CHAPTER ONE

GENERAL INTRODUCTION

Sugarcane (*Saccharum* species hybrids) is a perennial agricultural crop belonging to the family of Gramineae and grown primarily for the sucrose-containing juices expressed from its stalks. It is best planted commercially in tropical and sub-tropical areas throughout the world that are characterised by warm temperatures, humid climates, and deep fertile soils (Barnes 1974). The primary use for sugarcane is to produce sucrose, which can then be utilised in a range of products. Approximately 76% of the world's sugar supply is derived from sugarcane (www.Illovo.co.za); South Africa is the leading producer of sugar in Africa and is a major sugar exporter to the world market. In the year 2000 the country ranked 6th in the league of the world's top exporters, with shipments of 1.3 million tonnes, raw value (www.sugaronline.com).

In order to sustain the global competitiveness of this industry and further improve it, innovative approaches have to be explored to respond to the increasing challenge aimed at producing more sucrose per dry weight by overcoming yield-limiting constraints.

Sugarcane biomass yields are among the highest for any crop. However, record sucrose yields are more or less 60% of the theoretical maximum (Bull and Glasziou 1963). There is a considerable potential for increasing sucrose accumulation in sugarcane if the physiological and biochemical limits can be identified and modified (Moore et *al*. 1997). Attempts at this have been made through traditional breeding methods and optimization of growing conditions (Moore and Maretzki 1997). Although this has resulted in improved plants, the selection procedure is very long and arduous. Limits certainly exist in the major physiological processes of photosynthesis, sucrose transport, and metabolism. Enzymes involved in these key steps have been identified, characterised, and evaluated for their roles as key regulatory steps. Genes encoding these enzymes are being cloned and used to transform plants to modify enzyme activity and increase sucrose accumulation. The results suggest that control in major metabolic pathways is shared between enzymes and it is therefore more difficult to transform than had been previously appreciated (Moore et *al*. 1997).

A kinetic model describing sucrose accumulation in sugarcane was published (Rohwer and Botha 2001). This model was used to calculate the control coefficients of enzymes in the sucrose synthesis pathway for sucrose futile cycling (degradation and re-synthesis of sucrose), with a view to determining with reactions control this energetically wasteful process. It requires the rate equations of all reactions in the pathway and the kinetic parameters of every enzyme. The model was recently improved (Schäfer et *al*. 2004a) by substituting the generic parameters SuSy in the original model with experimentally determined kinetic parameters of SuSyC obtained by Schäfer (2004).

Despite extensive studies on sucrose accumulation in sugarcane, the biochemical processes controlling the yield of sucrose remain poorly understood. Therefore, an increase in sucrose content is likely to be achieved by a better understanding of these processes. In this study, therefore I will be focusing on enzyme activities in relation to sucrose accumulation in order to determine their possible implications for sucrose metabolism. Then, the corrected kinetic model for sucrose breakdown of synthesis for sucrose accumulation (Schäfer et *al*. 2004) will be expanded by incorporating enzymatic activities data to reflect changes in tissue maturation. If expanded successfully, the kinetic model for sucrose accumulation will then be used with the aim of identifying the most effective control points of processes that contribute to cytosolic sucrose accumulation in different internodes and genotypes.

Sucrose is a major product of photosynthesis in green plants, accounting for much of the CO_2 fixed during photosynthesis (Kruger 1997). It also serves as the principal long-distance transport compound in most plants and as a storage compound in some (including sugar beet, sugarcane and carrot) (Hartt et *al*. 1963; Komor 2000). Sucrose metabolism is governed by several enzymes (Quick and Schaffer 1996). Invertases (invertase, β-fructofuranosidase, EC 3.2.1.26) hydrolyze the disaccharide, producing free glucose and fructose. They are classified by solubility, cellular location, and pH optima. Sucrose Phosphate Synthase (SPS, EC 2.4.1.14) synthesizes Sucrose-6-Phosphate (Suc-6-P), which is then dephosphorylated by Sucrose Phosphatase (SPP, EC 3.1.3.24). In addition, Sucrose Synthase (SuSy, EC 2.4.1.13) is capable of catalysing both sucrose synthesis and degradation.

In plants there are two enzyme groups, the hexokinases (HK, EC, 2.7.1.1) and the fructokinases (FRK, EC 2.7.1.4), that can facilitate the reversible phosphorylation of hexoses towards glycolysis using a nucleotide triphosphate as phosphoryl donor. HK can phosphorylate both glucose and fructose, whereas FRK specifically phosphorylates fructose. In addition, the interconversion of fructose 6-phosphate and fructose 1,6-bisphosphate involves the regulatory metabolite fructose 2,6-bisphosphate and three enzymes: ATP-dependent phosphofructokinase (PFK; EC 2.7.1.11), PPi-dependent phosphofructokinase (PFP; EC 2.7.1.90) and Fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11). The focus of the present study is on PFP and PFK activities.

This investigation combines enzyme activity profiles assessment and kinetic modelling outputs. Thus, a detailed study of the enzyme activities governing sucrose metabolism in sugarcane and their correlation with sucrose content was carried out (Chapter 3). Then, enzyme activity data of different internodes will be incorporated to obtain a detailed model of every stage in the tissue maturation process and its performance assessed (Chapter 4).

CHAPTER TWO

ENZYME ACTIVITIES AND SUCROSE ACCUMULATION

2.1 Introduction

Sucrose is the most important low-molecular weight carbohydrate in higher plants. Together with starch it is the dominant assimilation product in leaves, and in most plants it is the major compound which is translocated in the phloem to the non-photosynthetic and storage tissues (Kruger 1997). Sugarcane has been subjected to detailed physiological and biochemical studies for years because it accumulates sucrose to high concentrations (Moore 1995 and reference therein). Information on where and how sucrose accumulation is controlled in the sugarcane plant is essential for developing strategies for agronomic or genetic regulation of partitioning.

2.2 Sucrose metabolism and enzyme activities in internodal tissue

2.2.1. Sucrose metabolism in sugarcane culm

During the growth phase of the maturation of internodes, sucrose accounts for 68-75% of the soluble solids (Welbaum and Meinzer 1990). This proportion can surpass 90% at the end of the crop cycle (Moore 1995). To understand the biochemical basis for increased sucrose accumulation within the storage parenchyma, current research emphasis on sugarcane has been focussed on studying expression levels of the enzymes responsible for its accumulation.

2.2.2. Enzymes involved in sucrose metabolism

2.2.2.1 Sucrose phosphate synthase

Sucrose synthesis occurs solely in the cytosolic compartment of the cell. There are two routes for the synthesis of sucrose. The first is catalyzed by the enzyme sucrose synthase (SuSy: UDP-glucose:D-fructose 2-α-D-glycosyl-transferase, EC 2.4.1.13), and

the second by sucrose phosphate synthase (SPS; UDP-glucose:D-fructose-6-P 2-α-D-glucotransferase, EC 2.4.1.14). Two enzymes could be responsible for sucrose synthesis, namely SPS and/or SuSy. It is widely accepted that sucrose is derived from hexose phosphates through the sequential reactions of UDP-glucose pyrophosphorylase, SPS and sucrose phosphatase (Stitt et *al*. 1987; Huber and Huber 1996). However, SuSy could also be involved in sucrose synthesis even though the equilibrium constant for SuSy formation of sucrose is only 5 compared with 3250 for SPS (See review of Moore 1995).

SPS is viewed as the main enzyme that synthesizes sucrose in the sink tissues (Wendler et *al*. 1990; Goldner et *al*. 1991; Huber and Huber 1996; Stitt 1997). In the younger internodes both SuSy and SPS contribute to sucrose synthesis. However, SPS activity becomes gradually more important in sucrose synthesis as the internodes get older, exceeding SuSy more that three-fold (Botha and Black 2000). Results of incubation analyses of internodal tissue slices carried out in a solution containing labelled glucose, and obtained after extracting and hydrolyzing the sucrose, showed that in young internodes 70% of the label was present in the glucose moiety of the disaccharide, and in mature internodes this declined to 50% (Botha and Black 2000). Additionally, pulse labelling experiments with sugarcane cell suspension cultures indicated that SuSy did not contribute substantially to sucrose synthesis and, as a consequence, it was suggested that sucrose synthesis is rather catalysed exclusively via SPS (Wendler et *al*. 1990; Goldner et *al*. 1991). Sucrose accumulation was found to be correlated with the difference between the activities of soluble acid invertase and SPS, amongst the progeny of a defined cross between a high-sucrose accumulating genotype (*Saccharum officinarum*) and a low-sucrose content genotype (*Saccharum robustum*) (Zhu et *al*. 1997; Lingle 1999).

2.2.2.2 Sucrose synthase

Once in the stem, sucrose can be catabolised by SuSy or one of three invertases prior to being used for cell growth, respiration, metabolism, or storage (Hawker 1985; Hawker et *al*. 1991).

SuSy activity is low in storage parenchyma cells but relatively high in the vascular strands from both immature and mature storage tissue. It has been suggested that SuSy does not catabolise sucrose in the metabolic compartment of parenchyma cells because a highly specific uridine diphosphatase which rapidly hydrolyzes UDP rendering it unavailable for transfer to glucose by SuSy (Moore 1995). It is generally accepted that SuSy's main role is the generation of UDPG from sucrose for the formation of cell wall materials and starch (Chourey et *al*. 1998). Also, SuSy activity in sugarcane has been associated with maturing internodes (Lingle and Smith 1991) and with more mature fully elongated internodes (Hawker and Hatch 1965; Buczynski et *al*. 1993). Some studies showed negative correlation between SuSy activity and sucrose accumulation rate (Lingle and Smith 1991) and others showing no correlation (Botha and Black 2000). The patterns of SuSy activity in relation to sucrose content sometimes differs considerably between studies: for example, in one study SuSy activity was more than twice as high in internode six than in internode three (Buczynski et *al*. 1993), while in another the relative activities between these internodes showed reverse distribution (Lingle and Smith 1991). The difficulty is based on the differences with the different experimental protocols, growing conditions and cane varieties.

The SuSy sucrose breakdown/synthesis ratios varied between internodes differing in maturity and indicated an increasing trend as internodes mature. The data from Schäfer et *al* (2004) are in accordance with the labelling data from Botha and Black (2000) that clearly demonstrated SuSy's involvement in sucrose synthesis in young internodes.

2.2.2.2.1 Sucrose synthase isoforms

In most plants investigated, SuSy is encoded by at least three genes which are differentially expressed among different genotypes, organs, tissues, or stages of development (Moore 1995). Two forms of SuSy (SS1 and SS2) are present in sugarcane internodes (Buczynski et *al*. 1993). However, three other forms of the enzyme were purified and characterised from sugarcane leaf roll tissue with significant differences in substrate K_m values and sucrose breakdown synthesis ratios (Schäfer 2004).

Both forms (SS1 and SS2) of sucrose synthase characterised in sugarcane internodes by Buczynski et *al* (1993) resemble each other very closely as shown by their kinetic and pH parameters. SuSy activity is highest at the neutral pH and both isoforms display similar K_m values for sucrose and UDP (uridine 5'-diphosphate). These results could be explained by the fact that genes coding for the two forms are also highly homologous (A. Suresh Kumar, personal communication). K_m values determined for sucrose were 3 and 19-fold higher than those for fructose for both SSI and SS2 isoforms, respectively (Buczynski et *al*. 1993). Immunoblot analyses indicated that all of the SuSy activity in the older internodes was due to SS1. This is largely as a result of down regulation of SS2. The mechanisms of regulation of transcription and translation of SuSy genes are not yet known.

2.2.2.3 Invertases

2.2.2.3.1 invertase isoforms

Sucrose can be hydrolyzed in three distinct cellular compartments: cell wall, metabolic compartment (cytoplasm) and the storage compartment (vacuole), through the action of invertases. Plant invertases (β-D-fructofuranosidase, EC 3.2.1.26) are divided into two groups defined by their pH optima. One acid invertase is located in the apoplastic space (cell wall invertase, CWI); another is located in the vacuole (vacuolar acid invertase, VAI or soluble acid invertase, SAI). Both display maximum activity at pH ranging from 4 to 5.5. The neutral (or alkaline) invertases (NI) exhibit maximal activity between pH 7 and 8 and is present in the cytoplasm (Tymowska-Lalanne and Kreis 1998).

