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Journal of Chromatography B, 845 (2007) 151–156
JOURNAL OF CHROMATOGRAPHY B
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Application of high performance anion exchange-pulsed amperometric detection to measure the activity of key sucrose metabolising enzymes in sugarcane

Peter L. Albertson *, Christopher P.L. Grof

CSIRO, Plant Industry, Queensland Bioscience Precinct, 306 Carmody Rd., St Lucia, 4067 Brisbane, Qld, Australia

Received 23 February 2006; accepted 2 August 2006
Available online 22 August 2006

Abstract

A novel method using an HPAE-PAD system, which is routinely applied to detect carbohydrates at low levels (ng per sample injection), has been applied to the measurement of key sucrose metabolising enzyme activities in partially purified extracts of sugarcane tissues. Extraction and assay procedures tailored for the HPAE-PAD system enabled the accurate measurement of enzyme activities in more mature internodes than had previously been possible using enzyme coupled assay methodology. A major advantage of the HPAE-PAD method is the capability to monitor a broad range of sugars in each assay and provides an overarching perspective of the mix of competing enzymes that may be operating simultaneously in crude extracts. The technique has been successfully applied to measuring the activity of key sucrose metabolising enzymes in sugarcane stem tissue that is generally low in protein and high in endogenous sugars, primarily sucrose.

Keywords: HPAE-PAD; Enzyme activity; Invertase; Sucrose phosphate synthase

1. Introduction

The investigation of sucrose metabolism in sugarcane was pioneered by researchers in Australia in the 1960s and 70s [1–3], and has been continued more recently by other groups around the world [4–12]. The measurement of the key sucrose metabolising enzymes throughout this period has routinely relied upon an enzyme coupled assay system for those enzyme reactions having substrates or products not readily measurable. This system couples secondary enzyme reactions to the product of the primary enzyme reaction (involving the enzyme of interest) to form another compound which can be quantified, usually spectrophotometrically. The amount of the secondary product is directly proportional to the sugar produced in the primary reaction. The enzyme coupling reaction can be performed after or simultaneously with the primary reaction. If the coupling reaction is performed afterwards, an aliquot of the primary reaction is combined with the coupling enzyme/s in a suitable buffer possibly resulting in dilution and loss of sensitivity. When the primary and coupling reactions are performed simultaneously it may be under sub-optimal assay conditions for one of the enzymes, resulting in the potential underestimation of primary enzyme activity [13]. There may also be difficulties selecting suitable coupling enzymes for the measurement of a particular enzyme activity. For instance other enzymes present in crude extracts may become active in the presence of the coupling reagents or the assay conditions are unsuitable, e.g. differing pH, effector molecules etc. These factors can influence the rate of the coupled reaction and therefore may not enable an accurate measurement of activity for the enzyme being studied.

HPAE-PAD is an established method of carbohydrate analysis. It is sensitive, selective, has a broad linear detection range and has been applied to measure carbohydrates in food [14,17], animal [15] and plant tissue [16,17]. In contrast, we have applied

Abbreviations: HPAE-PAD, high performance anion exchange-pulsed amperometric detection; UDP, uridine diphosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; PEG, polyethylene glycol; NI, neutral invertase; SAI, soluble acid invertase; SS(f), sucrose synthase forward; SS(r), sucrose synthase reverse; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-thanesulfonic acid

* Corresponding author. Tel.: +61 07 3214 2215; fax: +61 07 3214 2288.
E-mail address: peter.albertson@csiro.au (P.L. Albertson).

