure Chemical Industries Ltd. DEAE-cellulose was from Pharmacia Fine Chemicals, Co. Ltd. and DEAE-Toyopearl from Toyo Soda Mfg. Co. Ltd. All other chemicals were of analytical grade.

2.2. Crude enzyme preparation

Unless otherwise specified, all the operations were performed at 4 °C. Crude limonoid glucosyltransferase was extracted from albedo, as described by Cai et al. (1998), with some modifications. The albedo tissue was homogenized with 4 volumes of pre-cooled 0.1 M saline, containing 0.5% PVP and 1 mM PMSF (from 0.1 M stock solution in DMSO) in a tissue homogenizer at medium speed for 3 min. The homogenized slurry was stirred for 2 h and then filtered through gauze. The filtrate was centrifuged at 10,000×***g*** for 30 min. The clear supernatant was brought to 75% $(NH_4)_2SO_4$ saturation and allowed to stand for 1 h. The protein precipitate was collected by centrifugation and stored at −80 °C.

The precipitate was dissolved in a small volume of pre-cooled 10 mM Tris–HC1 buffer, pH 7.8 and dialyzed against the same buffer for 12 h with four changes of buffer. The dialysate was centrifuged to remove any insoluble material and the supernatant was used as crude enzyme preparation.

2.3. Enzyme activity and protein assay

Enzyme was routinely assayed, as described by Cai et al. (1998), at pH 7.8 in a 100 μl volume, with some modifications. The assay solution contained 1 mM limonin (with an open D-ring), 2 mM UDPG-2Na, 5 mM $MnCl_2$ and 20 mM Tris–HC1 buffer. After 1 h incubation at 37 °C, the reaction was stopped by boiling (5 min, 100 °C). The supernatant, collected by centrifugation (10,000×***g***, 15 min), was used for product assay by HPLC with a Nucleosil $_7C_{18}$ reverse phase column (4.6×250 mm). Elution was done by using a linear gradient of methanol in water (15–30%) within 35 min at a flow rate of 0.4 ml min^{-1}. Limonin glucoside was eluted at 13 min; the amount was calculated using standard limonin glucoside and the unit of activity was expressed as pkat. Protein was estimated by measuring absorbance at 280 nm or by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as standard.

2.4. Enzyme purification

The crude extract was further fractionated with ammonium sulfate. It was first brought to 35% saturation, centrifuged, supernatant collected and then brought to 75% saturation by an additional amount of $(NH_4)_2SO_4$. Protein precipitate was dialyzed against 10 mM Tris–HC1 buffer, pH 7.8 and chromatographed on a DEAE-cellulose column (2.6×42 cm) pre-equilibrated with the same buffer. After loading the enzyme, the column was thoroughly washed with the same buffer until the UV absorbance of the eluate returned to the baseline. The bound protein was eluted with a linear gradient of 0–0.5 M NaC1 in the same buffer at a flow rate of 0.5 ml min^{-1}. Active fractions were collected and concentrated by ammonium sulfate precipitation. The protein precipitate was re-dissolved in a small volume of 20 mM Tris–HC1 buffer, pH 8.3 and dialyzed against the same buffer. The supernatant collected by centrifugation was applied to a DEAE-Toyopearl column (1×29 cm) pre-equilibrated with the same buffer. Bound protein was eluted with a linear gradient of 0–0.4 M NaCl in the same buffer at a flow rate of 0.5 ml min^{-1}. Fractions containing the activity were pooled for characterization.

2.5. Optimum pH and temperature

The assay solution was incubated at different pH (ranging from 3.5 to 10.5) using a universal buffer under standard conditions and optimum pH was calculated from the pH-activity plot. The optimum temperature for the enzyme activity was determined by measuring activity at different temperatures from 10 to 80 °C.

2.6. pH and heat stability

For the determination of pH stability, the enzyme was incubated for 12 h at 4 °C in a universal buffer at pH 3.5–10.5 and the residual activity was assayed under standard conditions. Heat stability was measured by incubating the enzyme in 10 mM Tris–HC1 buffer (pH 7.5) for 30 min at 20–80 °C and the relative activity was determined under standard assay conditions.

