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Screenig and Some Properties of Limonoid Glucosyltransferase from Selected Citrus Varieties

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Abstract: Limonoid glucosyltransferase (LGTase) is an enzyme in citrus that converts limonoids into corresponding glucosides and eventually reduces limonoid bitterness. Albedo from seven varieties of citrus were screened for this enzyme and its storage properties were examined. The varieties studied were Buntan (*Citrus grandis* Osbeck), Banpeiyu (*C. grandis* Osbeck), Natsudaidai (*C. natsudaidai* Hayata), Ponkan (*C. reticulata* Blanco), Iyomikan (*C. iyo* hort.), Meiwa kumkuat (*Fortunella crassifolia* Swingle) and Hassaku (*C. hassaku* hort.). Banpeiyu exhibited the highest specific activity followed by Buntan and Iyomikan. Banpeiyu also contained the highest amount of albedo per fruit weight followed by Buntan. Crude enzymes from all the seven varieties showed higher stability in the pH range of 5.5 to 7.5. The enzymes retained the activity up to 1 year as precipitate in ammonium sulfate while the activity lost very rapidly when stored in 10 mM Tris-HCl buffer, pH 7.5 at -20 or -80°C. Storage at 4°C in the same buffer could retain about 60% of enzyme activity only up to 10 days. Saline was the simpler and preferable medium for extraction of the enzyme. Banpeiyu and Buntan were the most suitable varieties for extracting LGTase in terms of saline extractability, specific activity, and stability of the enzyme. The pattern of protein bands in the selected varieties in terms of molecular weight were also compared.

Key words: Citrus, limonoid bitterness, albedo tissue, UDP-glucose: limonoid glucosyltransferase

Introduction

Citrus fruits are popular for their nutritional and medicinal value. Juice from most of the citrus varieties are facing commercial problem due to its bitterness caused by limonoids, a group of chemically related, highly oxygenated, tetracyclic triterpenoids (Maier *et al.*, 1969). Among citrus limonoids, limonin and nomilin are the primary causes of bitterness. The limonoid content in citrus fruits reportedly decreases during the process of ripening (Hashinaga *et al.*, 1981). This decrease is considered to be due to conversion of limonoid to a corresponding glucoside. These non-bitter glucosides are one of the major secondary metabolites and are synthesized in the fruit tissue and seeds (Hasegawa *et al.*, 1991; Fong *et al.*, 1991). Activity of this glucoside synthesizing enzyme, limonoid glucosyltransferase (LGTase), has also been reported in albedo (Hasegawa *et al.*, 1997; Cai *et al.*, 1998). The glucoside levels are surprisingly higher than free limonoids in citrus juice (Fong *et al.*, 1989). Yet, the remaining free limoniods cause bitterness problem after juice preparation.

A number of postharvest methods have been developed to minimise the limonoid bitterness problem. These include using of cyclodextrin polymer, affinity columns and immobilized bacterial cells or enzymes either to remove the bitter principle or to convert it to non-bitter ones (Hasegawa and Pelton, 1983; Shaw, 1990). Some reports suggested that limonoids and their glucosides have many health benefits including prevention of cancer (Lam *et al.*, 1989, a, b; Miller *et al.*, 1989). Because of this, the researchers in recent days are emphasizing on finding an alternative to remove bitterness without affecting limonoids or their metabolites.

Enhanced activity of 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 which can then be used in column as immobilized cells to convert free limonoids in juice into glucosides. Both of these possibilities need isolation and characterization of LGTase and its gene. Therefore, the objective of this study was to get a sound idea of the richer and cheaper source of LGTase enzyme and its storage properties with respect to pH and temperature.

Materials and Methods

Plant Materials: Fruits from locally produced citrus varieties viz, Buntan (*Citrus grandis* Osbeck), Banpeiyu (*C. grandis* Osbeck), Natsudaidai (*C. natsudaidai* Hayata), Ponkan (*C. reticulata* Blanco), Iyomikan (*C. iyo* hort.), Meiwa kumkuat (*Fortunella crassifolia* Swingle) and Hassaku (*C. hassaku* hort.) were obtained from the whole sale market and stored at 7-10°C.

Chemicals: Tris (hydroxymethyl) aminomethane, dimethyl sulfoxide (DMSO), phenyl methyl sulfonyl fluoride (PMSF), and polyvinyl pyrrolidone (PVP) were from Wako Pure Chemical Industries Ltd. Limonin substrate and its glucoside were purified in the laboratory. All other chemicals used were of analytical grade.