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doi:10.1016/j.jchromb.2006.08.001

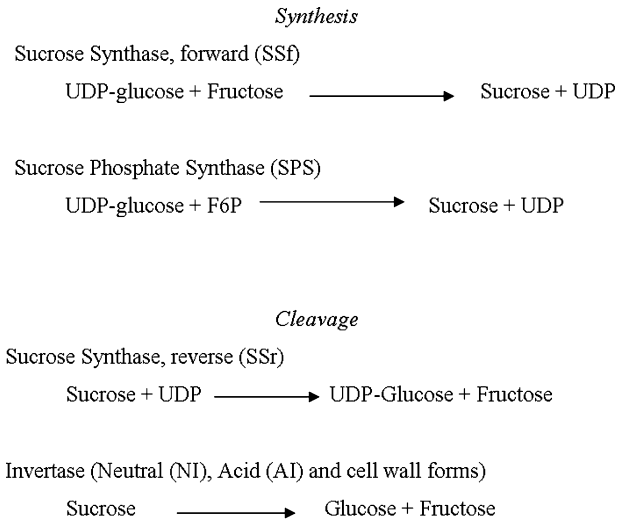

Fig. 1. Key enzymes involved in sucrose metabolism. Grouped by function into either sucrose synthesising or cleaving enzyme forms.

HPAE-PAD analysis to measure enzyme activity in sugarcane tissue. The aim of our research is to determine whether the variation in stalk sucrose content of progeny within a sugarcane population can be attributed to the level of activity for several key enzymes involved in sucrose metabolism.

In sugarcane, the allocation of sucrose produced through photosynthesis in the leaf is transported towards sink tissue, where some is used for growth or cellular maintenance and the remainder is transported to the stem parenchyma cells for storage. Sucrose transported to the stem undergoes continuous cleavage and re-synthesis, often referred to as "futile cycling". In young rapidly growing tissues (leaves and internodes at the top of the stalk), hydrolysis predominates whereas in maturing tissue (lower internodes) sucrose synthesis is favoured. There are several key enzymes that contribute to sucrose synthesis and cleavage in stem tissues. Sucrose synthesis is catalysed by sucrose synthase (SS forward (SS(f)); EC 2.4.1.13), a bi-directional enzyme, and sucrose phosphate synthase (SPS; EC 2.4.1.14) plus sucrose phosphate phosphatase (SPP; EC 2.4.1.14), whereas cleavage is catalysed by the different isoforms of invertase (cell wall, soluble acid and neutral; EC 3.2.1.26) and sucrose synthase operating in the reverse direction (SS(r) (EC 2.4.1.13)) (Fig. 1). Two of these enzymes, SS(r) and the invertases, have a common substrate, sucrose. The invertases may therefore be active in the SS(r) assay mixture (Fig. 1) and this must be taken into account in order to provide an accurate estimation of SS(r) activity. The balance of the activities of the sucrose synthesising and cleaving enzymes is believed to play a major role in regulating sucrose accumulation. The research we are conducting is focused on identifying biochemical markers for high sucrose accumulation in sugarcane. These markers would then be used to assist in the breeding by greatly reducing the 15–17 year period currently required to develop a new cultivar.

In this paper we describe the successful application of HPAE-PAD to monitor the activity of several key sucrose metabolising enzymes (SS(r), SS(f) and NI) in sugarcane stem tissue. Determining enzyme activity in this tissue is difficult because it is low in protein and high in fiber as well as endogenous sugars, particularly in mature internodes. We also briefly discuss the broader application of this method to measuring the activity of other enzymes, particularly those involved in carbohydrate metabolism, as it is able to detect numerous sugars at low levels and monitor them simultaneously.

2. Experimental

2.1. Solvents and chemicals

Solutions of NaOH were derived by diluting stock 5 M solution (Convol ampules, BDH Laboratory Supplies, Poole, UK) with de-ionised water (Millipore Corporation, Bedford, USA).

2.2. Standard solutions

Sugar standards (glucose, fructose and sucrose) were produced from analytical reagent grade chemicals (Roche, Boehringer-Mannheim and Sigma) dissolved in de-ionised water. HPAE-PAD calibration standards ranged from 15 to 750 ng, 20 to 1000 ng and 30 to 1500 ng per 10 μL injection for glucose, fructose and sucrose, respectively. Glucose calibration standards for the enzyme-coupled assay ranged between 375 and 7500 ng.