2.7. Electrophoresis

Purity and molecular weight of the purified enzyme were determined by native polyacrylamide gel electrophoresis (native PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE),

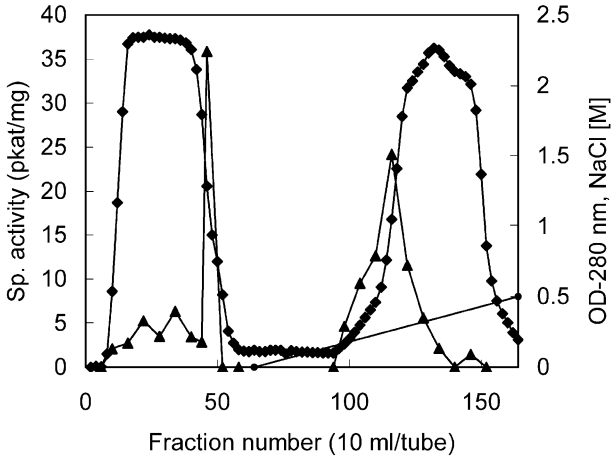

Fig. 1. Elution profile of DEAE-cellulose chromatography.(◆) OD at 280 nm; (Δ) enzyme activity; and (●) NaCl gradient.

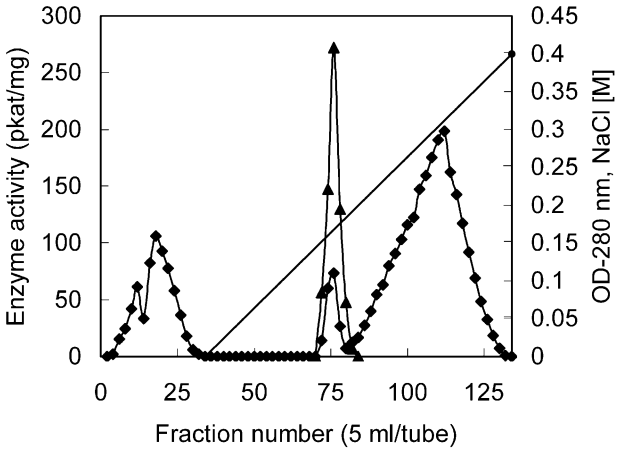

Fig. 2. Elution profile of DEAE-Toyopearl chromatography.(◆) OD at 280 nm; (Δ) enzyme activity; and (●) NaCl gradient.

Table 1
Purification of pummelo limonoid glucosyltransferase

Purification steps	Total protein (mg)	Total activity (pkat)	Specific activity (pkat/mg)	Yield (%)	Purification fold
Crude enzyme	548	658	1.2	100	1.0
35–75% $(NH_4)_2SO_4$ ppt.	407	651	1.6	98.9	1.3
DEAE-cellulose	68	603	8.9	91.6	7.4
DEAE-Toyopearl	0.9	195	216	29.6	180

respectively, as described by Weber and Osborn (1969) on 10% gel at pH 8.9. Before electrophoresis (SDS-PAGE), the enzyme was treated with 0.1% SDS in the presence or absence of 1% 2-mercaptoethanol at 100 °C for 3 min. The protein was stained with Coomassie Brilliant Blue. The molecular marker proteins used were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), trypsinogen (24 kDa) and trypsin inhibitor (20 kDa).

2.8. Kinetic analysis

Kinetic parameters for limonin were determined by measuring the initial reaction velocities at varying concentrations of limonin while maintaining the concentration of UDPG at 2 mM. Kinetics for UDPG were studied by measuring the initial reaction velocities at varying concentrations of UDPG while maintaining the concentration of limonin at 1 mM. The values of the Michaelis constant (K_m) and maximum velocity (V_{max}) were calculated from Lineweaver–Burk plots.

2.9. Metal ion effect

The influence of different divalent metal ions and EDTA on enzyme activity was determined by pre-incubation of the enzyme with 1, 5, and 10 mM metal ion or EDTA for 30 min at room temperature, followed by incubation with limonin and UDPG-2Na under standard conditions.

