FRUCTOKINASE ACTIVITY IN THE SUGARCANE CULM: EXPRESSION PATTERNS AND KINETIC PROPERTIES

by

SUZANNE WILMIEN HOEPFNER

Thesis presented in fulfilment of the requirements for the degree of

Master of Science

at the University of Stellenbosch

February 2001

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

February 2001

ABSTRACT

Five hexose kinases, two fructokinases and three hexokinases, were identified in sugarcane culm. Fructokinase, a fructose specific hexose phosphorylating enzyme, was further investigated. Two isoforms, FRK1 and FRK2, were found. The isoforms were purified to homogeneity and antibodies raised against each. Both FRK1 and FRK2 have pH optima of 8.0 and both are homodimers of 69 kDa, consisting of subunits of 33 kDa. FRK2 was subject to substrate inhibition by fructose concentrations exceeding 0.1 mM while FRK1 was not inhibited by 1.0 mM fructose. Sugarcane FRK2 is more sensitive to substrate inhibition than FRK2 from other plants. The reaction catalysed by FRK1 is ATP-specific. The FRK2 reaction can utilise a variety of nucleotide triphosphates and no substrate inhibition is apparent when assayed with UTP instead of ATP. We proposed the existence of two nucleotide triphosphate binding sites on the enzymes. One of the sites is an ATP-specific regulatory site while the other is a catalytic site with wide substrate specificity. Additionally two fructose-binding sites are proposed. One is a catalytic site and the other a allosteric regulatory site. Binding of fructose to the allosteric site is only possible if ATP is present in the regulatory ATP-binding site. Such a configuration could explain the kinetic properties of FRK2. Both fructokinase protein expression and total fructokinase activity decreased during development. Consequently the decrease in activity is the result of decreased expression and not inactivation of existing protein. The ratio of FRK2 to FRK1 activity is dependent on the developmental stage of the tissue. FRK1 appears to be the isoform that is preferentially expressed in mature tissue. Previous measurements of fructokinase activity in crude extracts have been inaccurate as a result of the divergent kinetic properties of the isoforms. Based on the findings in this project a novel method is proposed whereby both the activity of each isoform and total fructokinase activity can be accurately calculated using a mathematical equation.

OPSOMMING

Vyf heksokinases, twee fruktokinases en drie heksokinases, is in suikerrietstingel geïdentifiseer. Fruktokinase, 'n fruktose-spesifieke heksose fosforileringsensiem, is verder ondersoek. Twee isovorme, FRK1 en FRK2, is gevind. Die isovorme is gesuiwer tot homogeniteit en teenliggame is teen beide vervaardig. FRK1 en FRK2 het albei 'n pH optimum van 8.0 en beide is homodimere van 69 kDa, bestaande uit subeenhede van 33 kDa. FRK2 is baie gevoelig vir substraatremming deur fruktose, terwyl FRK1 nie gerem word deur konsentrasies selfs so hoog as 1.0 mM fruktose nie. Suikerriet FRK2 is meer sensitief vir substraatremming as FRK2 van ander plante. Die reaksie wat deur FRK1 gekataliseer word, is ATP-spesifiek. Die FRK2-reaksie kan 'n verskeidenheid nukleotiedtrifosfate benut en geen substraatremming kom voor wanneer die reaksie gemeet word met UTP in plaas van ATP nie. Gebaseer op dié bevindinge word 'n model voorgestel waar twee nukleotiedtrifosfaatbindingsetels op die ensieme voorkom. Een van die setels is 'n ATP-spesifieke regulatoriese setel, terwyl die ander 'n katalitiese setel met breë substraatspesifisiteit is. Verder postuleer ons twee fruktose-bindingsetels. Een van die setels is katalities en die ander een is 'n allosteriese regulatoriese setel. Binding van fruktose aan die allosteriese setel kan net plaasvind as ATP gebind is in die ATP-regulatoriese setel. So 'n konformasie kan die kinetiese eienskappe van FRK2 verduidelik. Die uitdrukking van fruktokinase-proteïen en die totale fruktokinase-aktiwiteit neem beide af gedurende ontwikkeling. Gevolglik is die afname in aktiwiteit die gevolg van verminderde proteïenuitdrukking en nie inaktivering van bestaande proteïen nie. Die verhouding van FRK1- tot FRK2-aktiwiteit is afhanklik van die ontwikkelingstadium van die weefsel. Dit wil voorkom of FRK1 die isovorm is wat by voorkeur in volwasse weefsel uitgedruk word. Vorige bepalings van fruktokinase-aktiwiteit in ru-ekstrakte was onakkuraat as gevolg van die uiteenlopende kinetiese eienskappe van die isovorme. Gebaseer op die bevindinge van hierdie projek stel ons 'n oorspronklike metode voor waarvolgens beide die aktiwiteit van elke isovorm en totale fruktokinase-aktiwiteit akkuraat bereken kan word met behulp van 'n wiskundige formule.

ACKNOWLEDGEMENTS

Firstly I would like to thank Professor Frikkie Botha for his support and enthusiasm. Thank you for always being willing to listen and help and for solving my biggest problems with the simplest solutions. This learning experience has far exceeded my expectations.

Thanks go to the staff and students at the Institute for Plant Biotechnology for their support and assistance during this project.

I am grateful for the financial support of the South African Sugar Association and the National Research Foundation and for the facilities provided by the University of Stellenbosch.