2.3. Plant enzymes: extraction and partial purification

Several stalks of a commercially grown sugarcane cultivar, Tellus, were harvested from the field (Kalamia, Australia) at 8 months of age. Several internodes were selected down the stem, separated and the rind removed. Internodes are numbered according to the description of Moore [18] whereby the first internode is that subtending the base of the youngest fully expanded leaf. The material was diced, 5 g subsampled for enzyme analysis and immediately frozen in liquid nitrogen and stored at −80 °C.

Enzymes were extracted from 5 g tissue in 50 mL extraction buffer (50 mM Hepes containing 1 mM EDTA, 1 mM EGTA, pH 7.5), as previously described [19]. The crude extract was concentrated by a two-step precipitation and resuspension procedure utilising 15% PEG (polyethylene glycol 8000 MWt, Sigma) (Fig. 2). A second PEG precipitation was required to remove residual sugars from the extract. The final pellet was resuspended in 2–3 mL extraction buffer and desalted on a Sephadex G25 column (50 mm × 10 mm) and stored on ice until required.

2.4. Enzyme assays and protein determination

Assays were subsequently conducted with 50 mM Hepes, pH 7.3. Substrates for the enzyme assays were well above reported K_m values enabling close to maximum enzyme activity (V_{max}).

2.4.1. Sucrose cleaving enzymes

Invertase and SS(r) assays were conducted in the presence of 125 mM sucrose, the SS(r) assay also included 15 mM UDP.

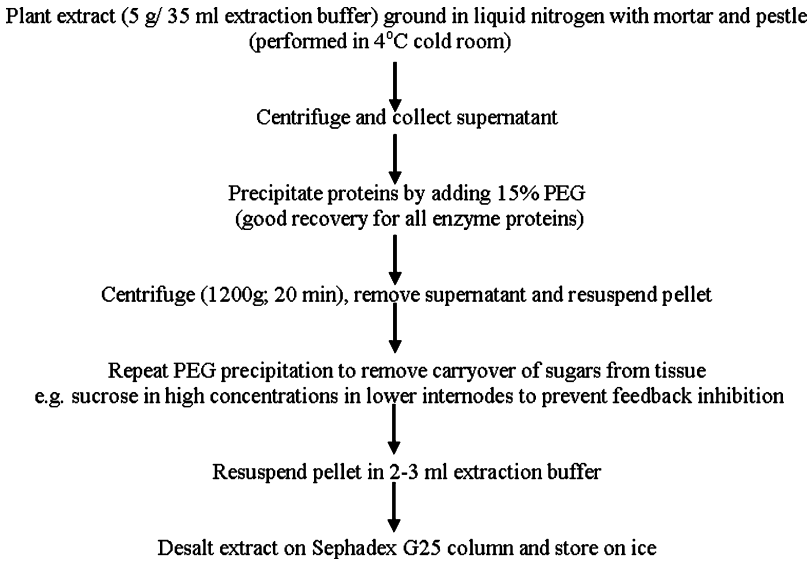

Fig. 2. Summary of the extraction and partial purification methodology.

2.4.2. Sucrose synthesising enzymes

SS(f) was conducted in the presence of 30 mM UDP-glucose and 15 mM fructose.

All assays were conducted for 30 min in a circulating water-bath (Grant W14, Grant Instruments (Cambridge) Ltd., Cambridge, England) at 37 °C. The enzyme assay was initiated by adding a 100 μL aliquot of crude extract to 200 μL of 2× assay buffer plus 100 μL of enzyme substrates. Aliquots at time zero (t_0) and 30 min (t_{30}) for all enzyme were stopped initially by freezing in liquid nitrogen and once all assays were completed were heated to 80 °C for 5 min. The acid invertase assay tubes were then neutralised with Imadazol [15] prior to heating. All tubes were then stored at −20 °C until the reaction products were analysed by HPAE-PAD. The difference in product present in the two samples ($t_{30} - t_0$) was then used to calculate enzyme activity.

Aliquots of partially purified extracts were frozen for subsequent protein determinations using the Bradford reagent [20] and BSA as the standard.

2.5. HPAE-PAD apparatus and chromatography

The HPAE-PAD system consisted of a Model 464 pulsed electrochemical detector, a Model 600 s controller and a Model 717 autosampler all from Waters, Milford USA. A computer was used for data acquisition using the Millenium® software (Waters).

