RESEARCH ARTICLE

ASSESSING THE EFFECT OF SOLUBLE ACID INVERTASE ACTIVITY IN POST-HARVEST SUGARCANE UNDER TROPICAL CONDITIONS

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Abstract

Sugarcane is a major source of sucrose in India. Sucrose accumulation is a key physiological mechanism controlled by the activity of invertase enzymes. High activity of invertase enzymes at cane maturity and during post-harvest period reduces the sucrose yield and recovery. This study, report the activity profile of sugarcane soluble acid invertase (SAI) and its effect on sucrose yield at cane maturity and post-harvest period. Differential activity of SAI activity in High (Co 11015) and low (BO 91) sugar tropical sugarcane cultivars resulted in significant rise of reducing sugars accumulation and reduction in sucrose yield. Among the two cultivars, BO 91 was found to be more susceptible for sucrose deterioration as compared to Co 11015. Significant increase in SAI activity to the fold of 4.45, 4.81, 2.95 and 3.52 at 0hr, 24hr, 48hr and 72 hr after harvest was observed in BO 91 as compared to Co 11015. Reducing sugar accumulation was significantly increased from 0 hr to 48 hr after harvest corroborating the effect of high invertase activity during post-harvest period. In contrast, sucrose content was significantly decreased to the fold of 1.1, 1.3 and 1.6 in BO 91 and 1.0, 1.13 and 1.16 in Co 11015 after 24hr, 48hr and 72 hr after harvest respectively. These results demonstrated the invertase activity profile during post-harvest period under tropical conditions and provided the scope for controlling the invertase activity and thereby enhancing the sucrose yield in sugarcane.

Key words : Invertase, sucrose, post-harvest, reducing sugar; sugarcane.

Introduction:

Sugarcane is an important commercial crop in more than 100 countries. It contributes almost 80% of total sugar produced from all the sugar crops. In India, 358 million tons (mt) of cane are produced annually which shares about 20 per cent of the area and 22.6 per cent of the world sugar production. Sugarcane is a C_4 grass that can accumulate sucrose in the stems to the levels exceeding 25% of the fresh weight (FAOSTAT 2014, http://faostatfaoorg/defaultaspx). Sucrose content and cane weight are the key factors which decide yield and quality of sugarcane and ultimately the income of sugarcane farmers. The major problem in sugarcane is degradation of sucrose at cane maturity and after harvest. One of the most important factors responsible for degradation of sucrose is the high activity of endogenous invertase enzymes. Degradation of sucrose by sugarcane invertases during cane maturity and processing reduces the sucrose yield and sugar recovery (Chandra et al., 2012). Thus, results to huge loss in revenue to farmers and sugar factory. When sucrose accumulation reaches to saturation level, the biosynthesis of sucrose is stopped which indicates the maturity of plant. Subsequently, due to the activity of invertase enzymes, stored sucrose is degraded and affect the stability of sucrose in the sink tissue. Also, after harvest, endogenous invertases are activated due to loss of moisture and lack of any physiological and biochemical control mechanism. Weight loss of canes 7-10% under subtropical conditions

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within 72 hrs after harvest, loss to growers exceeds > 100 rupees/ton (Solomon et al., 2009). Sugar mill with a capacity of 5000 ton cane per day crushing (TCD), 72 h stale cane leads loss of around 4 lakhs rupees per day on account of low sucrose recovery (Solomon, 2009).