2.2.2.3.2 Neutral invertase

Sucrose is believed to be hydrolysed in the mature storage parenchyma tissue by neutral invertase. NI is present at low levels in very young tissue and at greater levels in older tissue (Hatch et *al*. 1963; Batta and Singh 1986). Based on this, it has been proposed that SNI regulates sucrose movement from the vascular to storage tissue in mature internodes or that it is involved in the turnover of hexoses in mature tissues. However, further investigations have reported an almost opposite distribution of activity; with SNI increasing slightly up to the fifth internode followed by a steady decline in

activity as internode maturity increases. In addition, it was demonstrated that invertase is not expressed to the same level in all tissues throughout internodal tissues (Rose and Botha 2000).

In the light of these contradictory reports, a study was carried out recently (Bosch et *al*. 2004) to isolate and characterize the SNI gene, and analyse transcription patterns in different tissues by comparison to sucrose, glucose and fructose levels. Thus, the SNI cDNA was isolated using the *L. temulentum* clone as a probe and then subsequently identified. It showed 53% homology on an amino acid level compared to published sequences of neutral invertases from *D. carota* (Sturm 1999) and *L. temulentum* (Gallagher et *al*. 1998). Additionally, SNI transcript and protein levels were higher in the younger internodes with low sucrose concentration than in the mature tissues, suggesting that SNI could supply young tissues with hexoses.

2.2.2.3.3 Soluble acid invertase

Soluble acid invertase activity is reported to be high in the apoplast and vacuoles of young, actively growing internodes, and almost absent from the mature internodes. Cell-wall bound invertase is active in both young and older internodes (Hawker and Hatch 1965; Hatch and Glasziou 1963; Gayler and Glasziou 1972). This developmental variation in SAI activity reflects both the demand for hexoses during cell elongation, and the greater requirement for metabolic substrates in developing cells. SAI is also believed to mediate remobilisation of sucrose from storage for growth, or to maintain cellular processes during periods of stress.

In addition, transgenic sugarcane cells in which soluble acid invertase activities in different sub cellular compartments were lowered and subsequently increased sucrose accumulation (Ma et *al*. 2000). There is a strong indication that genetically modified sugarcane with reduced acid invertase might increase sucrose accumulation. However, such manipulation is yet to provide the expected limited control on sucrose metabolism.

2.2.2.4 Fructokinase and Hexokinase

As discussed earlier sucrose cleavage in plant tissues can be catalysed either by invertases or by SuSy. Invertases generate two monosaccharides, fructose and glucose, which must be phosphorylated before entering the glycolytic pathway. SuSy generates UDPGlc and fructose. Two enzyme groups, the hexokinases (HK, EC 2.7.1.1) and fructokinases (FRK, EC 2.7.1.4) can facilitate the irreversible conversion of hexoses to hexose-6-phosphate using a nucleotide triphosphate as a phosphoryl donor. HK can phosphorylate both glucose and fructose, whereas FRK specifically phosphorylates fructose (Doehlert 1989; Pego and Smeekens 2000).

Numerous studies have revealed that a given plant tissue contains a spectrum of three to five different hexose-phosphorylating activities (Turner and Turner 1980; Kruger 1997) which differ in their chromatographic and kinetic properties. Tissues previously investigated include pea seeds (Turner et *al*. 1977; Turner and Copeland 1981), spinach leaves (Baldus et *al*. 1981; Schnarrenberger 1990), maize endosperm (Doehlert 1989, 1990) and soybean nodules (Copeland and Morell 1985), avocado fruit (Copeland and Tanner 1988) and barley leaves (Baysdorfer et *al*. 1989). The functional significance of the different forms is only partly understood.

In sugarcane, sucrose and hexoses are taken up by the storage parenchyma cells. During sucrose accumulation there is significant cycling of carbon between the hexoses and sucrose. As a result, the intracellular hexose pool consists of a mixture of imported hexoses and the products of sucrose breakdown. Despite this, low fructose concentrations are maintained in mature tissue (Lingle and Smith 1991; Whittaker et *al*. 1997) and this suggests high fructose phosphorylating activity.

2.2.2.4.1 Fructokinase isoforms

Both FRK and HK expression have been poorly investigated and very little is known about their contributions to sugarcane metabolism. Recently, Hoepfner and Botha (2003) presented one of the first reports in which two fructokinase isoforms, FRK1 and FRK2, were detected and characterised in sugarcane culm tissue at different stages of development. The substrate kinetics of FRK1 and FRK2 differ largely. FRK1 activity was

saturated at 0.2 mM fructose (K_m = 28 ± 6 µM) and was not inhibited by 1.0 mM fructose. In contrast, FRK2 reached maximum activity at 0.1 mM fructose (K_m = 18 ± 5 µM), and was inhibited by 83% when assayed with 1.0 mM fructose (K_i = 160 ± 7 µM). Studies of the distribution of FRK isoforms showed that in mature internodal tissues, FRK1 appeared to become the preferentially expressed isoform. But overall, total FRK activity declined during maturation. In order to gain more insight into the distinctive role of HK and FRK isoforms in sugarcane metabolism, further investigations need to be conducted, these should include product inhibition and its physiological significance and also their intracellular distribution in sugarcane.

2.2.2.5 ATP and PPi-dependent phosphofructokinase

In many plant tissues glycolysis is the dominant pathway of carbohydrate oxidation and, under most conditions, it is the major drain on the hexose pool (Kruger 1997). The first committed step in this sequence is the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate catalyzed by phosphofructokinase (PFK; EC 2.7.1.11) and pyrophosphate:fructose-6-phosphate phosphotransferase in plants (PFP; EC 2.7.1.90). There is a good indication that PFK contributes to the entry of hexose phosphates into glycolysis and the reaction catalysed by this enzyme, under physiological conditions is believed to be far from equilibrium (Turner and Turner 1980; Geigenberger et *al*. 1993). In addition, there is a strong correlation between PFK activity and glycolysis in a wide range of plants (Ap Rees 1988). Furthermore, in all the tissues in which this enzyme has been measured, the results have shown that the activity of PFK is sufficient to catalyse the estimated glycolytic flux. Finally, the studies of carbohydrate metabolism in isolated pea chloroplasts and plastids provide evidence for the direct involvement of PFK in glycolytic plastids, since these organelles lack PFP.

In contrast, PFP catalyses a reaction that is near equilibrium. As a result attributing a precise role to this enzyme is complicated. PFP has been implicated in many processes, such as glycolysis, gluconeogenesis, regulation of the pyrophosphate concentration, equilibration of the hexose phosphate/triose phosphate pools (Frommer and Sonnewald 1995). Transgenic potato plants, in which PFP activity in the tubers is considerably decreased, showed no significant variation in phenotypic characteristic from control plants (Hajirezaei et *al*. 1994). Nevertheless, changes in carbon partitioning to sucrose

at the expense of starch synthesis and a decline in the triose phosphate to hexoses ratio, led to the hypothesis that PFP catalyses a net glycolytic flux in this tissue. Similar findings from the analysis of transgenic potato plants over-expressing a mammalian phosphofructo-2-kinase gene supported this previous assumption.

Recent investigations have shown a negative correlation between total PFP activity and sucrose content across a range of sugarcane varieties with different levels of sucrose. In addition, an increased PFP activity coincided with an increased rate of respiration and a decreased ability to accumulate sucrose (Whittaker and Botha 1999; Bindon and Botha 2002). Although these findings do not provide much indication about the role of PFP activity in sugarcane sink metabolism and sucrose storing ability, the authors think that this might be related to a long-term requirement for sucrose utilisation rather than storage, particularly in the lower sucrose storing varieties (Whittaker and Botha 1999). In the light of these findings, PFP might represent a potential target for molecular manipulation in sugarcane. Overall, transgenic plants with altered PFP and fructose-2,6-P_2 levels will certainly supply valuable insights to understanding the role of PFP activity in sugarcane.

2.2.3 Manipulating sucrose accumulation

The increase of sucrose content in commercial sugarcane varieties using traditional methods such as conventional breeding is believed to have reached its limits (Grof and Campbell 2001). However, the ability of sugarcane stem to contain a significant increase in sucrose accumulation on a fresh weight basis is estimated to be more than 25%, which represents double the current commercials yields (Bull and Glasziou 1963; Moore et *al*. 1997). From this perspective, there is a large scope to use modern gene transfer techniques to improve the sucrose accumulation load of sugarcane.

Although sugarcane sucrose metabolism remains poorly understood, there has been much progress made to elucidate the physiological and biochemical barriers in efforts to increase the amount of photoassimilate directed towards stored sucrose in the sugarcane stem. Three evident targets for molecular manipulation, aimed at increasing sucrose content stored in the stem, have been identified (Grof and Campbell 2001): manipulation of the sucrose synthesis process in the leaf and stem, improvement of

sucrose transport mechanisms and altering the expression or activity of the key enzymes responsible for the hydrolysis of sucrose in the cytosol or/and in the vacuole of the stem parenchyma. However, one of the major limitations to genetic engineering of sugarcane is the lack of suitable promoters required to drive strong constitutive or tissue/organ-specific expression of transgenes.

2.2.4 Kinetic modelling and Metabolic Control Analysis (MCA)

The kinetic characteristics of enzymes make available important information about their interactions with substrates, products and effectors. For example, K_m values are seen to provide an indication of the affinity of enzymes for their substrate, and conclusions about enzymes' physiological roles are based on these values. But, the kinetic parameters of separate enzymes do not by themselves provide much insight into the behaviour of a functioning metabolic pathway. Among the different approaches for studying and modelling metabolism, MCA (Kacser and Burns 1973; Heinrich and Rapoport 1974) is the preferred theoretical framework because it quantifies the degree of control of individual reaction steps on the steady-state pathway flux or metabolite concentrations. Therefore, MCA can be useful in helping to identify potential targets steps for metabolic manipulation, because the reaction in the pathway that have the most potential of modifying a target flux or metabolite concentration can be pinpointed.

The failure to significantly improve sucrose content using traditional approaches has led to a new strategy based on pathway analysis and kinetic modelling to study sucrose accumulation in sugarcane (Rohwer and Botha 2001). This model was designed using the program "WinSCAMP" (Sauro 1993) and it comprises 11 reactions that are directly or indirectly involved in sucrose metabolism. Enzymes with sucrose as substrate or product are included, while glycolysis and the enzymes phosphoglucoisomerase (PGI), phosphoglucomutase (PGM) and UDP-glucose pyrophosphorylase (UGPase) are included as a single "drain" reaction and a so-called "forcing function" respectively. The forcing function presupposes that the reactions catalysed by PGI, PGM and UGPase are close to equilibrium *in vivo*, which is supported by metabolite measurements in most tissues (Schäfer 2004). This model was used to calculate the control coefficients of enzymes in the sucrose pathway for sucrose futile cycling (cleavage and re-synthesis of

sucrose), with the aim of identifying which reactions control this energetically wasteful process.

Previously, similar models have been used successfully to study the control of different steps on mitochondrial respiration and it was predicted that overexpression of NADH oxidase is more successful than acetolactate synthase overexpression for increasing production of diacetyl by *Lactococcus lactis* (Hoefnagel et *al*. 2002). In plants, metabolic control analysis (MCA) has been successfully used to estimate the flux control coefficient of phosphoglucoisomerase on sucrose and starch production using *Clarkia xantiana* mutants with decreased levels of this enzyme (Schäfer 2004).

2.2.5 Current limitations of the kinetic model

The first version of the kinetic model describing the metabolic processes of sucrose accumulation and futile cycling in medium-mature (internode 5) sugarcane culm tissue has been completed and validated by comparison to independent experimental data (Rohwer and Botha 2001). Recently, Schäfer et *al* (2004) corrected it by replacing generic kinetic parameters of SuSy with experimentally determined kinetic parameters of SuSyC isoform from sugarcane leaf roll. However, the construction of the kinetic model involved a number of simplifications and assumptions.