Finally, I would like to thank my family and friends. In particular, thanks go to Wouter, Fletcher and Stephanie for their support and threats, but most of all for believing in me.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A_{340}	absorbance at 340 nanometre
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
CTP	cytidine 5'-triphosphate
Da	Dalton
DEAE	diethylaminoethyl
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
F6P	D-fructose-6-phosphate
Frk	fructokinase gene
FRK	fructokinase (ATP:D-fructose-6-phosphotransferase), EC 2.7.1.4
FW	fresh weight
g	gram
g	gravitational force
GK	glucokinase (ATP:D-glucose-6-phosphotransferase), EC 2.7.1.2
GTP	guanosine 5'-triphosphate
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HK	hexokinase (ATP:D-hexose-6-phosphotransferase), EC 2.7.1.1
IgG	immunoglobulin G
K_i	inhibition constant
Km	Michaelis constant (concentration of substrate that produces half maximal activation)
L	litre
m	metre
M	molar
mA	milli-Amperé
MES	2-[N-morpholino] ethanesulfonic acid
min	minute
mRNA	messenger ribonucleic acid
$NADP^+$	oxidised nicotinamide-adenine phosphate dinucleotide
NTP	nucleotide triphosphate
PAGE	polyacrylamide gel electrophoresis

Pi	inorganic phosphate
PPi	inorganic pyrophosphate
SDS	sodium dodecyl sulphate
SPS	sucrose phosphate synthase (UDP-D-glucose:D-fructose-6-phosphate 2-α-D-glucosyltransferase), EC 2.4.1.14
SuSy	sucrose synthase (UDP-D-glucose:D-fructose 2-α-D-glucosyltransferase), EC 2.4.1.13
TBST	tris-buffered saline containing Tween 20
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tween 20	polyoxyethylene sorbitan monolaurate
U	unit of enzyme activity (1 micromole substrate consumed per minute)
UDP	uridine 5'-diphosphate
UDPGlc	uridine 5'-diphosphate glucose
UTP	uridine 5'-triphosphate
V	volt

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Sugarcane is produced in 102 countries and sugar production for 1999/2000 is estimated at 133 million tons. Approximately 73 % of the sugar is produced from sugarcane and the remaining 27 % from sugarbeet. Most of the world's sugar production is consumed in the countries of origin, with approximately 25 % traded on the world market (http://www.illovo.co.za). The Southern African Development Community (SADC) is the ninth biggest sugarcane producer in the world, and the sixth largest exporter of sucrose. During the 1999 / 2000 season, the South African sugarcane industry contributed an estimated R1 300 million to the country's foreign exchange earnings (http://www.sasa.org.za).

To maintain international competitiveness in this highly volatile market, there is a continuous search for improvement of the crop. Previous advances in sucrose production have been through manipulation of the yield of sucrose per stem and the quantity of cane grown per unit of land (Moore and Maretzki 1997). However, over the past two decades sucrose production seemed to have reached a plateau that can not be overcome through conventional plant breeding. The difficulty of selecting for desired phenotypes in field trials may partly be blamed, but it is also possible that the genetic resources available in the germplasm have already been substantially or completely exploited.

Several lines of study are currently pursued in an effort to genetically manipulate sugarcane for improved sucrose load. One of these is the manipulation of key enzymes of metabolism. However, sound physiological data on limiting or co-limiting factors are an indispensable requirement for sensible gene-technology based breeding approaches (Komor 2000). Despite numerous studies the processes involved in sugarcane sucrose metabolism and subsequent accumulation of sucrose in the stem is poorly understood (Moore 1995 and references therein).

In an effort to determine the most viable target enzymes, much work has been done on the pathways of transport, metabolism and accumulation of sucrose in sugarcane. Recent work has indicated that both hexoses and sucrose are taken up by the storage

cells of sugarcane where they enter into a continuous cycle of sucrose synthesis and degradation (Komor 2000; Whittaker and Botha 1997). Consequently the intracellular hexose pool consists of a mixture of imported hexoses and the products of sucrose degradation.

The first step in the mobilisation of hexoses is phosphorylation by the hexokinases, a family of hexose phosphorylating enzymes that use a nucleotide triphosphate as a phosphoryl donor. A high rate of mobilisation of hexoses was previously observed in sugarcane (Whittaker and Botha 1997). As sugarcane tissue matures respiration decreases and this was at least in part ascribed to substrate limitation (Whittaker and Botha 1997). The possibility therefore exists that hexose mobilisation is reduced in maturing tissue, leading to a shortage of phosphorylated hexoses to enter the respiratory pathway.

Despite the potential importance of hexose mobilisation in the control of carbon flux in sugarcane internodal tissue, the enzymes involved in hexose phosphorylation have not been studied in detail before. To gain a better understanding of the role of these enzymes, information regarding their expression and kinetic properties are required.

The aim of this study was threefold. Firstly, to purify and kinetically characterise the fructokinase activity(s) in the culm. Secondly, to raise high titre, monospecific polyclonal antibodies against the purified isoforms of fructokinase, and thirdly, to determine the influence of tissue maturity on isoform presence and distribution.

CHAPTER 2

LITERATURE REVIEW

2.1 COMMERCIAL

In conjunction with sugar beet, sugarcane produces over 90 % of the world's sucrose. Sugarcane can accumulate sucrose to concentrations exceeding 25 % of its fresh weight or 60 % of its dry weight (Bull and Glasziou 1963).

Sucrose is the third most important agricultural export product in South Africa and a valuable source of revenue. During the 1999 / 2000 season 21.2 million tons of cane was crushed to produce 2.5 million tons of sucrose. About 50 % of this sugar was marketed in Southern Africa, and the rest exported to Africa, the Middle East, North America and Asia. Based on revenue generated through sugar sales on the local market, the Southern African region and preferential and world market exports, the South African sugar industry is responsible for generating direct income totalling R4 700 million. Based on actual sales and selling prices in 1999 / 2000, it is estimated that the industry contributed R1 300 million to the country's foreign exchange earnings.