2.10. Effect of amino acid modifying agents

Effects of diethyl pyrocarbonate (DEPC), iodoacetamide and citraconic anhydride (CA) on the activity of the enzyme were tested, as described by Bhattacharya and Dubey (2000) and Kotwal, Khire, and Khan (2000).

3. Results and discussion

3.1. Enzyme purification

The purification of pummelo LGTase is summarized in Table 1. The enzyme was recovered in the 75% $(NH_4)_2SO_4$ fraction from the crude protein of pummelo albedo tissue. It was re-fractionated in 35–75% $(NH_4)_2SO_4$ that raised the purity of the enzyme to 1.3-fold. Chromatography of this fraction on a DEAE-cellulose column gave two peaks of activity, one peak before addition of NaCl and another peak at about 0.25 M NaCl (Fig. 1). Both peaks of activity (fractions 47–49 and 98–139) were pooled, mixed and concentrated which raised the purification to 7.4-fold. Finally, DEAE-Toyopearl chromatography gave a single peak of activity at 0.15 M NaCl (Fig. 2). Active peak (fractions

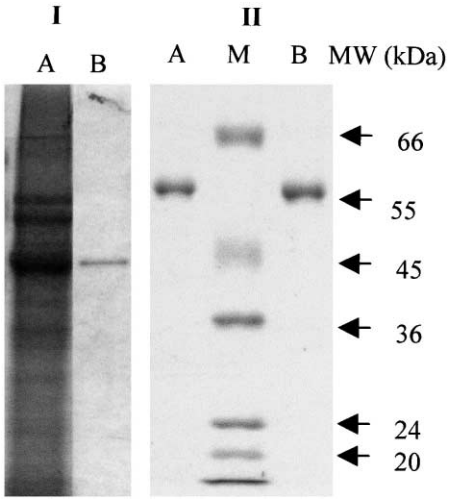

Fig. 3. Native (I) and SDS-PAGE (II) patterns of pummelo albedo LGTase. (I) lane A and B, crude and pure enzyme; (II) lane A and B, pure enzyme with and without 2-mercaptoethanol, respectively; lane M, molecular wt. marker proteins.

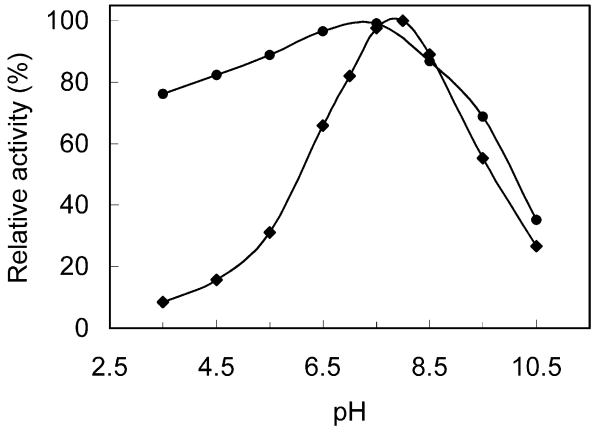

Fig. 4. Effect of pH on the activity and stability of limonoid glucosyltransferase.(◆) optimum pH; and (●) pH stability.

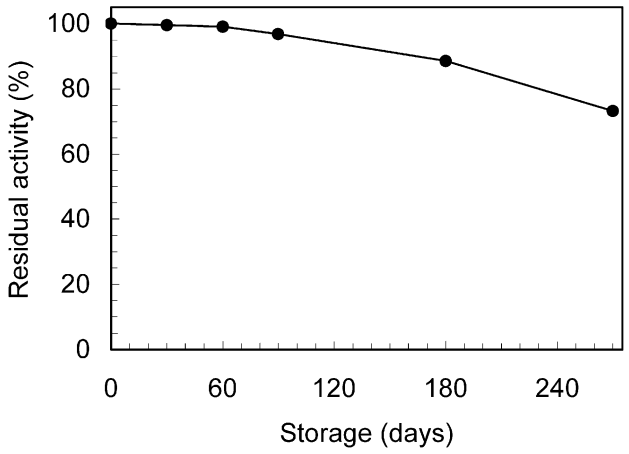

Fig. 5. Storage stability of LGTase at 4 °C in 10 mM Tris–HCl buffer, pH 7.5.