The model treats sucrose accumulation into the vacuole as a single reaction, but does not include the vacuolar metabolism explicitly. For example, acid invertase activity is known to occur in the vacuole, leading to the hypothesis that sucrose might be accumulated into the vacuole, hydrolysed by invertase, and the glucose and fructose cycled back into the cytosol. For future extensions of the kinetic model for sucrose accumulation, the vacuolar compartment should also be included. This is however a longer-term goal, because of the experimental difficulties encountered in obtaining separate data for the cytosol and the vacuole, and of the paucity of available data on the transport processes between these two compartments. Another simplification, involved the clamping of the concentrations of the metabolic cofactors ATP, ADP, UDP and P_i at experimentally determined levels. These cofactors contribute in many more enzyme reactions than those explicated in the model, and their steady-state concentrations will therefore be a function of all the reactions they participate in (Rohwer and Botha 2001).

Due to the lack of required data, a number of assumptions were made. For example, most of the kinetic parameters of enzymes (K_m and K_i values of substrates and products) were estimated.

In addition, it is well-known that trehalose is an important regulatory molecule of carbohydrate metabolism in some plants (Wingler 2002). Trehalose is synthesised via a two-step pathway from hexose phosphates. An aim for future model extension is to determine whether trehalose plays an equally important regulatory role in sugarcane. Also, I am currently incorporating the enzyme activities data measured in the different internodal tissues, in order to obtain realistic working models for each of the internodes. This should help explain why the pattern of sucrose accumulation differs between tissues of different stages of maturity.

Nevertheless, studies are underway to further extent the kinetic model by incorporating the complete glycolysis pathway down to the sucrose accumulation pathway in sugarcane culm tissue by using computer simulation softwares. This should help shed more light into the physiological role of PFP activity in relation to sucrose metabolism in sugarcane tissues.

CHAPTER THREE

DETERMINATION OF ENZYME ACTIVITY PROFILES IN RELATION TO SUCROSE CONTENT ACROSS SUGARCANE VARIETIES

3.1 Abstract

To gain a better understanding of the biochemical processes controlling sucrose accumulation; sucrose phosphate synthase (SPS), sucrose synthase (SuSy), invertases (acid and neutral), fructokinase (FRK), hexokinase (HK), pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (PFP) and ATP: D-fructose-6-phosphate 1-phosphotransferase (PFK) activities were determined in sugarcane varieties differing in sucrose content. Invertases and SuSy (sucrose cleavage direction) had peaks of activity in younger tissues then declined to lower levels. Neutral invertase had a higher specific activity than soluble acid invertase in both immature and older tissues. Furthermore, soluble acid invertase (SAI) and SuSy (cleavage direction) were inversely correlated to sucrose content in some varieties. Sucrose content was related to SPS in NCo376; Co331 and US6656-15, while the difference between SPS and SAI activities among genotypes was not. The phosphorylation activities of free hexoses were found to be higher in the younger tissues then declined in parallel with fructose and glucose concentrations. Evidently, the specific activity of internodal PFP differed substantially between the sugarcane varieties and was inversely related to sucrose content. PFK activity was similar to some extent or lower than PFP activity and no correlation was evident between PFK activity and sucrose content.

3.2 Introduction

Sugarcane ripening is associated with an increase in sucrose concentration in mature stalk tissue (Glasziou and Gayler 1972), and its accumulation is probably associated with the balance between the breakdown and synthesis reactions (Hatch et al. 1963; Batta and Singh 1986; Whittaker and Botha 1997; Sacher et *al*. 1963).

Despite significant differences in sucrose content between sugarcane varieties, the biochemical processes that account for these differences are not fully understood (Moore 1995). Numerous studies have been published and, depending on the units in which the results are expressed, e.g. tissue mass or protein basis, different patterns and considerable variation are evident across sugarcane varieties (Hatch et *al*. 1963; Gayler and Glasziou 1972; Lingle and Smith 1991).

Sucrose metabolism is governed by several enzymes (Quick and Schaffer 1996), and its hydrolysis in the storage parenchyma tissue is believed to be catalysed by one of the invertases. Neutral invertase occurs in the cytoplasm and results of recent study indicated the highest levels of sugarcane neutral invertase expression were found in the younger tissues (Bosch et *al*. 2004). SNI transcript and protein levels are higher in the young tissues, suggesting that SNI could contribute to the supply of hexoses (Bosch et *al*. 2004).

In contrast, soluble acid invertase activity is high in the apoplast and vacuoles of young actively growing internodes (Hatch and Glasziou 1963; Sacher et *al*. 1963). It was observed that SAI activity was correlated with stem elongation. In addition SAI activity is negatively correlated with sucrose concentration (Su et *al*. 1992; Vekataramana et *al*. 1991), suggesting that the enzyme controls sucrose concentration through the inversion of stored sucrose. Cell-wall bound acid invertase is active in both young (Hatch and Glasziou 1963; Sacher et *al*. 1963) and older internodes (Hawker and Hatch 1965).

Furthermore, sucrose accumulation is regulated by the sequential action of SPS and sucrose phosphatase (Hatch et *al*. 1963). SPS is involved in sucrose synthesis in the immature internodes and this is in agreement with published labelling studies (Botha and Black 2000).

SuSy is implicated in a wide variety of processes; including nitrogen fixation (Gordon et *al*. 1999), starch synthesis (Chourey et *al*. 1998) and carbon sinks activities (Huber and Akazawa 1986, Zrenner et *al*. 1995). In sugarcane, the enzyme is thought to favour the generation of UDPG from sucrose for the formation of cell wall materials and starch. However, it has also been associated with internodes elongation (Lingle and Smith 1991; Hawker and Hatch 1965; Buczynski et *al*. 1993) and could be involved in sucrose synthesis in younger internodes (Goldner et *al*. 1991; Goodwin and Mercer 1983; Schäfer et *al*. 2004).

To date, there have been few investigations into the enzymes responsible for the phosphorylation of hexoses in relation to sucrose metabolism in sugarcane. However, a recent study reported that two isoforms of fructokinase are present in sugarcane internode tissues (Hoepfner and Botha 2003) but their physiological role in sucrose metabolism is not yet clearly understood.

PFP activity has been studied (Lingle and Smith 1991) but the activity measurements obtained, were largely inconsistent. Consequently, the possible physiological contribution of this enzyme to sucrose metabolism in the developing sugarcane stalk could not be evaluated. Subsequently, after more detailed investigations, Whittaker and Botha (1999) found that PFP activity was negatively related to sucrose content in different sugarcane varieties, although its physiological significance does need further investigation. Consequently, data on the profiling of enzymes of sucrose metabolism is important for the study of its accumulation and for programmes aiming for its improvement.

The study reported here was to investigate specific enzyme activity profiles in a range of sugarcane varieties at various developmental stages, and to determine possible implications for sucrose metabolism.

3.3 Materials and Methods

3.3.1 Materials

Five sugarcane *Saccharum* spp. hybrid varieties (N30, N19, NCo376, Co331 and US6656-15) were obtained from the fields of the SASRI (South African Sugar Research Institute), Mt. Edgecombe, South Africa. Mature stalks from separate plants with approximately 12 internodes were randomly selected and harvested. Internodes were sampled to represent immature (internodes 3 and 4), medium-maturing (internodes 6 and 7), and older (internodes 8 and 9) culm tissue. The internode attached to the leaf with the uppermost visible dewlap was defined as internode no. 1 (Van Dillewijin 1952).

All coupling enzymes, co-factors and substrates used for enzyme assays and sugar determinations were purchased from Sigma-Aldrich S.A. (Pty) Ltd. (South Africa) or Roche Biochemicals (South Africa).

3.3.2 Protein extraction and enzyme assays

Crude extracts were prepared by grinding internodal tissues to a fine powder in liquid nitrogen. The ground tissue was extracted with ice-cold extraction buffer in a buffer volume to tissue mass ratio of 2:1. The standard extraction buffer contained 100 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2 mM EDTA, 10% (v/v) glycerol, 5 mM DTT, 2% (m/v) PVPP and Roche Complete^{TM} protease inhibitor. Extracts were filtered through nylon cloth and centrifuged at 10 000g for 10 minutes to remove coarse material. Extracts were then desalted using a Sephadex G-25 (Pharmacia PD-10) column equilibrated with desalting buffer (100 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2 mM EDTA and 10 % (v/v) glycerol). Aliquots of the eluant containing proteins were rapidly frozen in liquid nitrogen and stored at -80 ºC.

Invertase assays: Invertases were assayed at pH optima as determined by Hatch et *al* (1963) and the temperature optimum was determined in preliminary studies. Neutral invertase specific activity was determined at 30 ºC in a final volume of 250 µl. The final assay contained 50 mM HEPES (pH 7.0) and 125 mM sucrose. The same assay system was used for acid invertase except for a 50 mM citrate/phosphate buffer at pH 5.5. Results of preliminary experiments indicated that the reaction velocity under these

conditions was linear for at least 6h. The assay was stopped after a 2h incubation period by a stop solution of 2 M Tris and 22 mM $ZnSO_4$. The liberated glucose was determined enzymatically by measuring NAD reduction after incubation with hexokinase/glucose 6-P dehydrogenase (Huber and Akazawa 1986).

SPS and SuSy assays: SPS activity was determined by a method modified from Huber (1983). A 20 µl aliquot of the desalted extract was added to 50 µl of the reaction mixture. The assay mixture to determine SPS activity contained a final concentration of 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.2 mM NaF, 2 mM fructose 6-P, 10 mM Glc-6-P, 25 mM UDPG, and 20 µl desalted extract. For SuSy the assay mixture contained a final concentration of 100 mM Tris (pH 7.5), 15 mM $MgCl_2$, 20 mM UDPG and 10 mM fructose (Buczynski et *al*. 1993).

Reaction mixtures were incubated at 28 °C for 60 min and terminated by the addition of 70 µL of 30% (m/v) KOH. Tubes were immersed in boiling water for 10 min to remove free hexoses (Huber 1983). After cooling, a mixture of 0.14% anthrone in 80% H_2SO_4 was added and incubated 20 min at 40 °C. The concentration of the reaction product was determined spectrophotometrically at 620 nm.

Sucrose cleavage by SuSy was assayed as described by Huber and Akazawa (1986). The reaction mixture consisted of 100 mM Tris (pH 7.0) containing 2 mM $MgCl_2$, 2 mM NAD, 1 mM ATP, 320 mM sucrose, and 10 µl of a mixture of coupling enzymes consisting of 4 units of hexokinase, 4 units of phosphoglucoisomerase, and 4 units of glucose-6-P dehydrogenase in a total volume of 250 µl. The SuSy reaction was initiated by the addition of UDP to a final concentration of 1.5 mM.

FRK and HK assays: activities were measured spectrophotometrically in a 250 µl volume containing 50 mM HEPES-KOH (pH 8.0), 4 mM $MgCl_2$, 2.5 mM ATP, 0.33 mM NAD, 0.25 U glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 0.25 U of phosphoglucose isomerase (EC 5.3.1.9), 0.1 or 1.0 mM fructose. The reaction mixture used for the measurement of HK was the same as for FRK, except that fructose was replaced by 2 mM glucose and phosphoglucose isomerase was omitted. Activities were derived from the increase in absorbance at 340 nm.

PFP and PFK assays: PFP and PFK measurements were carried out at 30 ºC in a total volume of 250 µl. Activity was measured by following the oxidation of NADH at 340 nm. PFP activity was measured in the glycolytic (forward) direction according to Kruger et *al*. (1983). The reaction mixture used for the measurement of PFK was the same as for PFP, except that the PPi was replaced by 10 mM ATP, D-fructose-2,6-bisphosphate was omitted and 100 mM HEPES (pH 7.2) was used (Botha et *al*. 1988).

3.3.3 Sucrose extraction and measurement

Sugars were extracted from aliquots of frozen tissues in a ratio of 1 g to 10 ml 70% (v/v) ethanol diluted with HM-buffer (100 mM HEPES (pH 7.8), 20 mM $MgCl_2$) by incubating at 70 ºC. The samples were then centrifuged at 10 000g, for 10 min at room temperature. The supernatant was immediately used for sugar determination. Sucrose, glucose and fructose concentrations were determined enzymatically (Bergmeyer and Bernt 1974).