2.2 THE IMPORTANCE OF SUCROSE

Sucrose is the primary product of photosynthesis and accounts for most of the CO_2 absorbed by a plant during photosynthesis (Kruger 1997). It is the first photoassimilate to accumulate in photosynthetic cells from where it is translocated to the non-photosynthetic and storage tissues. Sucrose is the major form in which carbon is translocated in plants (Hartt *et al.* 1963; Hawker 1985; Komor 2000). Many plants contain sucrose as a storage compound. However, starch is usually the main storage molecule and sucrose occurs at low concentrations relative to starch. In contrast, sugarcane and sugarbeet both accumulate sucrose to high concentrations and contain virtually no starch (Komor 2000).

2.3 TRANSPORT TO STORAGE TISSUE

Photoassimilate transport, partitioning and accumulation form the basis for the productivity and success of any plant (Moore 1995). In plant cells sucrose is the major form in which assimilated carbon is transported to sinks (ap Rees 1988; Kühn *et al.* 1999).

The pathway of sucrose accumulation begins with the translocation of sucrose through the phloem sieve elements to the stem internodes (Moore 1995). The bulk of sucrose moves toward the plant base and roots, while smaller amounts move upward toward the apical meristem and immature sink leaves (Hartt *et al.* 1963; Hatch and Glasziou 1964). In sugarcane, sucrose arrives intact at the sink and is then broken down and resynthesized during movement into the storage cells (Hatch and Glasziou 1964; Moore 1995; Moore and Maretzki 1997). Sucrose is either metabolised in the cytosol to provide carbon skeletons and energy, or can be accumulated in the storage parenchyma cells and the apoplastic space surrounding them.

There are conflicting views on sugar transport through the plasmalemma of sugarcane storage parenchyma cells. Four possible methods for moving sugars from the phloem into the storage compartment can be postulated: (1) Sucrose moves from the phloem through the apoplast into the storage parenchyma cells (apoplastic unloading) (Sacher *et al.* 1963; Glasziou and Gayler 1972a); (2) Sucrose moves through plasmodesmata situated between the sieve-elements and the epidermis-like cells surrounding the phloem, and then through the apoplast, moving radially from the phloem to the storage parenchyma (Hawker 1985); (3) Sucrose is unloaded from the sieve elements into the storage parenchyma cells via plasmodesmata (symplastic unloading), after which sugars are leaked from the storage parenchyma into the apoplast (Glasziou and Gayler 1972b; Oparka and Prior 1988); (4) There is simultaneous symplast and apoplast transport through the storage parenchyma so that the sugars in the apoplast are a mixture of those from phloem unloading and from the leakage of storage cells.

Studies on sugarcane suspension cultures (Maretzki and Thom 1972; Komor *et al.* 1981) supported the apoplastic unloading hypothesis. The cells expressed high levels of acid invertase and took up reducing sugars but not sucrose. This was interpreted to mean that sucrose is unloaded from the phloem into the apoplast where it is cleaved by invertase and the hexoses then taken up by hexose carrier sites in the plasmalemma.

Although there is no known anatomical necessity for apoplastic transport in the sugarcane stem, early labelling, enzymatic and membrane transport research supported this idea and it became a integral feature of the sucrose accumulation model as proposed by Glasziou and Gayler (1972a) (Moore 1995).

Anatomically it was shown, however, that the sclerenchyma bundle sheaths and most of the storage parenchyma cells of the stem become lignified and suberised prior to the bulk of internodal sucrose storage (Jacobsen *et al.* 1992). These modified cell walls prevent apoplastic solute flow and are therefore barriers to the radial transfer of sucrose (Moore 1995). Two well-separated apoplastic spaces, one in the bundle, the other in the stem parenchyma (Jacobsen *et al.* 1992; Welbaum *et al.* 1992), have lead to the hypothesis that phloem unloading had to proceed symplastically and that unloaded sugars first appear in the cytosol of storage parenchyma.

Komor (2000) proposed a model for sugar transport in internode parenchyma that could accommodate both apoplastic and symplastic phloem unloading. According to this model, partial apoplastic transport may occur in immature, growing internodes since the cells only become lignified and suberised after cell expansion has ended. Symplastic transport would occur in the mature internodes. The driving force for symplastic transfer may be diffusion along a concentration gradient and/or bulk flow by hydrostatic pressure (Moore 1995; Komor 2000).

As a result of symplastic transport, unloaded sugars will first appear in the cytosol of the storage parenchyma. The sugars are then subjected to several cyclic processes in parallel: a metabolic cycle of sucrose hydrolysis and synthesis (see 2.5), a cycle of sugar efflux and uptake through plasmalemma-located transport systems (see 2.4), and a cycle of sucrose and hexose transfer into, and out of, the vacuole (see 2.8). The rate of these cyclic processes changes during the ripening of the internodes (Whittaker and Botha 1997; Komor 2000).

2.4 SUCROSE OR HEXOSE UPTAKE?

One of the major differences in opinion on sugarcane metabolism is whether the storage parenchyma cells take up sucrose or hexoses. Bieleski (1960) concluded that both sucrose and hexoses were taken up by slices of sugarcane stem tissue. In

contrast, Hawker and Hatch (1965) found that sucrose was stored more rapidly when glucose or fructose were fed to mature internode tissue slices than when sucrose was fed. This indicated a preferential uptake of hexoses. When either labelled glucose or labelled fructose were fed to tissue slices, sucrose labelled equally in both hexoses was recovered. These results showed that adequate intracellular isomerase activity was present to fully randomise the label prior to sucrose resynthesis (Hawker and Hatch 1965). Earlier results from studies on immature internode tissue slices (Sacher *et al.* 1963) indicated that a minimum of 70 % of the labelled sucrose entering the tissue was hydrolysed during movement into the storage compartment. This led the authors to deduce that sucrose hydrolysis by invertase in the apoplastic space was obligatory for sucrose storage.