Crude enzyme preparation: Unless mentioned otherwise, all the operations were performed at 4°C. Crude limonoid glucosyltransferase was extracted from albedo as described by Cai *et al.* (1998) and Hasegawa *et al.* (1997) with some modifications in brief. Fruits were peeled and the albedo was cut from the peel excepting the case of kumquat in which the albedo was used along with the peel. The albedo was then homogenized with 4 volumes of pre-cooled 0.1M NaCl containing 0.5% PVP and 1 mM PMSF in DMSO or with 50 mM Tris-HCl buffer, pH 8.0 containing 1 mM PMSF in DMSO, 5 mM DTT and 15 mM 2-mercaptoethanol in a tissue homogenizer for 3 min. The homogenate was kept stirring for 2 hrs and then filtered through gauze. The filtrate was centrifuged at 10,000 x g for 30 min. The clear supernatant was brought to 75% $(NH_4)_2SO_4$ saturation and let it stand for

Table 1: Protein and LGTase activity extracted with saline and Tris-HCl buffer from albedo tissue of different citrus varieties

Variety	Protein (mg/ gm albedo)		Specific activity (pKat/mg protein)	
	Tris-HCl buffer	Saline	Tris-HCl buffer	Saline
Buntan	0.66 ±0.02	0.71 ±0.03	1.76 ±0.01	1.85 ±0.01
Banpeiyu	0.70 ±0.01	0.73 ±0.04	1.80 ±0.01	1.93 ±0.05
Ponkan	1.02 ±0.11	1.31 ±0.06	1.44 ±0.05	1.50 ±0.02
Iyomikan	0.43 ±0.01	0.57 ±0.04	1.18 ±0.02	1.24 ±0.04
Hassaku	0.78 ±0.03	0.94 ±0.05	0.22 ±0.01	0.24 ±0.01
Natsudaidai	0.91 ±0.12	1.24 ±0.26	1.11 ±0.09	1.16 ±0.05
Kumquat*	0.40 ±0.01	0.41 ±0.05	1.69 ±0.03	1.80 ±0.05

* Peel was used for enzyme extraction. Protein was assayed by the method of Lowry *et al.* (1955).

Table 2: Separable albedo and LGTase activity in different citrus varieties

Variety	Separable albedo (gm% fruit wt.)	Activity (pKat/gm albedo)	
		Tris-HCl buffer	Saline
Buntan	29.3 ± 1.3	0.12 ±0.00	0.13 ±0.01
Banpeiyu	34.5 ±0.5	0.13 ±0.00	0.14 ±0.01
Ponkan	6.5 ± 1.4	0.15 ±0.02	0.20 ±0.00
Iyomikan	10.1 ± 1.3	0.05 ±0.01	0.07 ±0.00
Hassaku	7.4 ± 1.3	0.02 ±0.00	0.02 ±0.00
Natsudaidai	9.0 ± 1.3	0.10 ±0.01	0.14 ±0.02
Kumquat*	29.4 ± 1.6	0.07 ±0.00	0.07 ±0.02

* Peel was used for enzyme extraction.

Table 3: Relative activity after storage of LGTase as precipitate in ammonium sulfate

	Relative activity (%) after storage							
Variety	3 months		6 months		9 months		12 months	
	-20°C	-80°C	-20°C	-80°C	-20°C	-80°C	-20°C	-80°C
Buntan	98.7	99.1	97.8	97.9	97.2	97.1	94.9	95.0
Banpeiyu	99.8	100	96.9	97.3	96.6	96.9	95.4	96.2

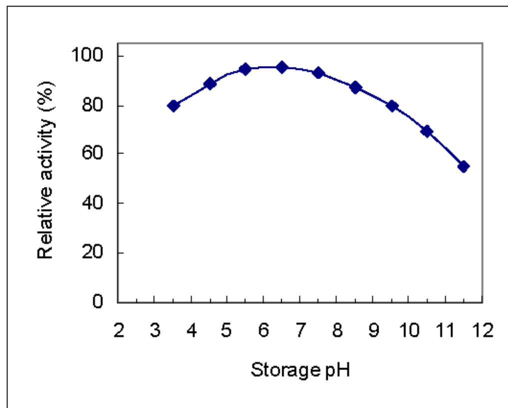

Fig. 1: Pattern of stability of crude limonoid glucosyltransferase stored at different pH

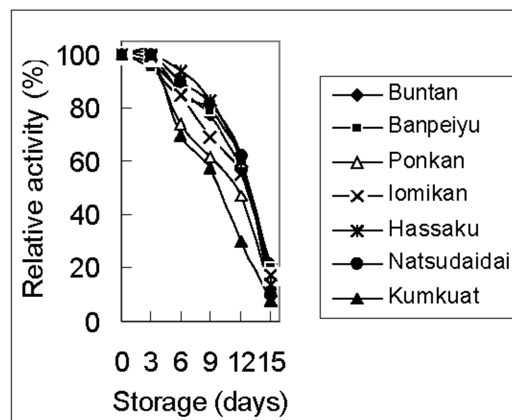

Fig. 2: Pattern of stability of crude limonoid glucosyltransferase in buffer at 4°C

1 hour. The protein precipitate was collected by centrifugation and was stored at -80°C.