The chromatographic separations were as follows and were adapted from a previously published report [21]. A model RCX-10 column (Hamilton Company, Reno, NV, USA), 250 mm × 4.1 mm (internal diam.) and 7 μm particle size, fitted with an inline RCX-10 guard column, 25 mm × 2.3 mm (internal diam. and 7 μm particle size) was used for the determination of sugars, primarily glucose, fructose and sucrose. The chromatography was performed at room temperature. The mobile phase, 75 mM NaOH degassed with helium (20 mL/min), was delivered at a flow-rate of 1.0 mL/min.

The PAD detector was operated using a gold electrode with a silver-siver chloride electrode at 2.0 μA in pulsed mode with the following potential settings: $E_1 = 80$ mV, $T_1 = 20$ cycles for 0.4 s; $E_2 = 730$ mV, $T_2 = 20$ cycles for 0.4 s; $E_3 = -570$ mV, $T_3 = 10$ cycles for 0.2 s.

Enzyme assay extracts were thawed and centrifuged in a bench top microcentrifuge (MicroMax, International Equipment Company, USA) at maximum speed for 5 min to pellet the denatured protein. An aliquot of the supernatant was transferred to a micro tube (1.5 mL) and for SS(f) and SS(r) assay mixtures was diluted three fold in de-ionised water. A sub-aliquot of 10 μL was injected onto the column and run for 15–20 min. Glucose, fructose and then sucrose eluted from the column with little or no background interference. Each set of ten samples was bracketed by five standards and the sugar concentrations were determined from the calibration curve.

2.6. Enzyme coupled methodology for the determination of sugar concentration

A number of neutral invertase assay mixtures were analysed using the HPAE-PAD and a coupled enzyme assay. The carbohydrate enzyme coupled assay method, involving the reduction of NADH to NAD^+ via coupling with glucose 6 phosphate dehydrogenase and hexokinase, was modified from a previously published report [22]. An aliquot of extract (30 μL) was added to each microtiter well and a further 270 μL enzyme coupling assay buffer mix was added, mixed and the reaction allowed to proceed for 15 min at room temperature. The microtiter plate reader used was a Thermo max from Molecular Devices Company (Sunnyvale, CA, USA). Measurements were conducted at 340 nm.

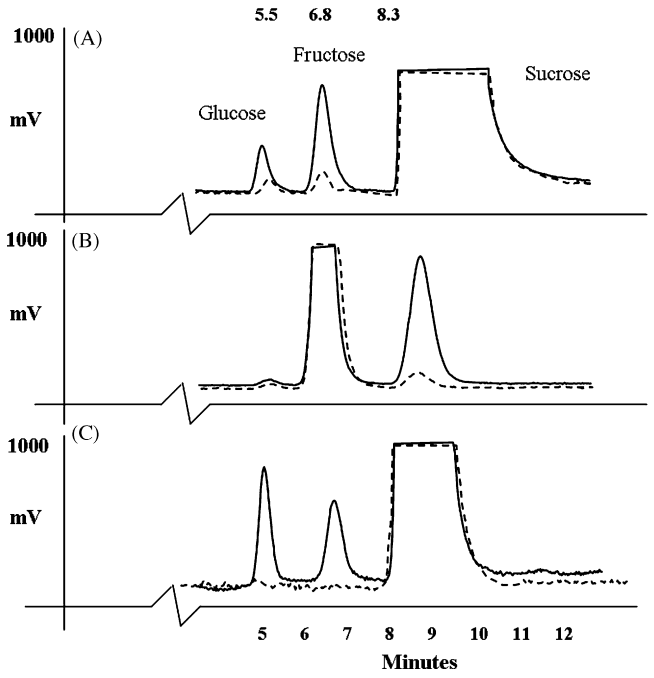

Fig. 3. Examples of HPAE-PAD traces for three enzymes (A) sucrose synthase (reverse), B) sucrose synthase forward and (C) neutral invertase (similar trace for acid invertase). Dashed and solid lines represent t_0 and t_{30}, respectively.