Reinvestigation of sugar uptake experiments with tissue slices (Lingle 1989) confirmed that cleavage of sucrose occurs in the apoplast. However, in stark contrast to the earlier experiments (Sacher *et al.* 1963; Hatch and Glasziou 1964), most of the sucrose was accumulated without randomisation of the supplied [^{14}C]fructosyl sucrose label. The conclusion was reached that sucrose could be taken up intact. This was supported by additional research where the bulk of sucrose was not cleaved in the apoplast and very little randomisation of the label was found in the accumulated sucrose (Thom and Maretzki 1992). The researchers interpreted their results as indicating that cleavage of sucrose was not necessary for its uptake. The authors emphasised that the two alternative pathways for sucrose uptake, either as intact sucrose or as its hexoses after cleavage, are not mutually exclusive. There could be two uptake systems on the plasma membrane, one for hexose and another for sucrose. A major difference between the later results showing the uptake of sucrose (Lingle 1989; Thom and Maretzki 1992), and the earlier results showing hexose uptake (Sacher *et al.* 1963; Hatch and Glasziou 1964), involves the treatments used for washing the tissue prior to the uptake studies.

When hexoses are taken up into the cytosol from the apoplastic space, this occurs through hexose carrier sites (Sacher *et al.* 1963; Bowen and Hunter 1972; Maretzki and Thom 1972; Komor *et al.* 1981). There is probably a separate hexose transporter for glucose and fructose (Moore 1995).

In young and maturing internodes the uptake of hexoses was shown to be much faster than sucrose uptake (Komor *et al.* 1996). Growing tissues are usually characterised by

high extracellular acid invertase activity, so that active transport systems, mainly hexose transport systems, absorb as much sugar as available for growth and cell expansion. Sucrose transport into the cell would be negligible in that situation because the bulk would be cleaved by invertase (Komor 2000). In mature storage tissue less invertase activity occurs and consequently less hexoses are formed through sucrose cleavage. Thus the only sugar available to be transported into the cell is sucrose (Komor *et al.* 1996).

2.5 SYNTHESIS AND DEGRADATION

Upon arrival in storage parenchyma cells in the sugarcane stem, sucrose is processed for use in cell growth, respiration, metabolism or storage (Hawker 1985). An enzymatic cycle of sucrose synthesis and degradation has been postulated (Glasziou 1961; Sacher *et al.* 1963; Glasziou and Gayler 1972a; Batta and Singh 1986; Wendler *et al.* 1990; Whittaker and Botha 1997).

2.5.1 Sucrose synthesis

Three enzymes, sucrose phosphate synthase, sucrose phosphatase and sucrose synthase, are associated with sucrose synthesis in green plants. These enzymes occur in the cytoplasm and cell walls (Hawker 1985).

2.5.1.1 Sucrose phosphate synthase (SPS)

UDPGlc + F6P → sucrose-6-phosphate + UDP

SPS (EC 2.4.1.14) is present in many plant tissues, especially those that synthesize sucrose (Hawker 1985). It is highly specific for its substrates, UDP-glucose (UDPGlc) and fructose-6-phosphate (F6P), and catalyses the production of sucrose-6-phosphate (Avigad 1982; Kruger 1997). This reaction is practically irreversible, especially when coupled to a reaction that drains sucrose-6-phosphate.

2.5.1.2 Sucrose phosphatase

sucrose-6-phosphate + H_2O → sucrose + Pi

Sucrose phosphatase (EC 3.1.3.24) dephosphorylates sucrose-6-phosphate that is produced by SPS, releasing inorganic phosphate (Pi) and sucrose (Avigad 1982; Kruger 1997). It is subject to partially competitive inhibition by sucrose, but is never completely inhibited (Hawker 1985).

2.5.1.3 Sucrose synthase (SuSy)

UDPGlc + fructose → sucrose + UDP

SuSy (EC 2.4.1.13) is the first enzyme capable of synthesizing sucrose that was discovered in plants (Hawker 1985). This enzyme catalyses the production of sucrose from fructose and UDPGlc. It is a readily reversible reaction *in vivo* in potato tubers and other plant tissues (Geigenberger & Stitt 1993; Viola 1996). It has been proposed that SuSy primarily participates in sucrose utilisation rather than synthesis (Hawker 1985; Wendler *et al.* 1990). A recent review, however, proposed that SuSy activity is developmentally influenced and that SuSy mainly synthesizes sucrose in immature storage tissue (Komor 2000).

2.5.2 Sucrose degradation

Sucrose is broken down to fructose, glucose and UDPGlc, depending on the prevailing pathway. Sucrose cleavage products can be channelled into respiration, biosynthesis or signalling processes. Hexoses need to be phosphorylated before they can be metabolised.

2.5.2.1 Invertase

sucrose → glucose + fructose

Invertase (EC 3.2.1.26) occurs in the cytosol, the vacuole and cell-wall-bound. The irreversible reaction catalyses the hydrolyses of sucrose to produce hexoses (Avigad 1982; Hawker 1985). Soluble acid invertase is found in the vacuoles of immature tissue. Membrane-bound acid invertase is associated with cell walls, while neutral invertase is found in the cytosol (Moore 1995).

2.5.2.2 Sucrose synthase (SuSy)

sucrose + UDP → UDPGlc + fructose

This enzyme has been discussed earlier and it was pointed out that it is thought to be primarily involved in sucrose cleavage. This theory is supported by the fact that SuSy is one of the major soluble proteins in growing plant tissues, where sucrose utilisation is important. SuSy mediated sucrose breakdown is inhibited by high fructose concentrations (Schaffer and Petreikov 1997a).