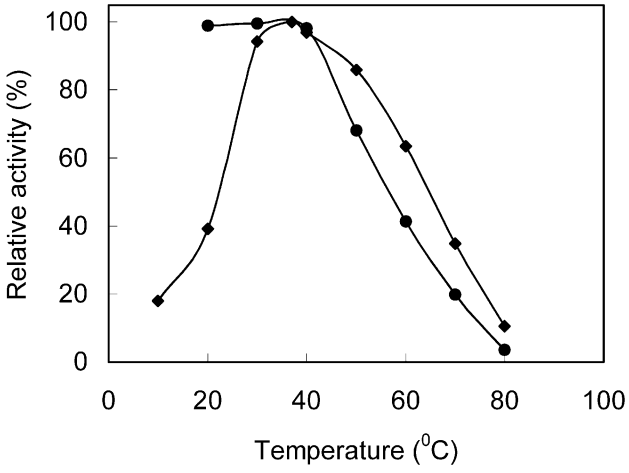

Fig. 6. Effect of temperature on the activity and stability of limonoid glucosyltransferase.(◆) optimum temperature; and (●) temperature stability.

72–80) was pooled and concentrated for characterization. This final step provided a 180-fold increase in the purification of LGTase. Purified LGTase gave a single band on PAGE (Fig. 3-I). The purification procedure was simple, yet 30% of the activity could be recovered by this procedure.

3.2. Properties of enzyme

SDS-PAGE of the purified enzyme, with and without 2-mercaptoethanol treatment, revealed a single protein band of the same mobility, suggesting that the enzyme consisted of a single polypeptide chain. Reference to the relative mobility of the molecular weight marker proteins, run in parallel with this enzyme on SDS-PAGE, gave a molecular weight of 55 kDa for the enzyme (Fig. 3-II). The molecular weight of pummelo LGTase is similar to that reported for flavonone glucosyltransferase from *Citrus paradisi* seedlings (McIntosh, Latchinian, & Mansell, 1990).

Limonoid glucosyltransferase from pummelo albedo showed optimum activity at pH 7.8. Activity decreased on both sides of the scale while a 63% decrease in activity was observed at pH 5.5 and 10.5. A similar pH optimum has been reported for *Citrus paradisi* flavonone glucosyltransferase, navel orange limonoid GTase and indoxyl-UDPG-glucosyltransferase (McIntosh et al., 1990; Hasegawa, Suhayda, Hsu, & Robertson, 1997; Marcinek, Weyler, Deus-Neumann, & Zenk, 2000). The enzyme was stable in a broad range of pH between 3.5 and 9.5 for 12 h incubation at 4 °C; the residual activity at pH 10.5 was 38% (Fig. 4). The enzyme was stored in 10 mM Tris–HCl buffer, pH 7.5 and residual activity was measured at different intervals. It retained 95 and 73% of the activity after 3 and 9 months, respectively (Fig. 5).

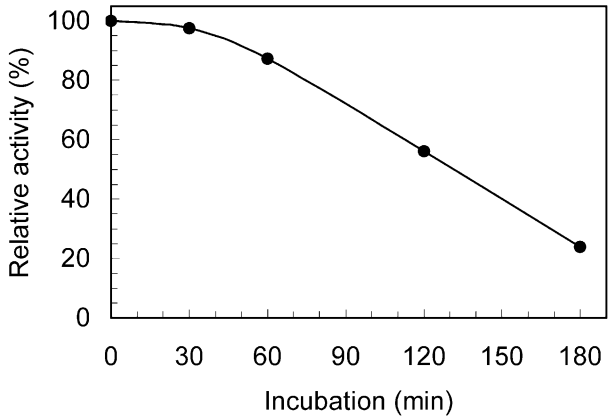

Fig. 7. Stability of LGTase at 40 °C in 10 mM Tris–HCl buffer, pH 7.5.