3. Results

3.1. HPAE-PAD chromatograms of three enzyme reactions: SS(f), SS(r) and NI

The HPAE-PAD traces showing the separation of the products for all enzyme assay reactions had extremely low background (Fig. 3). Glucose, then fructose and finally sucrose eluted from the column with retention times of approximately 5.5, 6.8 and 8.2 min, respectively. Enzyme reactions possessing these substrates at concentrations for near maximum activity (V_{max}) will cause overloading of the detector, e.g. fructose for SS(f); however the eluent concentration and flow rate permitted base line separation between the overloaded sugar substrate and the next eluted sugar (Fig. 3). As detailed in Fig. 1, the sucrose synthesising reaction, SS(f), requires UDP glucose and fructose as substrates. The HPAE-PAD methodology applied to monitor enzyme activity is unable to detect UDP-glucose, as determined empirically, therefore the chromatograms for the SS reactions do not contain UDP-glucose. Although HPAE-PAD can be applied to monitor phosphorylated sugars it was important in our study to detect the changes in glucose, fructose and sucrose from several key sucrose metabolising enzymes through a single analytical method. This was essential as the assays involved crude enzyme extracts where multiple enzymes are present and could be functioning simultaneously, e.g. SS(r) and NI. As the chromatographs of the enzyme products from SS(r) and NI reactions are quite different; NI produces equimolar fructose and glucose compared to SS(r) produces only fructose and UDP-glucose is undetected, it is possible to identify through the presence of glucose in an SS(r) chromatograph when these enzymes may both be active. The ability to measure several key sugars simultaneously also enabled the level of residual sugars carried over from the extraction process. High levels of these residual sugars could be present in the extract prior to assaying (t_0 sample) if the partial purification process (Fig. 2) was poor and could affect enzyme activity via feed-back mechanisms.

3.2. Calibration curves

Calibration samples were prepared at five concentrations for the HPAE-PAD. A block of standards (5) was performed prior to and after ten samples, with both blocks being used for the development of the calibration curve. The HPAE-PAD calibration curves, represented by the peak area versus standard sugar amount, were generated from linear regression analysis using the Millenium® software.

The enzyme coupled assay method had standards (6) of glucose ranging from 375 to 7500 ng/300 μL assay volume and the calibration curve plotted by linear regression (Microsoft® Excel software).

Calibration curves for both methods were obtained with a coefficient of determination (R^2) usually higher than 0.98. A comparison of glucose calibration curves using the two methodologies indicated the higher sensitivity of the HPAE-PAD system, with a detection limit of 10 ng compared to 150 ng for the enzyme coupled assay. Therefore, a small change in glucose resulted in a large change in peak area for the HPAE-PAD method and its calibration range was predominantly below that of the enzyme coupled assay calibration curve.

3.3. Application of the HPAE-PAD methodology

3.3.1. HPAE-PAD versus enzyme assay coupled methodologies to estimate Neutral Invertase activity

Estimates of glucose formed by NI using both methods were similar for the upper internodes (Int 1–3a and b), but not for the lower, middle internode (Fig. 4) which exhibited very low NI activity. NI activity decreases in more mature internodes as

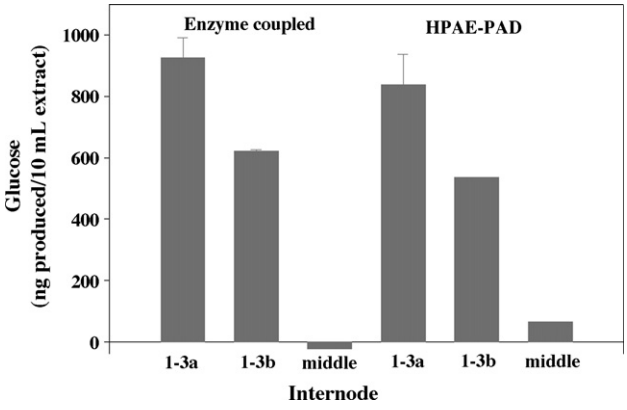

Fig. 4. Measurements of glucose produced from NI activity in two upper internodes (Int 1–3a and Int 1–3b)(pooled internodes 1, 2 and 3) and the middle internode (midway between the 10th and the bottom internodes) using the HPAE-PAD and enzyme coupled assay methods. Values are the mean ± S.E. of three replicates.