2.6 HEXOKINASES IN PLANTS

Mobilisation of hexoses can only occur after phosphorylation of the hexose sugars. Both hexoses taken up by the storage parenchyma (see 2.4) and hexoses resulting from cycling (see 2.5) need to be phosphorylated. Sucrose that is taken up intact will be available to both invertase and SuSy for breakdown. The continuous cycling of carbon between hexoses and sucrose will lead to continuous resynthesis of sucrose that will also be degraded. There would therefore be a constant demand for phosphorylation of glucose and fructose to allow for their mobilisation.

Hexoses are phosphorylated by the hexokinases, a family of enzymes that use a nucleotide triphosphate as phosphoryl donor for phosphorylation, catalysing the reaction:

hexose + ATP → hexose-6-phosphate + ADP

The hexokinase reaction occurs in all eukaryotic cells as the first step in the utilisation of hexoses. The subsequent steps vary, as the hexose-6-phosphate formed in this reaction may have different metabolic fates in different types of cell and in different physiological conditions. Although it could be argued that transport of hexoses into the cell is the initial step in their utilisation, the hexokinase reaction is the first dedicated reaction towards metabolising hexoses.

This family of hexose phosphorylating enzymes exhibits many different catalytic properties and varying tissue and intracellular distributions (Cárdenas *et al.* 1998).

Significant differences in the hexose specificity of the hexokinases have lead to the refinement of the term "hexokinase". The glucokinases (GK, EC 2.7.1.2) preferentially phosphorylate glucose, the fructokinases (FRK, EC 2.7.1.4) preferentially phosphorylate fructose and the hexokinases (HK, EC 2.7.1.1) can phosphorylate both glucose and fructose.

The hexokinases have been studied in a wide variety of bacterial, mammal and plant species. Only a few species, mostly bacteria, are known to contain true GK, i.e. enzymes specific for glucose. Most plants contain HK and FRK which differ in molecular mass and tissue distribution, and the enzymes often exist as a mixture of isozymes that differ in kinetic characteristics and molecular mass.

2.7 FRUCTOKINASE

During cycling of carbon between sucrose and its hexoses both SuSy and invertase take part in sucrose degradation and both produce fructose (see 2.5). FRK is the enzyme primarily responsible for fructose phosphorylation. Inadequate fructose phosphorylation capacity could theoretically lead to a build-up of fructose.

2.7.1 FRK isoforms

A recent review (Pego and Smeekens 2000) proposed a new naming system for the fructokinases. This system relies on the assumption that all FRK activity peaks studied in higher plants to date can be attributed to two different enzymes, FRK1 and FRK2. This nomenclature will be used throughout this manuscript.

All the plant species studied to date have multiple hexose kinase isozymes with at least one of these being a FRK. Hexose kinases from potato (Gardner *et al.* 1992; Renz *et al.* 1993), tomato (Martinez-Barajas and Randall 1996; Schaffer and Petreikov 1997a; Kanayama *et al.* 1998), pea seeds (Turner *et al.* 1977; Copeland *et al.* 1978), spinach leaves (Schnarrenberger 1990), maize (Doehlert 1989; Doehlert 1990), sugar beet (Chaubron *et al.* 1995), avocado (Copeland and Tanner 1988), rice embryo (Guglielminetti *et al.* 2000) and barley leaves (Baysdorfer *et al.* 1989) have been characterised, and these studies indicated that plant tissues typically contain three to five hexose kinases that differ in their physical and kinetic properties.

The active form of FRK is generally a homodimer of between 59 and 85 kDa (Kanayama *et al.* 1998; Martinez-Barajas and Randall 1996; Chaubron *et al.* 1995; Gardner *et al.* 1992), except in the cases of potato, where one study put the active form at 102 – 118 kDa (Renz *et al.* 1993), barley, where the active form of one isoform was a monomer of 37 kDa (Baysdorfer *et al.* 1989), and pea seed, where a monomer of 72 kDa was found (Copeland *et al.* 1984). Values obtained based on deduced amino acid sequences indicated a protein of 34.4 – 37.3 kDa (Taylor *et al.* 1995; Kanayama *et al.* 1997; Martinez-Barajas *et al.* 1997; see 2.7.3).

Relatively little is known about the existence, functions or genetic basis for tissue- or development-specific isozymes of plant glycolytic enzymes (Plaxton 1996). One FRK isoform that did not show developmental change was found in developing sugarbeet taproots (Chaubron *et al.* 1995). In tomato fruit the two FRK genes are differentially expressed (Kanayama *et al.* 1998) and total FRK activity in developing tomato fruit was influenced by development (Martinez-Barajas *et al.* 1997) as were the mRNA levels of each FRK gene (Kanayama *et al.* 1997). In potato three FRK isoforms were identified, and the isoforms show organ- and development-dependent changes. FRK1 and FRK2 were not separated from each other during this investigation (Renz *et al.* 1993).

2.7.2 Kinetics

In all plants that were studied (with the exception of avocado (Copeland and Tanner 1988)) at least one FRK that is inhibited by high fructose concentrations was found, while the other isoforms were insensitive to substrate inhibition. Recent work (Dai *et al.* 1997) indicated that the potato FRK gene showed substrate specificity for fructose when it was expressed in yeast cells and was inhibited by fructose concentrations exceeding 2 mM. The proliferation of the yeast was severely inhibited under high fructose conditions, which indicates that the inhibition of fructokinase activity has physiologically important effects.

Fructose as a substrate inhibitor presents a rather perplexing situation. If the enzyme and its substrate are in the same subcellular compartment, any accumulation of substrate will result in decreased phosphorylation and subsequently more fructose accumulation. Several studies have placed FRK exclusively in the cytosol (Tanner *et al.* 1983; ap Rees 1985; Copeland and Morell 1985; Copeland and Tanner 1988), except for one isoform from spinach that was associated with the chloroplast (Schnarrenberger 1990). In sugarcane suspension cells the hexoses appeared to be

almost completely located in the vacuole in the early culture phases, and then evenly distributed between cytosol and vacuole at the end of the cell culture cycle (Preisser *et al.* 1992). Unfortunately little is known about the intracellular distribution of sugars in intact sugarcane.