3.3.4 Protein determination

Protein was measured in desalted extracts using mouse immunoglobulin G (IgG) as a protein standard (Bradford 1976).

3.3.5 Data analyses

The mean and standard deviations were calculated for three separate replicates for the enzyme activities and six replicates for sugar concentration were calculated. The correlation between enzyme activity and sugar content was determined by linear regression using the Excel™ software package (Microsoft) and by Pearson correlation using SPSS 11.0 for Windows™.

3.4 Results

3.4.1 Sucrose content

In all the sugarcane varieties studied, sucrose concentration was low in the immature internode tissues and increased quite rapidly in the medium-maturing internode tissues

(table 1). Both glucose and fructose concentrations declined as the internodal tissue matured; glucose concentration was generally higher than fructose.

Table 1 Sugar content across varieties in tissues representing young (I_{3-4}) maturing (I_{6-7}) and older internodes (I_{8-9}). (Each value is the mean of ± SD from three separate extractions)

		Concentration (μmol g^{-1} FW)		
Variety	**internodes**	**Sucrose**	**Glucose**	**Fructose**
US6656-15	**I3-4**	75.46 ± 4.62	135.61 ± 15.26	110.3 ± 22.93
	I6-7	206.1 ± 16.95	113.31 ± 10.56	87.13 ± 7.73
	I8-9	280.2 ± 21.45	108.57 ± 9.65	81.73 ± 9.88
Co 331	**I3-4**	44.47 ± 28.75	234.36 ± 8.56	181.35 ± 3.81
	I6-7	190.16 ± 35.20	241.54 ± 20.18	172.32 ± 16.13
	I8-9	283.3 ± 27.61	235.71 ± 32.38	166.6 ± 4.93
NCo 376	**I3-4**	45.59 ± 18.65	378.45 ± 13.95	262.85 ± 8.28
	I6-7	407.98 ± 36.29	329.57 ± 9.19	188.36 ± 41.19
	I8-9	512.43 ± 92.84	190.21 ± 43.00	124.42 ± 21.56
N19	**I3-4**	71.11 ± 8.32	161.79 ± 16.52	123.13 ± 12.41
	I6-7	407.96 ± 67.17	87.32 ± 15.02	81.06 ± 5.88
	I8-9	625.77 ± 61.81	69.23 ± 17.89	67.92 ± 17.88
N30	**I3-4**	222.97 ± 13.78	341.45 ± 42.02	222.56 ± 37.34
	I6-7	501.83 ± 38.37	179.85 ± 36.04	86.36 ± 25.33
	I8-9	620.67 ± 52.07	121.70 ± 30.79	70.53 ± 23.28

3.4.2 Enzyme activity

SuSy activity in the synthesis direction increased from the younger tissues to the maturing tissues, and thereafter decreased in the older internodes in N30 and N19 while the reverse reaction activity of SuSy (cleavage direction) decreased with tissue maturity and therefore sucrose concentration in all of the genotypes. SPS activity increased steadily and was related to the internode's age in NCo376 and the low-sucrose content varieties Co331 and US6656-15 respectively. There was no significant correlation between SPS activity and internode maturation level in N19 and N30.

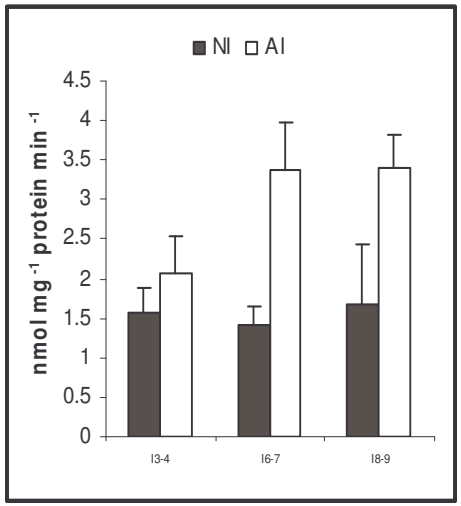
When the enzyme activities (difference in activities of SPS and SAI) and sucrose level, between all the range of internodes and the genotypes were compared simultaneously, no significant overall relationship was found (data not shown) (see Discussion, section 3.4).

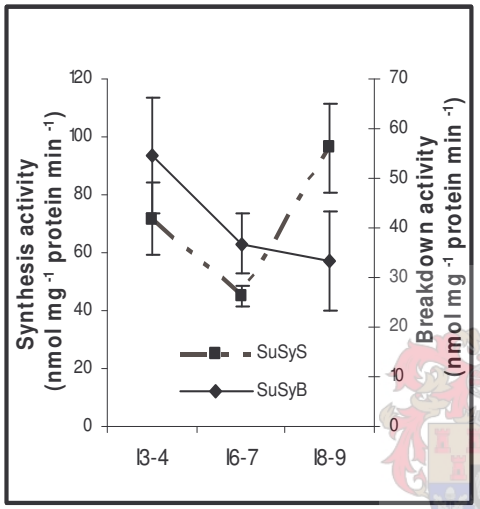
In addition, the activity of soluble acid invertase (SAI) varied with internode's age, with highest activities in the young internodes of N30. The higher SAI activity of young internodes of some genotypes decreased slightly (Figure 1), reaching low levels as the internode matured and accumulated sucrose. Neutral invertase activity data did not show distinctive changes in most of the varieties. The specific activities (given in nmol mg^{-1} protein min^{-1}) of both NI and SAI showed a low level of activity. Total fructose phosphorylation activity (Figure 1) decreased during maturation it was higher when assayed with 0.1 mM than with 1 mM. In most of the varieties except for NCo376, HK activity was found lower than FRK activity.

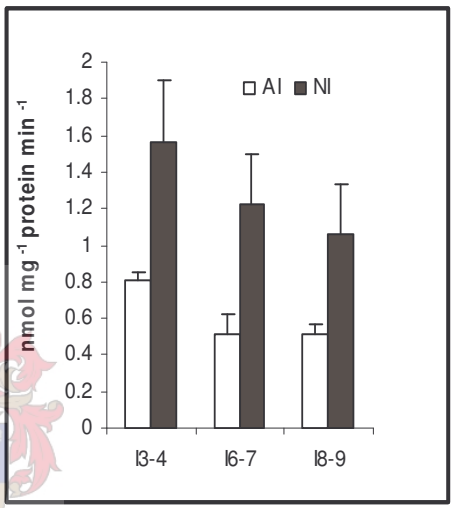
No indication of positive correlation has emerged from the studies of SPS activity in the commercial sugarcane varieties N19 and N30 respectively. SAI activity was correlated to sucrose concentration in N30 (r^2=0.81 and $p<0.001$), while NI correlated to sucrose concentration only in N30 (r^2=0.67 and $p<0.007$).