the hexose demands are reduced. The major reduction in NI activity occurs between internodes 1 and 10 (data not shown). Subsequently, lower internodes (11-bottom internode (23–33 internodes in mature cane)) have very little NI activity (data not shown). Therefore, for the bulk of the internodes along the sugarcane stalk the enzyme coupled assay method would be unable to effectively measure enzyme activity. The enzyme coupled assay method required 30 μL of sample in a total microtiter well volume of 300 μL. There is little scope to increase the sensitivity of this system by further increasing the sample volume as the background noise also increases substantially. In contrast, the samples for the HPAE-PAD method were originally diluted three-fold and only 10 μL was injected onto the column. By decreasing the dilution factor and or increasing the injection volume, even greater sensitivity can be achieved using the HPAE-PAD method. Alternative enzyme coupled assay techniques with very high (pmoles) sensitivity have been described elsewhere [23] which are as sensitive, if not more so, than the HPAE-PAD but may require enzyme cycling methods to increase sensitivity. These additional steps and possible background interference from the crude extract may limit their use. In contrast, the HPAE-PAD technique permits the measurement of several key sugars simultaneously with limited extract preparation and running time.

3.3.2. *Application of the HPAE-PAD system to the measurement of sucrose synthase (SS(f)and SS(r)) activity in sugarcane stem*

An initial examination of SS(f) and SS(r) activity in top and bottom internodes of a sugarcane stalk indicated a larger ratio of SS(f)/SS(r) activity in immature internodes (Fig. 5). With increasing internode maturity SS(f) declined and SS(r) increased resulting in a SS(f)/SS(r) ratio of approximately 1.0 (Fig. 5). A decrease in SS(f) activity in maturing sugarcane internodes has also been reported [24]. Varying SS(f)/SS(r) ratios may indicate regulation of sucrose metabolism down the stem and may be a result of post-translational modification of the enzyme, e.g. phosphorylation/dephosphorylation [25,26] or could indicate the presence of internode specific SS isoforms [13,27]. These results differ to a previously published report [13] using

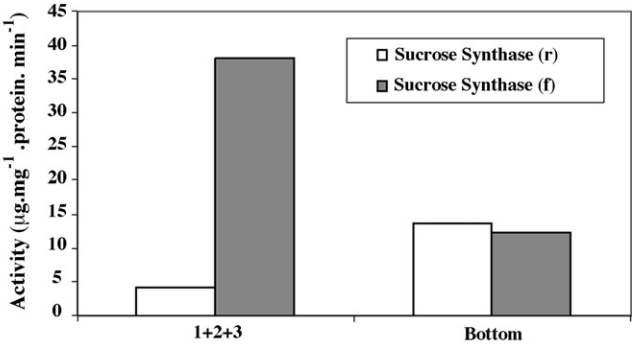

Fig. 5. Sucrose synthase activity in the reverse and forward directions for various sugarcane internodes determined by HPAE-PAD analysis. Internodes 1 + 2 + 3 are the upper three internodes including the meristem. Bottom internode was internode 23.

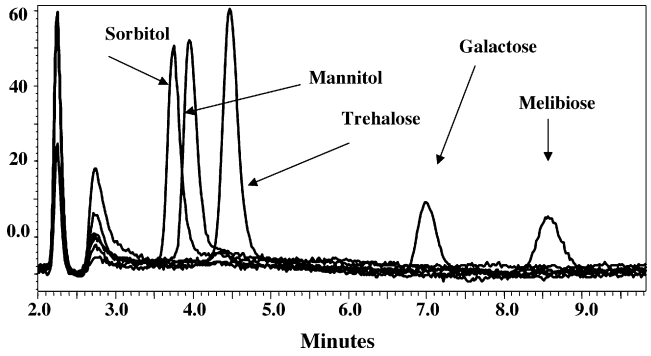

Fig. 6. Combined HPAE-PAD traces illustrating a few carbohydrate standards that can be monitored using the gold electrode. All samples are within the 100–200 ng/10 μL and were injected seperately.