The sucrose degradation reaction catalysed by SuSy is close to equilibrium *in vivo* and is subject to feedback inhibition by fructose (Wolosiuk and Pontis 1974 *in* Taylor et al 1995; Gardner *et al.* 1992; Trethewey *et al.* 1999). This means that FRK activity is necessary to sustain sucrose breakdown. In this scenario, inhibition of fructose metabolism would also lead to inhibition of fructose production, thereby slowing down the futile cycling between sucrose and hexoses.

It has been proposed (Huber and Akazawa 1986) that sucrose degradation via SuSy is integrated with the phosphorylation of fructose by UTP-linked FRK. In this model invertase and SuSy initiate separate sucrose degradation pathways. Invertase hydrolyses sucrose to its hexoses which are then subject to classical glycolysis. The proposed SuSy pathway involves cycling of uridylates and inorganic pyrophosphate (PPi) through SuSy, FRK, UTP-linked phosphofructokinase (EC 2.7.1.90) and UDPGlc pyrophosphorylase (EC 2.7.7.9). UDPGlc pyrophosphorylase, an effective PPi-scavenger, would consume PPi and form UTP. The UTP could then be utilised in the UTP-linked FRK reaction, thereby forming UDP for SuSy. The source of PPi is postulated to arise from the back reaction of pyrophosphate:fructose-6-phosphate 1-phosphotransferase (PFP; EC 2.1.7.90) (Huber and Akazawa 1986; Xu *et al.* 1989).

Several studies addressed UTP-utilisation by FRK following the conception of this hypothesis. Reports included FRK isoforms that are ATP specific (Baysdorfer *et al.* 1989; Schnarrenberger 1990; Renz and Stitt 1993), showing lower activity with other nucleotides and K_{mUTP} usually 5 to 10 times higher than K_{mATP}. Several FRK isoforms showing no ATP specificity under non-limiting UTP conditions were found (Copeland and Tanner 1988; Doehlert 1990; Schnarrenberger 1990; Gardner *et al.* 1992; Renz and Stitt 1993; Chaubron *et al.* 1995; Martinez-Barajas and Randall 1996). In most cases, however, the K_{mUTP} was between 5 and 50 times higher than the K_{mATP}. Without knowing the exact concentrations of ATP and UTP in the cytosol, the extent to which the UTP-linked FRK reaction functions cannot be assessed.

2.7.3 Genetics

One FRK gene that was present in low copy number was found in potato (Taylor *et al.* 1995). In tomato two divergent genes encoding FRK are expressed (Kanayama *et al.* 1998). It was suggested that one of the genes (*Frk2*) codes for the fructose inhibited isoforms of FRK, while the other (*Frk1*) codes for the fructose insensitive "housekeeping" isoforms that are ubiquitously expressed and supply F6P for glycolysis. Post-translational modification or differential splicing of the proteins was thought to lead to the different characteristics of isoforms that are encoded by the same gene (Taylor *et al.* 1995; Martinez-Barajas *et al.* 1997; Kanayama *et al.* 1998; Pego and Smeekens 2000). The open reading frame of the *Frk1* gene codes for a protein of approximately 37 kDa (Kanayama *et al.* 1997) and the *Frk2* gene for a protein of approximately 34.8 kDa (Taylor *et al.* 1995; Kanayama *et al.* 1997; Martinez-Barajas *et al.* 1997).

It is possible that FRK gene expression may be responsive to carbohydrate status. Kanayama *et al.* (1998) showed that both FRK genes are sugar regulated in tomato cotyledon tissue. If this is also true in sugarcane, where hexose levels can exceed 20 mM (Whittaker and Botha 1997) in immature tissue, the sugarcane Frk genes could be optimally induced in young tissue.

Alignment of the deduced amino acid sequences of *Arabidopsis* and tomato FRK1, *Arabidopsis*, tomato and sugarbeet FRK2, and *Arabidopsis* FRK3 revealed significant homology amongst all six plant proteins. Two motifs that are unique to FRK and are not conserved in other sugar kinases were identified and this domain was proposed to represent the fructose substrate recognition site (Kanayama *et al.* 1997; Pego and Smeekens 2000). Two highly conserved ATP-binding sites were also identified. One of these was identified by Pego and Smeekens (2000) and the other by Kanayama *et al.* (1997). The latter contains the GD-motif that is essential for activity and is perfectly conserved between the FRK2 proteins. A few mutations occurred in the FRK1 and FRK3 sequences.

2.7.4 Regulation

In barley leaf (Baysdorfer *et al.* 1989) and maize kernels (Doehlert *et al.* 1988) the maximum activity of HK and FRK was low, even compared to other glycolytic enzymes. This finding lead to the suggestion that hexose phosphorylation, and specifically FRK activity, could be a rate-limiting step in carbohydrate metabolism. The same conclusion was reached when potato tubers were investigated (Viola 1996). It was

found that the regulation of fructokinase *in vivo* could represent a limiting factor in the utilisation of sucrose in developing potato tubers. SuSy and FRK function in an apparently co-ordinated manner in the sucrose to starch metabolic pathway (Ross *et al.* 1994; Schaffer and Petreikov 1997b) and it has been proposed that the processing of sucrose through SuSy and FRK may represent an important control point for starch synthesis in potato tubers (Viola 1996).

2.7.5 Signalling

Sugars are central regulatory molecules controlling physiology, metabolism, cell cycle, development and gene expression (Jang *et al.* 1997) by repressing or activating genes involved in processes including photosynthesis, glyoxylate metabolism, respiration, starch and sucrose synthesis and degradation, nitrogen metabolism, pathogen defence, wounding response, cell cycle regulation, pigmentation and senescence (Sheen 1990; Jang and Sheen 1994; Jang *et al.* 1997; Smeekens and Rook 1997).