The precipitate was dissolved in small volume of pre-cooled 10 mM Tris-HCl buffer, pH 7.8 and dialyzed against the same buffer for 12 hrs with 4 changes of buffer. The dialysate was centrifuged to remove any insoluble material and the supernatant was used as crude enzyme preparation.

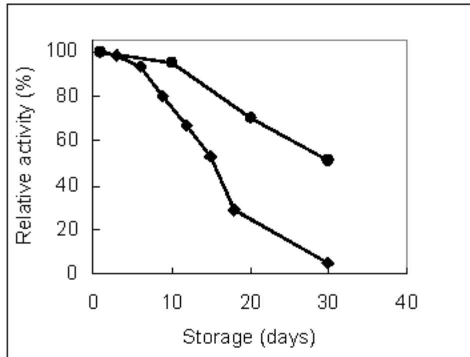

Fig 3: Pattern of stability of crude limonoid glucosyltransferase in buffer. (♦) -20°C and (●) -80°C

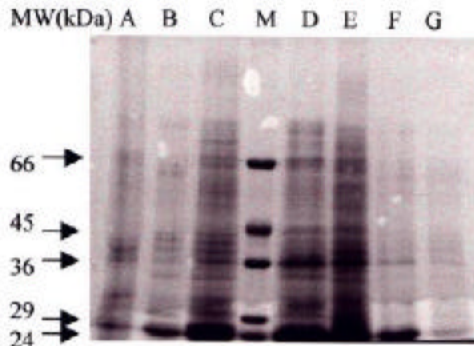

Fig. 4: SDS-PAGE of proteins extracted by saline from albedo of selected citrus varieties. Lane A, B, C, M, D, E, F, and G contain proteins from kumquat, Hassaku, Natsudaidai, Marker, Banpeiyu, Buntan, ponkan, and Iyomikan respectively. Arrows indicate the molecular weights in kDa of the marker proteins in the lane M

Enzyme 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, 10 mM Mg^{2+} and 20 mM Tris-HCl buffer. After 1 hr incubation at 37^0C, the reaction was stopped by boiling (5 min, 100^0C). The supernatant collected by centrifugation (10,000 x g) was used for product assay by HPLC with a Nucleosil $_7C_{18}$ reverse phase column (4.6 x 250 mm). Elution was done by using a linear gradient of methanol in water from 15 to 30% in 35 min at a flow rate of 0.4 ml min^{-1}. Limonin glucoside eluted at 13 min. The amount of product formed was calculated from the standard graph of limonoid glucoside and the unit of activity was expressed as pkat.

Protein estimation: Estimation of protein was regularly carried out by measurement of absorbance at 280 nm or by the method of Lowry *et al.* (1955) using BSA as standard.
2.6

pH stability: The crude enzymes were tested for their stability at different pH. For the purpose, an aliquot of the crude enzyme preparation was saved for assay of fresh activity and the rest was divided into 8 portions and dialyzed separately in a universal buffer at pH 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, and 10.6 for 24 hrs. The dialysates were then re-dialyzed in 10 mM Tris-HCl buffer, pH 7.0 for 12 hrs, centrifuged and the supernatants were used for assay of activity and protein measurement.

Enzyme storage: Crude proteins after extraction were stored either in Tris-HCl buffer solution or as precipitate in ammonium sulfate at 4, -20, and -80°C. Their relative activity were determined at different time intervals and compared with that of the fresh extract.

Electrophoresis: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Weber and Osborn (1969) on 10% gel at pH 8.9. Protein was stained with coommassie brilliant blue. Bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) and trypsinogen (24 kDa) were used as molecular marker protein.

Results

In order to compare the extractability of the desired enzyme, we applied two different extraction medium - Tris-HCl buffer and 0.1 M saline. It was found that, total extractable protein in 0.1 M saline was 3-36% higher than in buffer, while the specific activity was about 4-9% higher in case of saline extracted protein. Ponkan had the highest percentage of extractable protein followed by Natsudaidai and Hassaku. Though Hassaku had higher value of extractable protein, it had the lowest LGTase activity. Banpeiyu exhibited the highest specific activity in both saline and buffer extracted proteins followed by Buntan and kumquat (Table 1).