the enzyme coupled assay system which showed a SS(f)/SS(r) ratio >4.0 for mature and immature internode tissue. However, they did not account for NI activity. SS(r) activity shows changes in sucrose or fructose, whereas the presence of a glucose peak would indicate invertase activity. The discrepancy between the reported SS(f)–SS(r) ratio [13] and our results for stem tissue may also be due to the differing varieties used and internode/stalk maturity.

The advantage of the HPAE-PAD system to monitor several sugars at low levels has enabled measuring SS(f) and SS(r) activity in very mature sugarcane internodes (internodes >20 (meristem = 1)) which, to our knowledge, has not been performed previously. The HPAE-PAD trace of many sugars also provided a valuable tool by indicating possible enzyme activity occurring simultaneously with SS(r).

3.3.3. *Sugars detected by the HPAE-PAD*

An example of an HPAE-PAD trace for several sugars is shown (Fig. 6). These sugars were detected using the conditions described earlier and may not be optimal for each sugar. The potential to apply the HPAE-PAD to determine enzyme activities associated with the formation or loss of these sugars is great, particularly when enzyme coupled assays cannot be applied due to interference by other enzymes utilising the same substrate or products.

4. Discussion

The described HPAE-PAD method was adapted from the measurement of soluble sugars [17] and applied to the measurement of sugars produced by enzyme activity in sugarcane stem tissues. The extraction procedure involved concentrating the protein and removing low molecular weight compounds. In particular, the double PEG precipitation procedure (described in Fig. 2) was effective in removing residual sugars as the t0 HPAE-PAD traces illustrate (Fig. 3) and therefore prevented the possibility of product feed-back inhibition on enzyme activities. The HPAE-PAD system was applied to monitor the low level of sugars produced by the primary enzyme. The superior sensitivity of the HPAE-PAD system was evident when compared to a commonly used enzyme coupled assay system for the measurement

of neutral invertase. Although alternative enzyme coupled assay techniques with greater sensitivity have been developed for the measurement of sugars [19] these require incubation periods in excess of the HPAE-PAD run time and still only monitor a single sugar.

Mature sugarcane internodes are low in protein but high in fiber and endogenous sugars, which has often prevented the accurate measurement of *in vivo* enzyme activity in this tissue. We have successfully utilised the HPAE-PAD for the measurement of key sucrose metabolising enzymes in all sugarcane stem tissue. However, the application of this methodology has much broader application because the HPAE-PAD is capable of measuring many carbohydrates at low levels. It could be applied to accurately measure the activity of numerous other enzymes involved in carbohydrate metabolism not suited to coupled enzyme assays. For example in yeast, trehalose is a major storage carbohydrate and consequently the enzyme catalysing its synthesis, trehalose phosphate synthase, is highly active [28]. Although recently detected in plants [29] trehalose is only present in very low concentrations due to the high activity of trehalase [30]. The HPAE-PAD could be readily applied to measure both the synthesis and breakdown of trehalose.

5. Conclusion

To our knowledge, the application of the HPAE-PAD to the measurement of enzyme activity has not previously been reported. It has been successfully applied to examine low enzyme activities in complex extracts from sugarcane internodal tissue from extremes of the developmental spectrum. It has measured the products directly in the absence of coupling enzymes. The HPAE-PAD system can also monitor many sugars at low concentrations and this may have wider application in accurately measuring the activity of enzymes previously unsuited to enzyme coupled assays.

Acknowledgements

We thank Carl Loveridge (Queensland Univesity of Technology), Terry Morgan (CSR, Kalamia) for providing invaluable assistance and John Lunn (CSIRO, Plant Industry, Canberra) for valuable discussion and comments. We also thank the Sugar Research and Development Corporation (SRDC) for supporting aspects of this research.

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