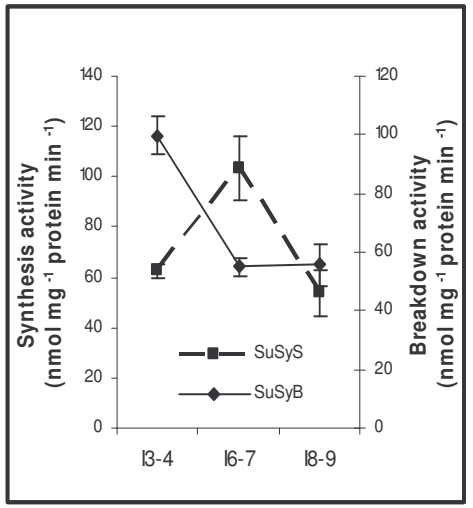
A. US6656-15

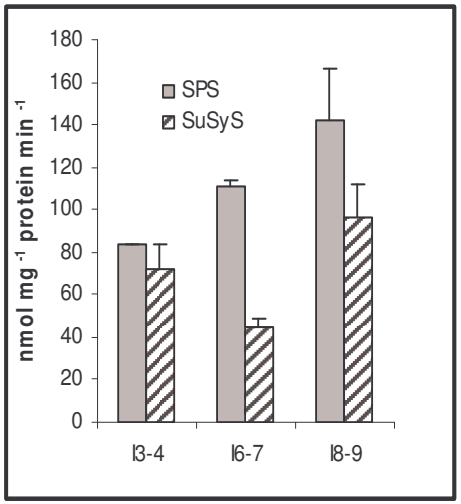
B. Co331

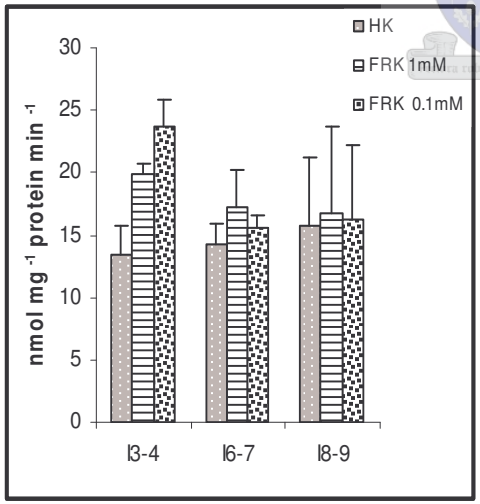


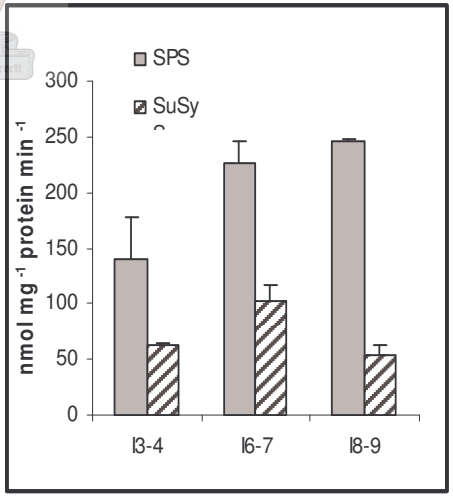


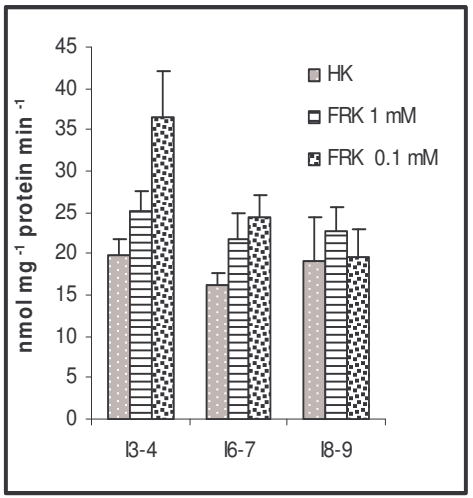




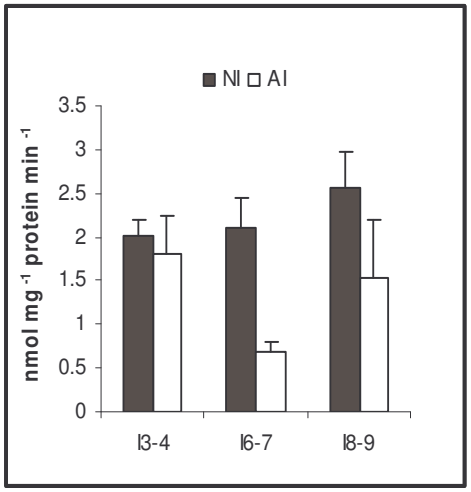


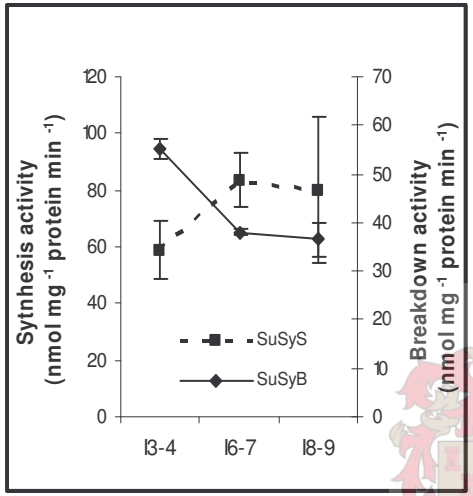


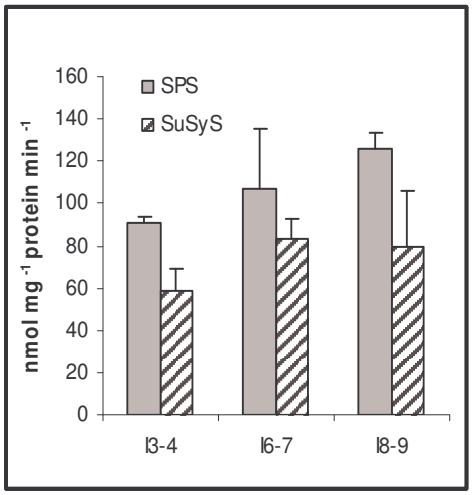


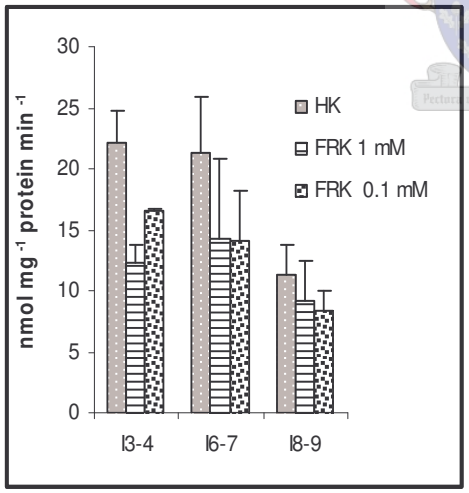


C. NCo376

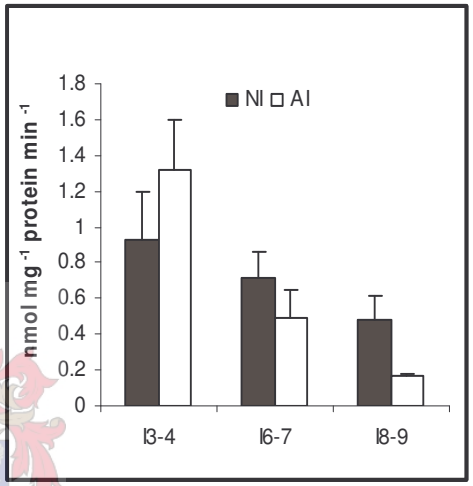


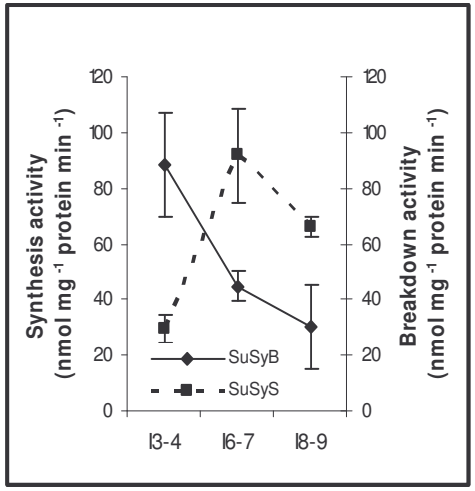


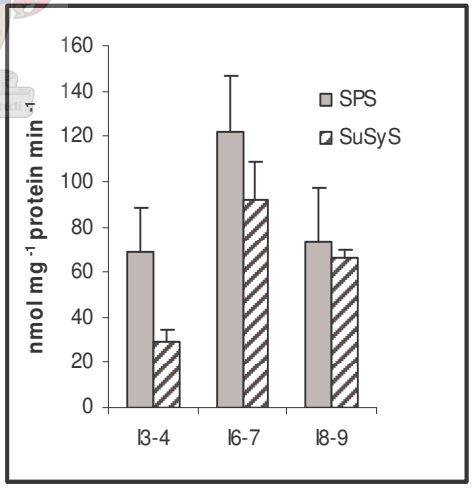


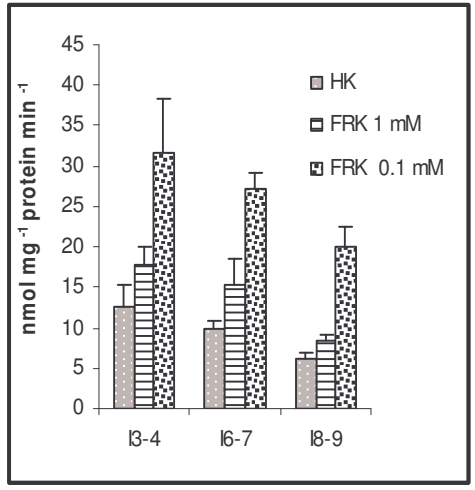


D. N30

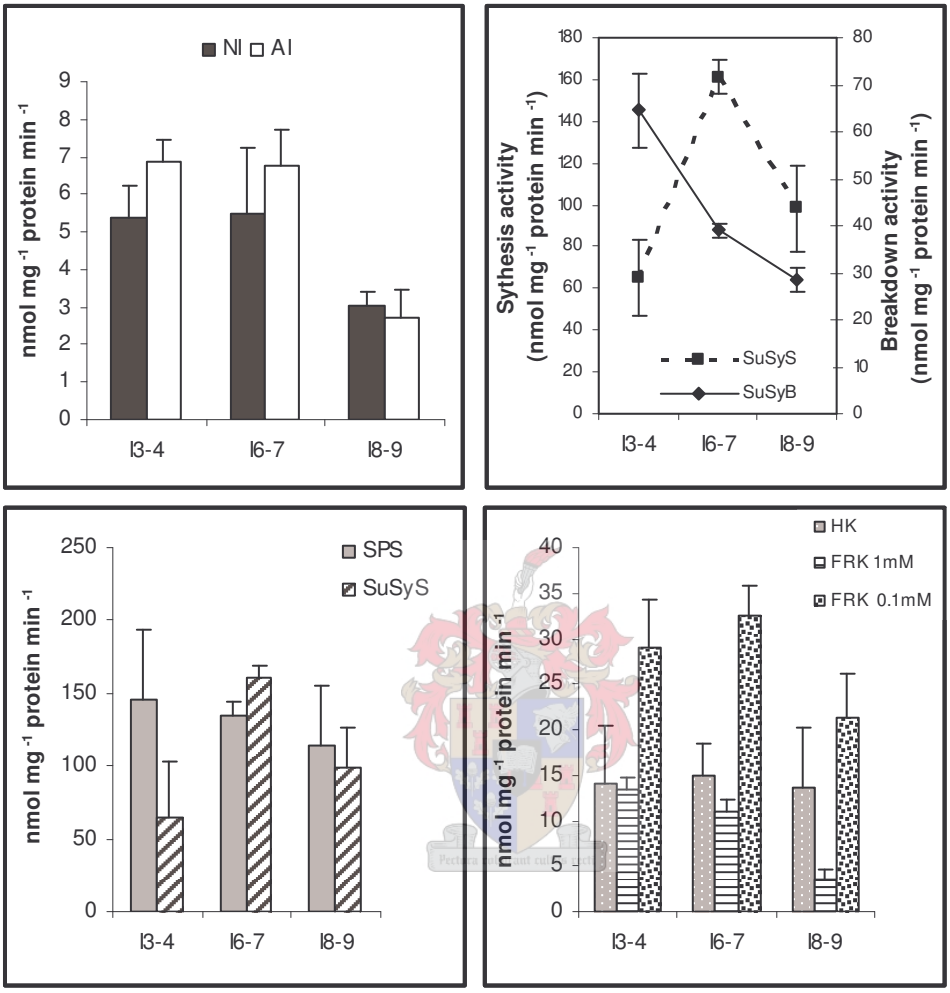








E. N19



SuSyS stands for sucrose synthase (synthesis direction) and SuSyB stands for sucrose synthase (breakdown direction).

Figure 1 Enzyme activities across varieties in tissues representing young (I_{3-4}), maturing (I_{6-7}) and mature (I_{8-9}) internodes. Each value is the mean of ± SD from three distinct measurements.

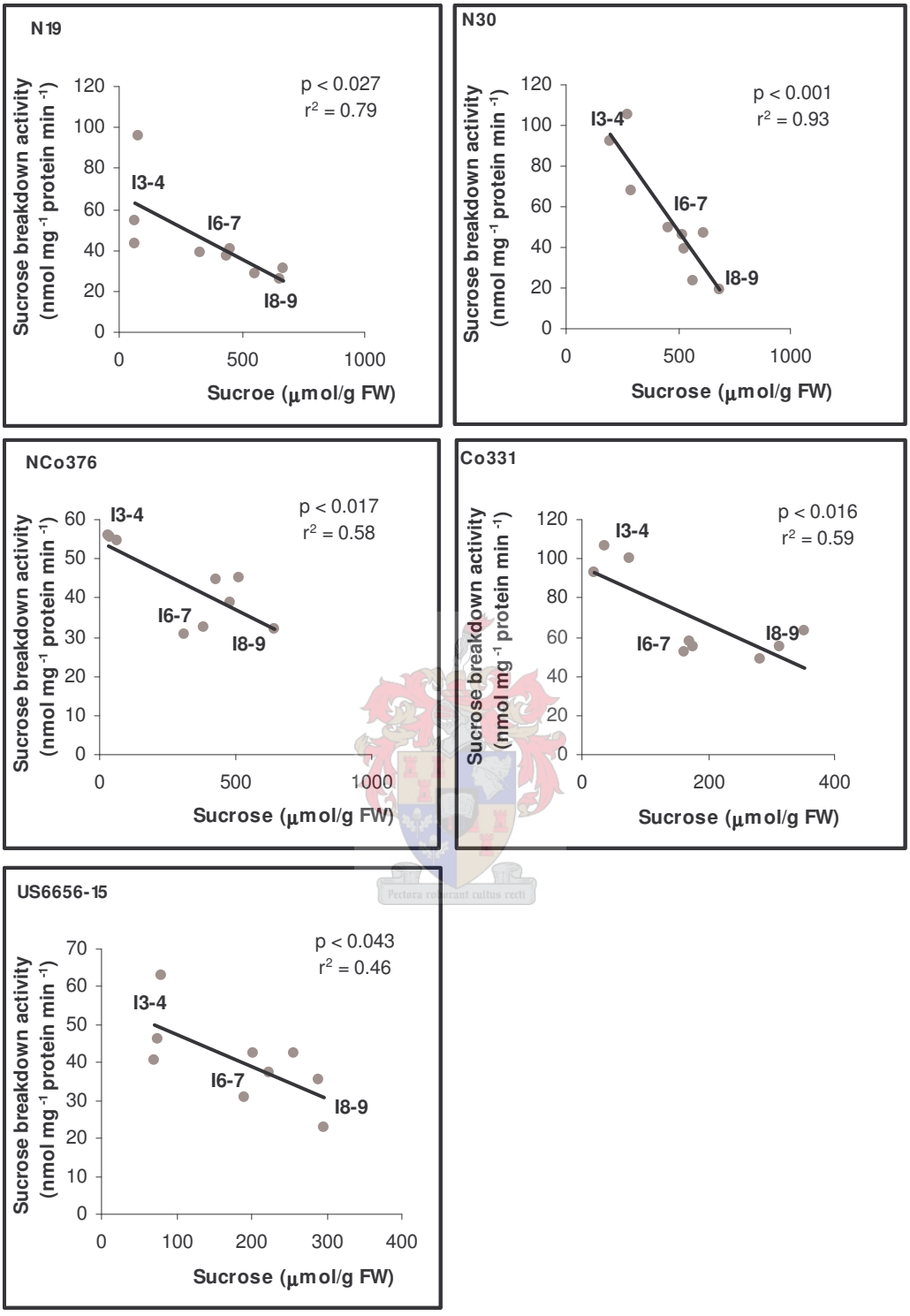


Figure 2 Sucrose concentration and SuSy breakdown activity in tissue representing young (I_{3-4}), maturing (I_{6-7}) and older (I_{8-9}) internodes of sugarcane.

The total catalytic activity of SuSy (breakdown direction) was generally related to sucrose content in most of the cultivars (Figure 2).