Table 2
Effect of metal ion and EDTA on the activity of limonoid glucosyltransferase

Reagent	Relative activity (%)		
	1 mM	5 mM	10 mM
None	100	100	100
Ca^{2+}	92	104	87
Co^{2+}	95	130	100
Cu^{2+}	67	42	49
Fe^{2+}	99	110	106
Hg^{2+}	67	67	66
Mg^{2+}	92	74	78
Mn^{2+}	98	133	123
Zn^{2+}	108	113	112
EDTA	0	0	0

Pummelo LGTase showed activity over a temperature range of 30–60 °C with the optimum at 37 °C. This temperature optimum corresponds to that reported for naringenin UDP-glucosyltransferase from grapefruit seedlings (McIntosh & Mansell, 1990). The enzyme was stable at temperatures up to 40 °C for 30 min incubation (Fig. 6). Rapid inactivation occurred above 40 °C. Time-dependence of thermal stability was studied by incubating the enzyme at 40 °C for different periods of time and the residual activity was calculated. Activity gradually decreased above 30 min incubation, whereas only 56% of activity remained after 2 h incubation (Fig. 7).

3.3. Enzyme activation or inhibition

Effect of divalent metal ions on the glucosyltransferase activity was determined at pH 7.8 (Table 2). Mn^{2+}, at 5 mM, enhanced pummelo LGTase activity up to 33% compared to the activity observed in the absence of metal ion or EDTA. Mn^{2+} also reportedly stimulates navel orange limonoid GTase, at 50 μM, by 66% compared to the activity observed with 100 μM EDTA (Hasegawa et al., 1997). Co^{2+} (5 mM) also stimulated pummelo LGTase by 30%. Mn^{2+} at 1 mM and Co^{2+} at 1 and 10 mM had no effect on the enzyme. EDTA, a metal chelating agent, at 1 mM completely inhibited the enzyme activity, indicating that the metal ion is essential for the enzyme activity. On the other hand, Cu^{2+} and Hg^{2+} inhibited the enzyme by 58 and 34%, respectively. Inhibition by Cu^{2+} and Hg^{2+} indicates that thiol, carboxyl groups or histidine residues are directly or indirectly involved in the catalytic mechanism of the enzyme (Kundu & Das, 1970).

Table 3
Effect of amino acid modifying agent on the activity of LGTase

Reagent	Residual activity (%)	
	1 mM	2 mM
None	100	100
Diethyl pyrocarbonate	24	0
Iodoacetamide	98	96
Citraconic anhydride	90	56

The effect of other amino acid modifying agents was studied and the results are shown in Table 3. DEPC, at 1 and 2 mM, exhibited 76 and 100% inhibitory effect, suggesting the involvement of histidine or tyrosine in catalysis. Treatment with iodoacetamide did not affect the enzyme activity, indicating that cysteine residues are not involved in catalysis. Citraconic anhydride at 2 mM showed 44% inhibitory effect on the enzyme. This might be indicative of an indirect role of lysine/arginine in catalysis. More thorough study is needed to precisely identify the catalytic residues of pummelo LGTase.

3.4. Kinetic constants

Kinetic studies of the enzyme were carried out under standard conditions. Apparent K_m and V_{max} values determined from Lineweaver–Burk plots, were 65 μM and 2 μM $min^{-1}mg^{-1}$ protein for limonin and 200 μM and 0.5 μM $min^{-1}mg^{-1}$ protein for UDPG, respectively. Some flavonoid GTases have K_m values of 50–70 μM and 100–500 μM for flavonoids and UDPG, respectively (Hrazdina, 1988; Reed, Davin, Jain, Deluca, Nelson, & Underhill, 1993). Another flavonone specific 7-O-glucosyltransferase has a K_m value of 62 μM for naringenin (McIntosh et al., 1990) that closely resembles the K_m value for limonin of LGTase.

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