Invertases [EC 3.2.1.26; β-fructosidase], a family of enzymes that hydrolyse sucrose into glucose and fructose, have been proposed to carry out critical functions during sucrose accumulation in sugarcane (*Saccharum officinarum* L.) (Moore, 1995). Sucrose stored in sugarcane internodes is resynthesised from the breakdown products of translocated sucrose that is primarily cleaved by invertase (Hatch and Glasziou, 1963). In sugarcane, sucrose unloaded from the phloem passes through three distinct cellular compartments: apoplastic space (cell wall), metabolic compartment (cytoplasm) and storage compartment (vacuole) (Sacher *et al.*1963; Chandra et al., 2011). Each compartment contains a characteristic invertase isoform; acid invertases located in the apoplastic space (cell wall invertase, CWI), vacuole (vacuolar acid invertase, VAI, or soluble acid invertase, SAI), and a neutral invertase (NI) present in the cytoplasm. Most of the earlier studies focused on SAI and relatively few considered the other isoforms, CWI and NI (Botha et al., 2001). Therefore, general conclusions drawn from those studies are tenuous. In recent years, genetic engineering has been used as a precise tool for identifying the key enzymes in metabolic pathways. Invertase has been a main target for molecular manipulation in a number of plants, including *Arabidopsis* (von Schaewen *et al*. 1990), tobacco (von Schaewen *et al*. 1990; Sonnewald *et al.* 1991), tomato (Ohyama *et al.* 1995), potato (Bussis *et al.* 1997) and carrot (Tang *et al.* 1999). Eggleston and Legendre (2003) advocated that the enhanced activity of acid invertases could be due to mobilization of cell invertase, possible synthesis of cut induced invertase and decreased activities of sucrose synthesizing enzymes induced by pH change. Sucrose enhancement through transgenic approach necessitates complete understanding of mechanism of sucrose accumulation process and its regulation in sugarcane (Watt *et al*., 2005). Although many efforts to control invertase enzymes (both SAI & SNI) activity in sugarcane through transgenesis and despite having successful transgenic events, there was no significant increase in sucrose accumulation (Botha *et al*., 2001;Rossouw *et al.,* 2007; Ma *et al*., 2000). This might be due to the regulatory feedback mechanism between culm (sink) and leaf (source) during sucrose accumulation. To understand the biochemical mechanism of sucrose degradation at cane maturity and after harvest, we studied the activity of soluble acid inveratse activity and its effect on sugar yield in tropical sugarcane cultivars (Co 11015 and Bo 91) during post-harvest period.

Materials and Methods

Plant materials

Two sugarcane cultivars namely Co 11015 (high sugar) and BO 91 (low sugar) were used for this study. Replicated samples of twelve months old mature canes were drawn from the experimental field at Sugarcane Breeding Institute (SBI), Coimbatore, Tamil Nadu. The two varieties with three replicates were taken and the surface cleaned. Then the juice was collected using the sugarcane crusher from 0hr to 72hrs time intervals. The juice extracts were centrifuged at 10000×g for 5 minutes at 4ºC and the supernatant was stored at 4ºC until further analysis.

Invertase Activity Assay

Invertase enzyme activity was evaluated using commercial invertase assay kit (Biovision, USA) as per the manufacturer's protocol. An aliquot of purified juice sample in a 40 µl of reaction volume adjusted with hydrolysis buffer (supplied with kit) were added in a 96-well plate along with background control. 10µl of 0, 2,4,6,8 and 10 nM glucose standards were added to separate wells of the plate and adjusted to 50ul reaction with hydrolysis buffer. The same volume of reaction buffer was used as the assay blank in separate wells. Substrate was added to each well (10µl of 20 mM sucrose) followed by incubation for 20 min at 37^0 C. After incubation, the reaction mixture containing 36 µl of assay buffer, 2 µl of enzyme mix and 2 µl of dye reagent (all supplied with the kit) was prepared and 40 µl of reaction mix was added to each of the blank, sample and standard wells followed by incubation for 30 min at 37^0 C in darkness. After incubation, absorbance was recorded at 570 nm using a microplate reader and the amount of glucose liberated was calculated from the glucose standard curve. The enzyme activity was calculated and expressed as nmoles of glucose formed per minute per ml of juice.

Reducing sugars estimation

Reducing sugar content was determined as per the method described elsewhere (Somogyi, 1952). Briefly, 100 µl of juice samples were taken in a test tube and volume was made up to 2 ml with distilled water. Then added 1ml of alkaline tartrate reagent and incubated at boiling temperature for 10 min. Samples were cooled to room temperature followed by addition of 1ml of arsenomolybdic reagent. Finally, reaction volume was made up to 10 ml with distilled water followed by absorbance at 620 nm after 10 min incubation at room temperature. Amount of reducing sugars were calculated using glucose standard curve.

Sucrose content determination

Sucrose content in the juice samples were estimated using resorcinol-hydrochloric acid (HCl) method. An aliquot (5µl) of juice sample was taken in a test tube and volume made up to 100µl with distilled water and then 500µl of 1% resorcinol and 1.5ml of 30% HCl was added. The resulting reaction was incubated at 80º C for 10 min and the reaction was stopped by incubating the tubes on ice.The absorbance was measured at 520nm against a blank (without sample). Sucrose content in the juice samples was measured using standard curve prepared at different concentrations (0.2-1 mg) of sucrose.

Results and Discussion

In order to study the activity profile of SAI during post-harvest period, invertase activity assay was performed in two different sugarcane cultivars namely Co 11015 (high sugar) and Bo 91 (low sugar) during the post-harvest period (0, 24, 4 and 72 hr). These high and low sugar cultivars were selected understand the varietal susceptibility and differential activity of invertase enzymes during post-harvest period.