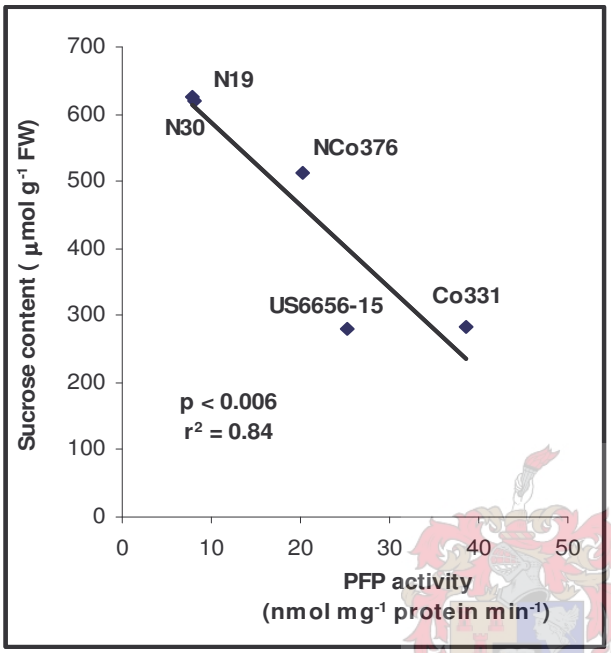


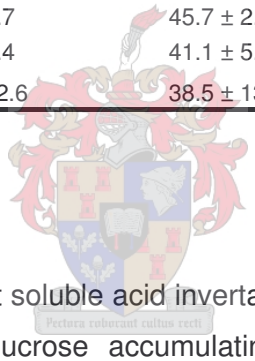
Figure 3 Correlation between mean PFP activity in internodes (I_{8-9}) and sucrose content among sugarcane varieties. Each value is the mean ± SD of three distinct measurements.

Between the five different varieties, specific PFP activity of internodes (I_{8-9}) was negatively associated with sucrose content (Figure 3). In addition, PFP activity in the immature internodes declined with the ability of the sugarcane varieties to accumulate sucrose (Table 2). The most apparent change was seen in the young internodal tissue (I_{3-4}). This is illustrated by the level of PFP activity particularly in US6656-15; Co331 and NCo376 respectively. In contrast, there was no significant difference in the activity level of PFP between N30 and N19.

PFK activities in NCo376, Co331 and US6656-15 were generally higher and different from those of N30 and N19 in the younger and older tissues (Table 2). In addition, the highest PFP/PFK ratio was found in the low sucrose-accumulating varieties Co331 and US6656-15 and inversely the lowest was observed in the high storing variety N19.

Table 2 PFP and PFK activities and PFP/PFK ratios in maturing (I_{6-7}) and older internodal tissues (I_{8-9}). (Each value is the mean of ± SD from 3 separate plants).

	PFP and PFK activities (nmol mg^{-1} protein min^{-1})					
	PFP		PFK		PFP/PFK	
Variety	I3-4	I6-7	I3-4	I6-7	I3-4	I6-7
N19	11.7 ± 3.8	8.6 ± 0.9	15.9 ± 0.5	19.7 ± 3.2	0.74	0.43
N30	10.1 ± 1.4	12.8 ± 1.3	16.1 ± 1.1	12.8 ± 1.4	0.67	0.97
NCo376	15.8 ± 1.7	23.6 ± 5.7	45.7 ± 2.4	36.1 ± 3.2	0.35	0.71
Co331	48.1 ± 0.8	33.3 ± 2.4	41.1 ± 5.1	35.2 ± 9.9	1.20	0.83
US665615	61.6 ± 10.4	31.9 ± 12.6	38.5 ± 13	24.9 ± 5.9	1.84	1.57



3.5 Discussion

Previously it was reported that soluble acid invertase was present in younger but not mature tissue of high sucrose accumulating varieties and that neutral invertase showed no particular distribution pattern related to species (Hatch and Glasziou 1963). Our data undoubtedly show that invertases are present in low but significant amounts down the sugarcane stem tissues; from low to high sucrose containing varieties and tissues. Changes in NI activity in relation to sucrose concentration between internodes were generally not significant in most of the genotypes. In contrast, neutral invertase activity correlated to sucrose concentration in N30 (r^2=0.67 and $p<0.007$). This is agreement with results of a recent study carried out by Bosch et *al* (2004).

However, the conditions at which enzyme assays are performed are often vastly different from those inside the cell. In vitro measurements are made at high

substrate to enzyme concentration ratio. Enzymes are assayed in isolation from other enzymes, and molecules that may interfere with enzyme activities in the cell are absent from the assay. Therefore, the reduced total catalytic activity of both NI and SAI measured from the crude extract preparation may be due to the limitations attached to the *in vitro* enzyme assay methods.

Zhu et *al* (1997) showed a significant negative hyperbolic correlation between SAI activity and sucrose concentration among genotypes. In contrast, in our study a significant linear negative correlation was found between acid invertase activities and sucrose concentration in commercial sugarcane variety N30 (r^2=0.81 and p<0.001).

The genotypes examined by Zhu et *al* (1997) were parents and progeny of a high sucrose × low sucrose inter-specific cross. It was observed that sucrose content was correlated to the difference between SPS and acid invertase. Although a negative correlation was found between SAI and sucrose concentration in commercial variety N30, there was no existing relationship between sucrose concentration and the difference between SPS and SAI activities in this study. The high level of SPS activity could have been used as an "inhibitory mechanism" to prevent acid invertase activity to increase, influencing sucrose accumulation. Further, the lack of such correlation in this study might possibly reflect the polyploidy nature of the genotypes selected.

SPS is regulated by a variety of mechanisms that operate at different levels and in different time frames (Stitt et *al*. 1987; Huber and Huber 1996). There are three distinct mechanisms that control the enzymatic activity of SPS protein: (i) regulation of gene expression (Huber and Huber 1996), (ii) allosteric control by Glc-6-P (activator) and inorganic phosphate (inhibitor) (Doehlert and Huber 1985), and (iii) covalent modification via reversible phosphorylation (Huber and Huber, 1996). SPS is regulated in synthesizing organs or tissues, including

those adapting to cold and drought, fruits, etiolated cotyledons, germinating seeds, sugarcane (*Saccharum officinarum*) stems and beta-roots (*Beta vulgaris*) (Huber and Huber 1996). Most warm-climate plant species are sensitive to brief exposures to low, non-freezing temperatures. Increased SPS activity can be attributed to protein phosphorylation state of SPS that possibly led to its activation due to cold effects. Although, the details of the regulation circuits have not been elucidated (Huber and Huber 1996); one of these factors or their additional effects may possibly be responsible for the increased SPS activity obtained.

In most of the cultivars there was an inverse correlation between sucrose content and SuSy activity. The highest SuSy was mostly associated with low sucrose levels. This could imply a role for SuSy in sucrose degradation. The cleavage activity of SuSy exceeded the hydrolytic activities of acid and neutral invertase. In addition, the glucose to fructose ratio was higher than one; this may suggest that sucrose could be degraded in the cytosol by SuSy prior to its importation in the vacuole. However, it is important to notice that the presence of hexoses is the product of sucrose degradation by invertases and SuSy activities, hexose movement and the hexose phosphorylation by HK and FRK activities.

However, it is reported that SuSy activity is directly associated with the phloem tissue (Hawker and Hatch 1965; Schäfer et *al*. 2004) and recognised that it might be implicated in sucrose breakdown for the generation of UDPG and a possible preservation of energy via this nucleoside activation of hexose moiety (Huber and Akazawa 1986).

A more detailed study of SuSy activity within different regions of internodes, with the exception of internode three, did not indicate any significant variations. Also, sucrose breakdown/synthesis ratios increased as the internodes aged. This is believed to account for the presence of different SuSy isoforms in different

internodes (Schäfer et *al*. 2004). Although much progress has been made towards elucidating the role this enzyme in sucrose accumulation, the question about isoform localisation and distribution among internode tissues is not fully understood.

SuSy in higher plants is also subject to a variety of possible regulatory mechanisms on the gene level (e.g. isoform differences) as well as the protein level. The following examples illustrate this. SuSy is generally inhibited in the sucrose cleavage direction by both Ca^{2+} and Mg^{2+} ions; for rice this inhibition is however mild (less that 20% inhibition at 10 mM (Huang and Wang 1998)). In pear fruit SuSys are more sensitive, with an average of about 40% inhibition at only 5 mM (Tanase and Yamaki 2000). However, in the sucrose synthesis direction Mg^{2+} ions have a stimulatory effect, with activation ranging from 40 to 60% in the rice and pear SuSys respectively, also at 5 mM concentration. In sugarcane, few studies have focussed on the physical/biochemical properties and fine regulation received little attention, therefore a high level of SuSy activity may well be the result of an activation by endogenous or/and exogenous factors.

Results obtained in this study showed that SuSy activity (synthesis direction) increased between young and maturing internode tissues; the higher specific activity of SuSy in the young internodes may support the fact that it is a good indicator of a sink for sucrose import for respiration and other biosynthetic activities as suggested by Botha and Black (2000). This does not exclude the fact that the enzyme may also be implicated in sucrose synthesis as it has been demonstrated by radiolabelling studies of sugarcane variety N19 (Botha and Black 2000).

An increased in SPS activity was associated with sucrose concentration in NCo376; Co331 and US6656-15 respectively. In accordance with this, pulse labelling experiments with sugarcane suspension cultures and tissue disks

indicated that SPS is the most important enzyme responsible for the synthesis of sucrose (Hatch et *al*. 1963; Batta and Singh 1986; Wendler et *al*. 1990; Terauchi et *al*. 2000).

In general, although on a whole stalk basis SuSy activity (synthesis reaction) was not related to sucrose concentration in all the sugarcane genotypes. We can assume that in most of genotypes both SPS and SuSy may have contributed to sucrose storage in the early stages as suggested by earlier experiments by Botha and Black (2000).

The phosphorylation of glucose and fructose is an important step in regulating the supply of hexose sugars for biosynthesis and metabolism (Whittaker and Botha 1997). Relatively few investigations have been carried out into the physiological functions of FRK and HK in sucrose accumulation in sugarcane. Reduced FRK and HK with maturation indicate a decrease in the amount of hexoses. However, these results do not clearly reveal the role of these enzymes.

Across sugarcane varieties, PFP activity in immature internodes varies slightly between high sucrose yielding varieties and significantly in the low sucrose accumulating varieties. The changes of *in vitro* PFP activity in internodal tissues (I8-9) are inversely related to sucrose content. These findings are in agreement with these of Whittaker and Botha (1999). Previously, it was found that respiration was positively correlated to PFP, suggesting that the enzyme is involved in the regulation of respiratory flux (Whittaker and Botha 1999). Hence, one can postulate that PFP is involved in the regulation of the balance of carbon supply to sucrose and the demand for carbon in respiration and biosynthesis in sugarcane as indicated by Bindon and Botha (2002). Furthermore, transgenic sugarcane plants with reduced PFP expression led to an increase in sucrose accumulation and a significant decrease in the triose-phosphate intermediates and an increase in the hexose phosphate pool (Van der Merwe 2005). These

findings indicate that PFP catalyses a net glycolytic reaction, especially in the medium-mature internodal tissue.

3.6 Concluding remarks

The main features of the regulation of sucrose accumulation in this study were found to be centred on three points of control in the sucrose metabolism pathway. (i) The maturation of sugarcane internodes coincides with an increase in SPS in most genotypes. This trend has been observed by others (Hatch et *al*. 1963; Batta and Singh 1986; Wendler et *al*. 1990; Botha and Black 2000) and this emphasizes the key role of this enzyme in sucrose accumulation. (ii) SuSy activity (cleavage direction) correlated negatively with sucrose concentration and hence with tissue maturation, in most of the varieties. This finding appears to indicate that SuSy could also be implicated in sucrose metabolism. (iii) It is well established that *in vitro* PFP activity is inversely correlated to sucrose content in sugarcane varieties differing in the amount of sucrose. Results of Van der Merwe (2005) have shown that reduced PFP expression led to an increase in sucrose accumulation.