Comparison of separable albedo and LGTase activity in different citrus varieties showed that Banpeiyu had the highest percentage of separable albedo among the varities tested followed by Buntan. Kumquat had the negligibly least amount of separable albedo but the value shown in Table 2 was, in fact, the combined weight of albedo and the peel. Ponkan showed the highest value of specific activity per weight of albedo followed by Banpeiyu, Buntan, and Natsudaidai respectively (Table 2).

Incubation of crude LGTase at different pH for 24 hrs retained almost 95% of activity in the pH range of 5.5 to 7.5. The loss in activity was higher above pH 10 and almost 45% of activity lost at pH 11.5 within 24 hrs of storage at 4°C (Fig. 1).

The crude extracts from different varieties were stored at 4°C in Tris-HCl buffer, pH 7.5 and activity was measured every 3 days. Loss in activity of crude enzymes from all the varieties showed a parabolic trend except that of kumquat. Activity of the extracts remained almost unaffected up to 3 days and then decreased slowly up to 9 days. There was a rapid decrease in activity after 9 days and more than 75% of enzyme activity was lost within 15 days of storage (Fig. 2).

The storage properties of the enzymes were also studied in Tris-HCl buffer at -20 and -80°C. All the enzyme preparations showed the same linear trend in loss of activity and almost all the activity lost within 30 days of storage (Fig. 3). This loss in activity was accelerated when the enzymes were repeatedly thawed and frozen. Storage-activity plot for -80°C showed that Buntan and Banpeyiu lost 50% of activity within 30 days. Other varieties were not studied at -80°C. However, universal buffer, pH 7.0 allowed frozen storage (data not shown).

The enzyme extracts from the variety Banpeiyu and Buntan were stored as precipitate in ammonium sulfate at -20 or -80°C. Almost 95% of the activity retained up to one year (Table 3).

The crude proteins extracted from different varieties were subjected to SDS-PAGE in non-reducing condition. All the crude extract contained bands of similar molecular size indicating that the MW of the enzymes might be the same irrespective of variety (Fig. 4).

Discussion

Limonoid glucosides have been isolated and identified from several citrus varieties (Ozaki *et al.*, 1991; Hasegawa *et al.*, 1989; Bennet *et al.*, 1989), and the location of synthesis has been reported in seeds and flesh of some citrus varieties (Hasegawa *et al.*, 1991; Fong *et al.*, 1992). There are fewer reports on *in vitro* LGTase activity in albedo extract of citrus (Cai *et al.*, 1998; Hasegawa *et al.*, 1997). It is obvious from our study that citrus in general possess this enzyme in albedo in addition to its occurrence in seeds and flesh. Although pummelo is reported to have surprisingly low level of limonoid glucosides in its juice (Hasegawa *et al.*, 1996), we found Buntan and Banpeiyu, two cultivars related to pummelo, contained high LGTase activity in their albedo extract. Since albedo is directly attached to flavedo through segment membrane, it seems to play a partial role in glucosylation of limonoids during the course of ripening.

Comparison of the protein bands on SDS-PAGE though did not give any concrete idea of the MW of the enzyme, it may give some idea of the presence of protein bands in the possible region that can help deciding the possible methods for purification. Plant GTase that use various small molecules as glucose acceptor are reported to have MW in the range of 40 to 60 kDa and that of navel orange albedo LGTase in the range of 56 to 58 kDa (Hasegawa *et al.*, 1997).

Investigation on effect of pH on the storage of enzyme for 24 hrs showed that pH 5.5 to 7.5 is suitable for storage. We selected pH 7.5 for the storage of the enzyme at different temperature, but the enzymes were unstable at 4, –20, and -80°C in Tris-HCl buffer. Similar result is reported in case of partially purified LGTase from navel orange albedo (Hasegawa *et al.*,1997). The inactivation is postulated to be due to the high chloride ion concentration. But the chloride ion up to 10 mM did not affect naringin UDP-glucosyltransferase activity (McIntosh and Mansell, 1990). In our study the extraction saline containing 100 mM chloride ion apparently did not have any adverse effect on the activity compared to buffer that contained 10 mM chloride ion. There may be some contaminating phenolics or other compounds associated with the crude or partially purified enzyme that causes loss in activity during storage. Our study showed that optimum pH for storage is around neutrality and universal buffer, pH 7 allowed frozen storage. Storage of the enzyme as precipitate showed to be the best possible way to keep it active even for a year. It can be concluded from our study that Buntan and Banpeiyu can serve as promising cultivars as a source for isolation and characterization of LGTase and its gene for further study at molecular level. A brief idea of the properties of crude enzymes obtained from this study may further help in its purification.

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