The enzyme assay experiments showed that SAI activity at 0 hour of the harvest was 43.2 and 9.7 $nMmin^{-1}ml^{-1}$ of juice in BO91 and Co11015 respectively. There was a significant increase in the activity of SAI from 24 to 72 hr after harvest in both the cultivars. An activity of 105.5 (24 hr), 128.13 (48 hr), 171.5 (72 hr) nM $min^{-1}ml^{-1}$ of juice in Bo 91 and 21.9 (24 hr), 43.34 (48 hr), 48.16 (72 hr) nM $min^{-1}ml^{-1}$of juice in Co11015 was recorded.

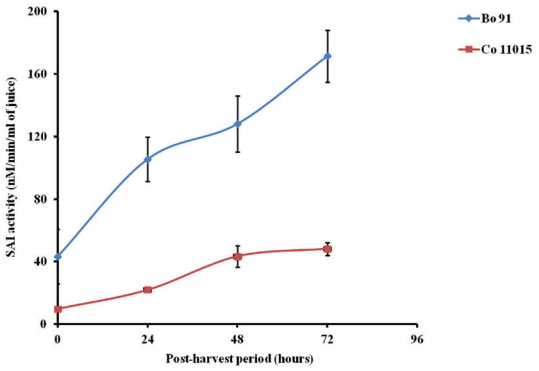

Fig. 1. Time course activity of soluble acid invertase (SAI) enzyme during post-harvest period

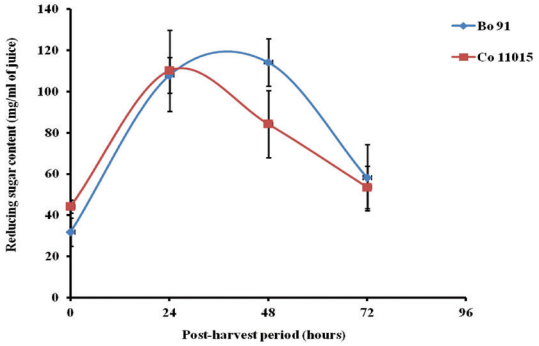

Fig. 2. Reducing sugar accumulation during post-harvest period under tropical conditions

When we compare these varieties for fold change enzyme activity Bo 91 exhibited 4.45, 4.81, 2.95 and 3.52 fold higher SAI activity than Co11015 at 0, 24, 48 and 72 hours after harvest respectively (Fig.1). It is evident that BO 91 is most susceptible variety for sucrose deterioration as compared to the Co11015. This is mainly due to the differences in the maturity time where Co 11015 is an early maturing and high sucrose accumulating variety than BO 91.

Analysis of reducing sugars concentration during the post-harvest period is an important indicator to test any variety for its susceptibility to sucrose deterioration. The results revealed that there was significant increase in reducing sugar content from 0-24 hours after harvest in both the cultivars and stabilized after 48 hours of harvest. The reducing sugar content at harvest (0 hr) was 31.8 and 44.21 mg ml^{-1} in BO 91 and Co 11015 respectively. There was significant increase in reducing sugar content of 107.9 mg ml^{-1}(24 hours) and 114.23 (48 hours) mg/ml in BO91and 110.21 (24 hours), 84.24mg ml^{-1}(48 hours) mg/ml in Co1 1015. After 72 hours of harvest the reducing sugar content was drastically reduced to 58.2 and 53.56mg ml^{-1}in BO 91 and Co11015 respectively. This has clearly showed that the trend in the accumulation of reducing sugars due to invertase activity was significantly increased from 0 to 48 hours in both high and low sugar cultivars (Fig. 2).

In contrast to the results of reducing sugar accumulation, sucrose content in the juice samples of high and low sugar cultivars was decreased from 0 to 72 hours after harvest. The sucrose content at harvest (0 hr) was 177.9 and 205.7 mg ml^{-1} in BO 91 and Co11015 respectively. But, there was a decrease in sucrose content to 157.3, 136.8 and 110 mg ml^{-1}in BO 91 and 201.3, 180.5 and 176 mg ml^{-1}in Co 11015 after 24-72 hr after harvest