CHAPTER FOUR

EVALUATION OF THE KINETIC MODEL FOR SUCROSE ACCUMULATION OF SUGARCANE CULM TISSUE

4.1 Abstract

With the view to evaluate the corrected kinetic model for sucrose accumulation in sugarcane culm (Schäfer et *al*. 2004), the steady state concentrations of metabolites (sucrose, glucose and fructose) were calculated by incorporating the Vmax values of the enzymes of different internodes and genotypes. Cytosolic sucrose concentration declined with tissue maturation in varieties Co331; NCo376 and US6656-15 and simultaneously glucose and fructose concentrations increased. In parallel to that, SPS and SuSy fluxes declined with the internode's age. Therefore, the kinetic model failed to reflect the increasing maturity on the stem when changes on enzyme activities were incorporated. Convincingly, the steady state concentration of sucrose calculated both by the original and corrected model in the younger internodes was in agreement to the experimental value of sucrose in variety N19. Although, the model has been constructed from the available data; some improvements are required to allow it to reflect the maturation stage between different internodes.

4.2 Introduction

The alteration of carbon partitioning by conventional breeding techniques has made a major contribution to increasing sucrose content of sugarcane over the past decades, mainly by overcoming productivity barriers in both the source and the sink (Moore et *al*. 1997). The noticeable failure to make significant progress in improving the sucrose load of commercial varieties during these recent years has led to the speculation that a yield plateau has been reached (Botha and Rohwer 2001; Grof et *al*. 2001). Therefore, there is a significant potential for increasing sucrose accumulation if the physio-biochemical limits can be identified and modified (Moore et *al*. 1997).

Molecular manipulation programs require a better understanding of the metabolic regulation processes. For instance, enzymes defined as 'rate limiting' can be significantly reduced, e.g. cytosolic pyruvate kinase (Gottlob-McHugh et *al*. 1992) or over expressed several-fold, e.g. phosphofructokinase (Burrell et *al*. 1994) with no substantial change in metabolic fluxes. In contrast, enhancing the activity of one enzyme can lead to a considerable increase in pathway flux; e.g. a 10-fold amplification of threonine aldolase activity in crude extracts from the filamentous fungus *Ashbya gossypii* led to a 9-fold increase of riboflavin production (Monschau et *al*. 1998).

Therefore, several theoretical frameworks for studying metabolism, such as biological system theory (Savageau 1991), metabolic control analysis (MCA) (Kacser and Burns 1973) and metabolic design (Kholodenko et *al*. 1998) have been developed to analyse multi-enzyme systems predictively and quantitatively. Of these approaches, MCA is a theoretical framework by which the effect of changes in enzyme activity on the fluxes, concentrations, and other relevant variables characterising biochemical systems at steady state can be determined (Chassagnole et *al*. 2001; Niederberger et *al*. 1992).

In the case of sugarcane, a detailed kinetic model was first developed to study factors determining sucrose accumulation and futile cycling (Rohwer and Botha 2001). This model was subsequently corrected by substituting the estimated kinetic parameters of SuSy in the original model with the experimentally determined parameters of the SuSyC isoform (Schäfer et *al*. 2004).

The objective of this section was the incorporation of enzyme activity data for different internodes (immature, medium-mature and older) to obtain a detailed model of every stage in the tissue maturation process, then to evaluate the kinetic model in order to identify the most effective control points of processes that contribute to cytosolic sucrose metabolism across maturing internodal tissues and various varieties with varying capacities to store sucrose.

4.3 Methods

4.3.1 Enzyme activities and modelling

Maximal enzyme activities were taken as determined across sugarcane varieties and at various development stages (Chapter 3). The conversion of the enzyme activities from values in (nmol/min/g FW) to values in (mM/min) is based upon the percentage of water content per gram of fresh weight in the internodes. Tissue water content declined from 84% at the 2nd internode to a minimum of 70% by the 20th internode (Welbaum and Meinzer, 1990). For example 80% of water content in internode 5 was assumed to correspond to an intracellular volume of 800µl, with 10% cytoplasmic and 90% vacuolar volume (Komor 1994).

The program WINSCAMP v1.2 (Sauro 1993) was utilised for kinetic modelling, using the corrected (Schäfer et *al*. 2004) model for sucrose accumulation in sugarcane culm tissue. The enzyme activity datasets for each variety were incorporated into this model in order to analyse its cytosolic sucrose accumulation patterns and then pinpoint potential control points in sugarcane

culm tissue. This model comprised of 11 reactions that are either directly or indirectly involved in sucrose metabolism. Enzymes involved in the sucrose metabolism pathway were included whilst others, specifically glycolysis and the enzymes phosphopgucoisomerase, phosphoglucomutase and UDP-glucose pyrophosphorylase (UGPase) were included as a single reaction and so called 'forcing function'. This model can be viewed at http:// jjj.biochem.sun.ac.za

4.4 Results

The model for sucrose accumulation (Schäfer et *al*. 2004) was used in this study to obtain the metabolites concentration (sucrose, glucose, and fructose). The Vmax values of enzymes (Table 3) were incorporated into the model and the metabolite concentrations were calculated.

Table 3 Measured Vmax values of the enzyme-catalysed reactions (values are given in mM/min).

Values are the converted Vmax of enzymes in the internodes, based on the percentage of water content per gram of fresh weight assuming a 10% cytosolic volume.

Variety	Internodes	NI	SuSy-B	SuSy-S	SPS	HK	FRK
US6656-15	I3-4	0.035	1.213	1.595	1.870	0.298	0.527
	I6-7	0.018	0.463	0.562	1.390	0.179	0.216
	I8-9	0.022	0.442	1.270	1.871	0.208	0.213
Co331	I3-4	0.007	0.716	0.448	1.007	0.143	0.262
	I6-7	0.005	0.338	0.635	1.394	0.100	0.150
	I8-9	0.004	0.350	0.338	1.541	0.119	0.123
NCo 376	I3-4	0.017	0.475	0.505	0.784	0.190	0.143
	I6-7	0.019	0.346	0.759	0.974	0.194	0.128
	I8-9	0.022	0.316	0.693	1.089	0.098	0.072
N30	I3-4	0.008	0.728	0.243	0.565	0.104	0.260
	I6-7	0.007	0.431	0.882	1.175	0.095	0.261
	I8-9	0.006	0.390	0.857	0.944	0.080	0.260
N19	I3-4	0.038	0.463	0.465	1.045	0.101	0.208
	I6-7	0.033	0.235	0.971	0.808	0.090	0.196
	I8-9	0.024	0.224	0.774	0.895	0.108	0.168

Table 4 Predicted flux values of the enzyme-catalysed reactions (values are given in mM/min).

Variety	Internodes	NI	SuSy	SPS	HK	FRK
US6656-15	I3-4	0.0159	-0.0277	0.2490	0.2217	0.3165
	I6-7	0.0038	0.1000	0.0631	0.1415	0.1253
	I8-9	0.0061	0.1026	0.0806	0.1655	0.1381
Co331	I3-4	0.0012	0.0614	0.0921	0.1108	0.1902
	I6-7	0.0003	0.0954	0.0074	0.0796	0.0446
	I8-9	0.0001	0.0505	0.0191	0.0946	0.0146
NCo 376	I3-4	0.0034	0.1261	0.0271	0.1500	0.0659
	I6-7	0.0038	0.1313	0.0243	0.1532	0.0570
	I8-9	0.0001	0.0734	0.0014	0.0715	0.0111
N30	I3-4	0.0014	0.0645	0.0634	0.0794	0.1712
	I6-7	0.0010	0.0605	0.0808	0.0756	0.1957
	I8-9	0.0007	0.0531	0.0816	0.0637	0.2068
N19	I3-4	0.0034	0.0988	0.0310	0.0797	0.1148
	I6-7	0.0024	0.1054	0.0154	0.0718	0.0918
	I8-9	0.0019	0.1049	0.0128	0.0876	0.0664

The predicted fluxes of SuSy in the direction of sucrose synthesis reaction are positive values and in the direction of sucrose cleavage reaction are negative values.

The predicted flux values (Table 4) were much lower than the Vmax values (Table 3). The most significant finding was that; in both sets of data (predicted fluxes and measured Vmax values) NI, HK and FRK activities decreased with internode tissues maturation. These results are consistent with NI, HK, and FRK activity profiles across sugarcane genotypes (See Chapter 3; Figure 1). HK and FRK had the highest flux values, which declined steadily with tissue maturity. Surprisingly, in the young internodes the predicted SPS flux values were reasonably high, and decreased substantially with tissue maturity in most of the genotypes. In addition, SuSy flux values from the model calculation indicated that the enzyme was probably involved in sucrose synthesis between the young and medium-mature internodes, as suggested in the literature (Moore 1995; Botha

and Black 2000). The model predicted that for the genotypes US6656-15; Co331 and NCo376, sucrose concentration was at its peak in the younger internodes (Table 5). In these respective varieties, sucrose concentration was reduced during the maturation of internode tissues. In contrast, the commercial varieties N30 and N19 did not show any significant variation in the amount of sucrose calculated at steady-state. Simultaneously, there was a gradual increase in glucose and fructose content and glucose concentration was generally much higher than fructose specifically in US6656-15, NCo376 and N30.

Table 5 Calculated metabolite concentrations and futile cycling (all values refer to *cytosolic* metabolite concentrations).

		Concentration (mM)			Percentage (%)
Variety	**Internodes**	**Sucrose**	**Glucose**	**Fructose**	**Futile cycling**
US6656-15	I3-4	25.85	8.74	0.20	7.18
	I6-7	18.95	25.90	6.24	2.32
	I8-9	21.53	18.85	4.45	3.32
Co331	I3-4	17.97	39.23	2.54	0.76
	I6-7	11.41	64.10	25.02	0.28
	I8-9	7.47	49.63	81.08	0.14
NCo 376	I3-4	17.62	22.77	11.80	2.19
	I6-7	17.88	21.86	12.62	2.45
	I8-9	8.08	74.05	57.88	0.07
N30	I3-4	14.55	64.98	4.42	0.62
	I6-7	16.35	69.48	1.98	0.52
	I8-9	15.49	87.16	1.57	0.38
N19	I3-4	14.48	67.67	7.98	2.58
	I6-7	13.44	77.02	10.67	1.96
	I8-9	13.09	57.46	16.13	1.63

Table 6 compares the steady-state concentrations of the variable metabolites calculated by the original model with those determined experimentally in the younger tissue of sugarcane variety N19. In general, the values of sucrose

concentration agreed well. In contrast, glucose and fructose were twice as low. The data obtained from the corrected version of the model indicated that the sucrose concentration was 58% higher, while the glucose concentration was twofold higher than the experiment value, and fructose was reduced by more than 90%.

Table 6 Kinetic model validation: comparison of the calculated and the experimentally determined metabolite concentrations
(all the values refer to *cytosolic* concentrations of variety N19 in the younger internodal tissues).

Metabolites	Concentration (mM)			
	$original_1$	$original_2$	corrected	Experiment
Fructose	40.58	49.72	7.98	17.59
Glucose	30.11	65.35	67.67	23.11
Sucrose	10.41	7.94	14.48	10.16

Model variants are as follows: $original_1$ (predicted from the original model when query with Vmax value of enzymes taken from literature); $original_2$ (original model predictions when query with Vmax value of enzymes measured. See Table 4); corrected (model predictions with K_{eq} and K_i values of SuSy corrected and Vmax value of enzymes from Table 4) and Experiment (see Table 5).

4.5 Discussion

The aim of this investigation was to study the validation of the kinetic model for sucrose accumulation in sugarcane culm tissue. Originally, the model for sucrose synthesis and breakdown in the culm (Rohwer and Botha 2001) was constructed to approximate the steady-state for sucrose accumulation in the cytosol for medium-mature sugarcane culm tissue (internode 5) of variety N19. Table 6 clearly indicated that the predictions (from both the original and modified model) for sucrose accumulation in the younger internodal tissues agreed with that of the

sucrose concentration experimentally determined. These findings consistently validate the analytical properties of this tool.