Hexose kinases act as sugar sensors, mediating diverse sugar responses (Jang *et al.* 1997; Dai *et al.* 1999). HK, the best known of this family, catalyses the first reaction in glycolysis, regulates the pathway at the enzyme level, and is involved in sugar-mediated regulation of gene expression (Pego and Smeekens 2000).

HK and galactokinase have sugar sensing capability in yeast and are involved in signalling cascades. The system might be partially conserved in plants and mammals (Pego and Smeekens 2000). Sugar-insensitive *mig* mutants in *Arabidopsis* lack a specific FRK activity because of a null mutation in the *Frk2* gene. The correlation between this mutation and sugar-insensitivity is under investigation (Pego and Smeekens 2000). FRK activity is associated with sugar-mediated gene regulation in mammals and modulates the glucose sensor in the pancreatic islet cells (Pego and Smeekens 2000).

The role of FRK in sugar responses of plants is not known, but is under investigation (Pego and Smeekens 2000). It is possible that FRK2, the widespread FRK isoform that is subject to substrate inhibition by fructose, is not so much a metabolic enzyme as a regulatory entity. Tomato *Frk2* mRNA was predominantly found in immature fruit and levels decreased during development and ripening. *Frk1* mRNA showed the opposite pattern in fruit with levels increasing during maturation, but was expressed throughout the plant (Kanayama *et al.* 1997).

2.8 STORAGE OF SUCROSE

Most plants use sucrose as a temporary storage product in storage organs, with starch being used for long term storage. Sugarcane stems and sugarbeet roots are exceptions to this, accumulating sucrose to high concentrations and containing little or no starch. Sucrose accumulation by sugarcane has been subjected to detailed physiological and biochemical studies since about 1960 (Moore 1995). During elongation of the sugarcane stem, and for a period of time following full elongation, the internodes accumulate increasing concentrations of sucrose. Development is a continuous process resulting in a gradient of maturation and sucrose accumulation down the stalk to a point where full maturity and stable, high sucrose concentration is reached (Moore 1995).

2.8.1 Source or sink control

The rate of sucrose accumulation could be regulated at the level of the source (rate of photosynthesis) or the storage sink (rate of storage). Under conditions favouring sucrose accumulation, the sugarcane stem of high yield varieties can store sucrose exceeding 62 % of the dry weight or 25 % of the fresh weight (Bull and Glasziou 1963), which approaches 800 mM concentrations. Some of the wild relatives of sugarcane store less than 2 % of the fresh weight as sucrose. Yet the photosynthetic rates of *S spontaneum* exceeds that of *S officinarum* and hybrid cultivars (Irvine 1975). On this basis, the differences in sucrose storage appear to be regulated at the level of the sink or within the translocation system between the source and sink (Moore and Maretzki 1997; Moore *et al.* 1997; Komor 2000).

Initially all developing plant organs are sinks that depend on imported photosynthate to sustain their respiration and growth. During maturation the internodes switch from importing photosynthates to support growth to importing them for storage. The developmental or metabolic events that regulate this switch from growth to storage are not yet known (Moore and Maretzki 1997).

2.8.2 Storage

Sucrose concentration in the storage cells is mainly controlled by the safe compartmentation of sucrose into the vacuoles and vacuolar acid invertase activity (Komor 2000). Most of the sucrose in storage parenchyma cells will be located in the vacuole since 90 % of the cell volume is comprised of vacuole. In sugarcane

suspension cells the concentration of sucrose in the vacuole and cytosol is the same (Preisser *et al.* 1992). If this is also true in intact tissue, the fate of 90 % of the sucrose in the cell will be determined by conditions inside the vacuole.

The rate of sucrose movement across the tonoplast will also be influential. If there is a rapid cycling of vacuolar and cytosolic sucrose the total sucrose pool will be in contact with both vacuolar and cytosolic enzyme activities. The permeation rate for sucrose is 70 times faster in suspension cell vacuoles compared to vacuoles from internodal storage cells (Williams *et al.* 1990; Preisser and Komor 1991; Komor 2000).

In suspension cells, where the permeation rate for sucrose through the tonoplast is high (Preisser and Komor 1991), sucrose will be subjected to degradation by both vacuolar and cytosolic invertases and sucrose concentrations will remain low. In immature internodal storage tissue the permeation rate will be low (Williams *et al.* 1990), but vacuolar acid invertase activity will be high. In more mature internodal tissue however, where the permeation rate for sucrose through the tonoplast is low and vacuolar acid invertase activity has decreased, sucrose that succeeded in reaching the vacuolar compartment will stay there in safety. The low transfer rate through the tonoplast may, of course, increase the probability that sucrose will be hydrolysed by cytosolic invertase before it can cross into the vacuole. However, since the cytosol is also the place of sucrose synthesis and resynthesis, a steady level of sucrose will be maintained (Komor 2000).

Sugarcane storage tissue maintains a low gradient of solute concentrations between the apoplast and the cytosol (Hawker 1965; Glasziou and Gayler 1972b; Moore 1995). In mature tissue there would be an even distribution of sucrose in the apoplast, cytosol and vacuole.

2.9 SUCROSE METABOLISM IN SUGARCANE CULM

Plants are able to use many different sources of carbohydrate, including starch, stored sucrose, stored hexoses and incoming sucrose and hexose. During the growth phase of internodes 10 and older, sucrose accounts for 68 – 75 % of the soluble solids. This value can exceed 90 % at the end of the crop cycle (Moore 1995). Knowledge of where and how sucrose storage is regulated in the sugarcane plant is critical for

developing strategies for agronomic or genetic regulation of partitioning. However, data to characterise sucrose partitioning and accumulation are incomplete (Moore and Maretzki 1997).