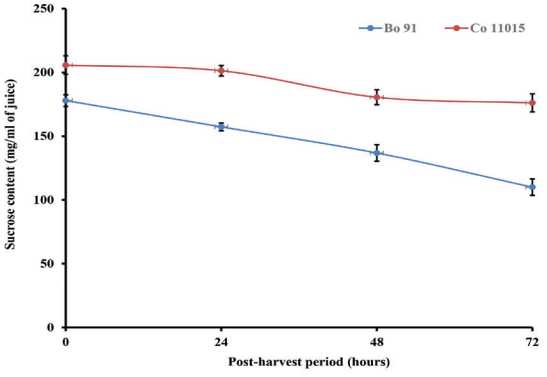

Fig. 3. Time course reduction of sucrose yield during post-harvest period under tropical conditions

respectively. Definite trend in reduction of sucrose was observed to the fold of 1.1, 1.3 and 1.6 in BO 91 and 1.0, 1.13 and 1.16 in Co 11015 after 24, 48 and 72 hr after harvest respectively (Fig. 3).

However, decreased reducing sugars and sucrose content was not coincided with the results of increased SAI activity after 72 hours of harvest. Although high SAI activity persists after 72 hours of harvest, plant has to maintain threshold levels of sucrose: reducing sugars ratio in order to maintain cell solute potential. Nevertheless, detailed study is required understand the relationship between SAI activity and sugar accumulation in sugarcane at crop maturity and during post-harvest period.

Post-harvest deterioration of sucrose in sugarcane is most common problem especially in sub-tropical India. Numerous studies have been conducted to assess the cause for deterioration of sucrose during post-harvest period in sugarcane (Solomon et al., 1990, 2006, 2009; Suman et al., 2000; Singh et al, 2008; Eggleston et al., 2009; Chandra et al., 2014). Adverse climatic conditions like high temperature leads to loss of moisture in harvested cane and resulting in reduced physiological activity and high activity of endogenous invertase enzymes affect the cane quality (Risk and Normand, 1968 and 1969; Solomon et al., 1990; Batta and Singh, 1991; Solomon, 2009). Surplus cane production causes the extension of milling season during summer months and deteriorates sucrose content under sub-tropics (Singh et al., 2008). Solomon *et al*. (2007) reported that 13.0 kg sugar loss per ton cane milled and loss of over 1.0 unit pol (percent cane) due to harvest-to-milling delays.

Previous studies have clearly demonstrated that activity of invertase enzymes is the key factor which decides the extent of sucrose losses during post-harvest period in sugarcane. Chandra *et al*. (2014) reported that acid invertase enzymes are involved in the degradation of sucrose during cane maturity and post-harvest. In addition to endogenous invertases, micro-organisms that colonize and grow in cut sugarcane stalks also produce invertases that further contribute to sucrose loss. It was reported earlier that increase in acid invertase activity to the fold of 1.38, and 4.75 after 48 and 240 hours of staling compared to freshly harvested cane during late milling season and application of anti-microbial formulations at the time of harvest slowed the rate of sucrose breakdown but losses were still substantial (Singh *et al*., 2008). Batta and Singh, (1991) reported seven fold increase in activity of acid invertase compared to four fold increase in neutral invertase after 12 days of storage. Solomon *et al*. (1990) observed that the increase of both acid and neutral invertase activity after 72 hr of storage of cane, with a corresponding rise in the level of invert sugar. Eggleston and Legendre (2003) also reported that enhanced activity of acid invertase could be due to possible synthesis of cut induced invertase and decreased activities of sucrose synthesizing enzymes induced by pH change. Increased activity of acid invertase enzyme and enhanced dextran accumulation was noticed during storage of harvested cane from 24 to 96 hr as compared to the freshly harvested crop (Solomon, 2009; Eggleston and Legendre, 2003).

In conclusion, post-harvest sucrose losses in sugarcane is a major problem in India. The action of invertases at cane maturity when the sucrose concentration in the sink tissues reaches saturation, affect the sucrose stabilization and reduces the sucrose yield. This study clearly demonstrated the activity profile of soluble acid invertase enzyme during post-harvest period. This is the first report to study the SAI activity and its effect on sucrose deterioration in sugarcane under tropical conditions. Further studies are required to understand the mechanism of futile regulation of invertase enzymes in sugarcane. One such

approach would be the post translation suppression of inverases enzyme activity by specific inhibitory proteins through sub-cellular targeting and *in planta* overexpression in sugarcane. This would be a novel strategy to validate the futile role of invertase enzymes in regulation of sucrose accumulation and also provide new insights into the understanding the mechanism of feedback regulation of sucrose metabolism and sink capacity in sugarcane.

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