With respect to the prediction outputs, in genotypes US6656-15, Co331 and NCo376, sucrose concentration declined from immature tissue (I_{3-4}) to medium-mature tissue (I_{6-7}) by 25.6% in US66, and by 32.6% in Co331. Furthermore, from medium-mature internodes (I_{6-7}) to older internodes (I_{8-9}) the sucrose content decreases by 41% in Co331 and 52.04% in NCo376. Concurrently, glucose and fructose increased (see Table 5 for details).

Although, SPS regulation in sugarcane is poorly understood (Grof and Campbell 2001), both Wendler et *al* (1990) (in cell suspension culture) and Botha and Black (2000) (in internodal tissue) have reported a positive correlation between SPS activity and sucrose accumulation. However, the high levels of both SPS and SuSy activities are not reflected on calculated sucrose concentration patterns and levels. The predicted flux patterns in internodal tissues of HK, FRK and NI suggest that these enzymes are the main contributors of hexoses and hexoses-phosphate supplies for sucrose synthesis in the kinetic model.

It is well reported that sugarcane tissues do contain different SuSy isoforms (Buczynski et *al*. 1993) in sugarcane internodes. Schäfer et *al* (2004) reported the presence of at least three isoforms of SuSy in sugarcane leaf roll tissue with significant differences in substrate K_m values and sucrose breakdown synthesis ratios. The observation that SuSy contributes to sucrose synthesis in young sugarcane tissue (Botha and Black 2000) was found consistent with the presence of SuSyA leaf roll with its low breakdown/synthesis ratio of 0.079 (Schäfer 2004). In contrast, SuSyB and SuSyC breakdown/synthesis ratios results at saturating substrate concentration were 0.38 and 0.49 respectively. For example, the kinetic model with experimentally determined K_{eq} and K_i values of SuSy increased sucrose concentration by more than 65% compared to the

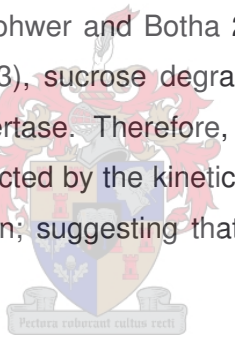
"generic" values of SuSy in the original model (Schäfer et *al*. 2004). Therefore, the simultaneous expression of different enzyme isoforms may add to the regulatory capabilities that plants have over their metabolism. The discrepancies observed between patterns of sucrose concentration experimentally determined and calculated from the model could partly be attributed to SuSy isoforms kinetic differences.

SPS was modelled with a reversible ordered, bi-reactant mechanism, with UDP-glucose binding first and UDP dissociating last from the enzyme (Rohwer and Botha 2001). The predicted SPS flux results at substrate saturation declined with tissue maturation. This resulted to the steady-state concentration of sucrose observed from the model predictions (See results section). The construction of the kinetic model for sucrose synthesis and breakdown is a first approach at describing sucrose metabolism in sugarcane quantitatively. To achieve that, a number of assumptions were made: for example, kinetic parameters such K_m and K_i values of subtracts and products were estimated; such is the case of SPS reaction (Rohwer and Botha 2001). These estimated K_i and K_m are likely to be incorrect and therefore should to a certain extent account for the rapid decline in the fluxes of SPS during internode tissue maturity.

Changes in the calculated steady-state concentrations of glucose and fructose seemed to reflect enzyme activity variations (see Chapter 3; Fig. 1) and the predicted Vmax values of HK, FRK, SuSy and neutral invertase. In both US66 and Co331, SuSy activity (breakdown activity) declined substantially between younger and medium-mature tissues by 32.5% and 44.93% respectively while, between younger and older tissues FRK activity decreased by 34.37% in US66 and by 45.9% in Co331. Similarly, HK activity decreased by more than 47% between internodes (I6-7) and (I8-9) in the NCo376 variety.

Previously, Rohwer and Botha (2001) indicated that decreased hexokinase levels reduced futile cycling and there was a decrease in the fluxes of sucrose accumulation rate. The same findings were noticeable with HK and FRK activities, as mentioned in the results. Therefore, it could also be postulated that overexpression of the fructose or glucose transporter or vascular sucrose importer are the most promising targets as hypothesized by Rohwer and Botha (2001).

There is a continuous degradation and re-synthesis of sucrose (futile cycling) in the sugarcane culm tissue (Glasziou and Gayler 1972; Moore 1995; Rohwer and Botha 2001). In the kinetic model, the percentage of futile cycling in the cytosol is defined as a continuous sucrose hydrolysis by neutral invertase and sucrose synthesis by SPS and SuSy (Rohwer and Botha 2001). However based on the findings of this study (Chapter 3), sucrose degradation is catalyzed mainly by SuSy but also by Neutral invertase. Therefore, viewed in this light the low percentage of futile cycling predicted by the kinetic model could be substantiated by these differences of definition; suggesting that SuSy may contribute in this wasteful process.



The steady-state concentration of fructose is several times reduced; compared to glucose when the model is queried with experimentally determined SuSy parameters (Schäfer et *al*. 2004). From an experimental standpoint, glucose and fructose values are to a certain extent equal. However, more than 90% of glucose and fructose in the younger internode tissues might be present in the vacuole (Vorster and Botha 1999). Therefore, the low values obtained with the modified model are not necessary incorrect as indicated by Schäfer et *al* (2004). Only metabolite measurement techniques than can differentiate between the cytosol and vacuolar compartment can resolve this issue (Schäfer et *al*. 2004; Rae et *al*. 2005).

4.6 Conclusion

In light of the results presented in this chapter, there is still some improvement that needs to be made in order to extent the kinetic model and use it efficiently as a predictive tool for the future selection and engineering of sugarcane varieties for improved agricultural production. Such improvements consist of obtaining in addition to enzymatic changes during tissue maturation, the correct values of kinetic parameters (K_m, K_i) and the reaction mechanisms of all enzymes implicated. Furthermore in terms of extension, experiments are underway in our group to include the vacuolar compartment and information about the trehalose metabolism into the kinetic model.

CHAPTER FIVE

GENERAL CONCLUSION

Developing new strategies with high prospects of success for genetic manipulation of key reaction steps in sugarcane sucrose metabolism require a good understanding of the physiological and biochemical processes involved. The results of this research study have made a modest contribution to our knowledge, in the sense that it poses important questions about key enzymes directly involved in sucrose accumulation and their potential to increase sucrose content in sugarcane. New insights were gained into the physiological role of individual enzyme activity in the process of sucrose accumulation. Calculated steady-state concentrations of sucrose from the kinetic model were in good agreement with the experimental value of the medium-mature sugarcane culm tissue of variety N19. We incorporated into the kinetic model Vmax value of enzymes from sugarcane sampled across its length, in an attempt to expand the model to reflect enzyme activity changes during tissue maturity. However, the kinetic model failed to predict the expected sucrose accumulation patterns in different genotypes. Viewed in this light, this study has contributed to our understanding of the regulation of sucrose metabolism in sugarcane and, simultaneously, has paved the way for further directed research projects.

There were a number of interesting findings that may have advanced our understanding of the relationship between enzyme activities and sucrose accumulation in developing tissue. The maturation process of sugarcane internodes correlated with a decreased of SAI in some genotypes. This enzyme has been studied extensively and the findings of this study are in accordance with what has been reported in literature (Su et *al*. 1992; Vekataramana et *al*. 1991; Terauchi et *al*. 2000). Detailed biochemical analyses were carried out on the progeny of a cross between high and low sucrose accumulating varieties

(Zhu et *al*. 1997). They concluded that soluble acid invertase activity above a critical threshold level prevented sucrose accumulation in the stem. It is proposed that SAI seems to be a good target for genetic manipulation for increasing sucrose accumulation. While transgenic sugarcane with reduced SAI activity may lead to an increase in sucrose load, this can equally result to the alteration of the revival of fully functional plant since hexose molecules are important for the remobilization of sucrose for growth. Such an investigation will undoubtedly provide important answers.

The maturation of sugarcane internodes corresponds with an increased in SPS activity in the following genotypes US6656-15, Co331 and NCo376. This pattern has been observed by others (Hatch et *al*. 1963; Batta and Singh 1986; Wendler et *al*. 1990; Botha and Black 2000) and highlights the role of SPS in sucrose accumulation. Therefore, changes in the ratio of SPS:SAI with increasing maturity of the stem may dictate the timing of the sucrose accumulation process and hence the final sucrose concentration as indicated by Grof and Campbell (2001). Also attempts are being made to up-regulate SPS activity through phosphorylation (Huber and Huber 1996). This attempt will help gain more insight into the physiological function SPS in sucrose accumulation.

The decrease in SuSy activity (cleavage direction) together with hexoses concentration could potentially indicate that the enzyme may play a significant role in the regulation of sucrose accumulation. It is well established that *in vitro* PFP activity is inversely correlated to sucrose content in sugarcane varieties differing in the amount of sucrose. Furthermore, a recent study (Van der Merwe 2005) has revealed that reduced PFP expression led to an increased of sucrose accumulation.

One of the main objectives of this study was to evaluate the corrected kinetic model by Schäfer et *al* (2004) to identify the most effective control points of

processes that contribute to sucrose accumulation across maturing internodal tissues of various varieties. After incorporating the changes in enzyme activities to reflect tissue maturity, the model was not able to anticipate the pattern of sucrose accumulation in various genotypes. However, the predictions of the model agreed with experimental value obtained for sucrose concentration in the medium-mature tissues of variety N19.

Both the high level of SPS and SuSy activities measured did not quantitatively account for the observed predicted sucrose concentrations in the genotypes investigated. In line to that, SPS and SuSy predicted fluxes declined significantly with the increasing maturity of the stem. Rohwer and Botha (2001) showed that decreased hexokinase levels reduced futile cycling; concurrently decreased the flux of sucrose accumulation. The same conclusions were reached in this study with both HK and FRK activities. There is a good indication that over-expression of the fructose or glucose transporters or vascular sucrose importer, and reduction of invertase are the most promising prospects for improving agricultural production of sucrose in sugarcane varieties. Futile cycling in the cytosol defined as a continuous sucrose cleavage by neutral invertase and sucrose synthesis by SPS and SuSy, declined with increasing maturity of the stem. However, results (Chapter 3) of SuSy (cleavage reaction) possible role in sucrose metabolism indicated that the enzyme may be mainly responsible for sucrose degradation. Therefore, the low percentage of futile cycling reported in this study may not reflect its correct physiological level.

With respect to the enzyme profiles (Chapter 3), further studies based on functional analysis of enzymes directly or indirectly involved in sucrose metabolism are required. Recently, some genes encoding enzymes associated with sugar metabolism and sugar transport in sugarcane have been identified (Zhu et *al*. 2000; Carson and Botha 2002; Grivet and Arruda 2002 and Casu et *al*. 2003). Therefore, functional analysis, knowledge on the localization and their

expression and activity will be very critical to gain better insight into the contribution of these enzymes in sucrose accumulation. This makes possible much more informed decisions on manipulation strategies for yield improvement.

With respect to the kinetic model, there have been conflicting reports about the equilibrium constant of SPS. Goodwin and Mercer (1983) found that K_{eq} for SPS formation of Suc-6-P was 3250 while Kruger (1997) reported 10. In addition, most of the enzymes in the original model used estimated K_i values equal to the substrate K_m values. Schäfer (2004) reported that K_i obtained for the SuSyC isoform differ significantly from the corresponding K_m value. It would therefore be useful to replace estimated K_i with their experimentally determined measured values. Such changes, although assumed to be of no consequences, could potentially have substantial effects on the model and its outcomes. It would be therefore advantageous to determine their correct values.

It is well documented that plant tissues do contain different isoforms for the same enzyme. The simultaneous expression of different enzyme isoforms may add to the regulatory capabilities that plants have over their metabolism. Such changes have proved to be very critical especially; given some of the interesting results gained in recent study (Schäfer et *al*. 2004), it would be helpful to obtain further kinetic data on SuSy isoforms and subsequently others enzymes. Indeed, SuSy isoforms distribution immature, maturing and mature internode tissues should be investigated then included in the model and their effects tested for the improvement of the kinetic model outputs. Therefore, this approach may prove to be very insightful for improving the kinetic model.

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