2.9.1 Carbon partitioning

Plant metabolism relies heavily on sugars as metabolic substrates, storage compounds, signalling molecules, osmotic regulators and as substrates for the synthesis of structural polymers (Renz *et al.* 1993). The hexokinase enzyme family phosphorylates the hexoses that are formed through cleavage of sucrose, as well as the hexoses already present in the cell, creating a pool of hexose phosphate. A pool of UDPGlc is also formed. UDPGlc is generated during sucrose cleavage by SuSy and through conversion of glucose-1-phosphate by UDPGlc pyrophosphorylase. The hexose phosphates and UDPGlc form a central pool from which several metabolic pathways diverge.

The sugars may enter any of a variety of pathways and the requirement for phosphorylation of glucose and fructose varies, depending on which carbohydrate is being utilised and on how and why it is being mobilised. In higher plants, the pathways that consume the bulk of mobilised hexose phosphates are respiration and synthesis of storage and structural polysaccharides (ap Rees 1988). In sugarcane the accumulation of sucrose as storage molecule is the major drain on hexose phosphates. During the ripening phase approximately half of the imported carbon is diverted to stored sucrose (Bindon 2000; Komor 2000).

In plants glycolysis acts as the primary pathway that fuels respiration (Plaxton 1996). This pathway is, to a varying degree, present in all organisms. It not only generates ATP and reductant that is needed for metabolism, but also produce intermediates for biosynthetic processes. A significant proportion of the carbon that enters the plant glycolytic and tricarboxylic acid cycle pathways is not oxidised to CO_2, but is utilised in the biosynthesis of numerous compounds such as secondary metabolites, isoprenoids, amino acids, and fatty acids. The biosynthetic role of glycolysis and respiration is particularly important in actively growing autotrophic tissues (Plaxton 1996 and references therein).

Carbon partitioning studies found that sucrose accumulation in maturing culm tissue coincided with decreased partitioning of carbon to respiration, amino acids, water-

insoluble matter and phosphorylated intermediates (Whittaker and Botha 1997). It was assumed that the insoluble fraction consisted mainly of fibre, and that increased partitioning to sucrose could be the result of redirection of carbon away from fibre synthesis toward sucrose. Recently it was shown that incorporation into fibre was overestimated and that 16 % of incoming carbon was allocated to fibre synthesis (Bindon 2000). However, this was the only significant sink to undergo marked change with the transition from immature tissue, therefore it is possible that the increase in sucrose storage in maturing internodes involves redirection of incoming carbon from fibre synthesis.

Increased partitioning of carbon to sucrose was inversely related to the partitioning to amino acids (Wendler *et al.* 1990; Whittaker and Botha 1997). Bindon (2000) found that the reduced respiratory flux observed in mature tissue was due to a reduction in allocation to protein and amino acids. Therefore, although mature tissue is characterised by reduced carbon partitioning into protein, it can not be directly correlated to the regulation of sucrose accumulation. Young internodes are undergoing development and are characterised by high growth rates (Moore 1995; Lingle 1999). It could therefore be expected that they should display a higher rate of protein synthesis than more mature internodes would, which are fully elongated.

2.9.2 Mobilisation of hexoses – futile cycling

There is simultaneous synthesis and degradation of sucrose, irrespective of whether the sugarcane cells are mobilising their sucrose, actively growing, rapidly storing sucrose or maintaining a high stable sucrose content (Whittaker and Botha 1997). The rate of cycling depends strongly on the age of the internode, with highest cycling occurring in young internodes and cycling successively decreasing with ripening of the internodes (Vorster and Botha 1999; Komor 2000).

The rapid cycling of sugars in non-photosynthetic cells has been referred to as a "futile cycle" (Dancer *et al.* 1990a), because the simultaneous synthesis and degradation of sucrose appears to involve some wastage of energy. However, it is recognised that these cycles allow cells to respond in a highly sensitive manner to small changes in the balance between the supply of sucrose and the demand for carbon for respiration and biosynthesis (Wendler *et al.* 1990).

As a result of this cycle of synthesis and degradation, small changes in the metabolite pools and enzyme activities of either of these pathways would result in larger changes in the net rate of sucrose storage. Increased sucrose accumulation by sugarcane could thus be the result of an increase in sucrose synthesis, or decrease in sucrose degradation (Moore 1995; Whittaker and Botha 1997).

On the whole plant level, the ability to turn over sucrose between the various compartments would allow for the rapid removal of photoassimilates from the source to prevent a sink inhibition of source activity. Over the longer term, turnover would allow for the remobilization of stored sucrose as a food supply for stimulating rapid regrowth following stress (Moore 1995).

2.9.3 Decrease in respiration with maturity

Analysis of glycolytic and respiratory metabolites in internodes of different age revealed that no shift in metabolite concentrations or metabolite ratios was obvious during ripening, except a decline in hexoses (Whittaker and Botha 1997). The diversion of sugar consumption away from respiration towards sucrose storage was attributed to a restriction of "substrate availability". The same had been found for sugarcane suspension cells (Wendler *et al.* 1990; Komor 2000). This could indicate that the affinities of the two pathways differ, with the sucrose synthesis pathway having the highest affinity for hexose phosphates.

2.9.4 Enzymatic control

Sucrose accumulation in the sugarcane stem is correlated with the difference between the activities of soluble acid invertase and SPS (Zhu *et al.* 1997; Lingle 1999). This is the only significant correlation of sucrose accumulation rate and enzyme activity that has been found. Transformation of sugarcane suspension cells with soluble acid invertase-antisense increased the intracellular concentration of sucrose. Transformation of sugarcane suspension cells to increase neutral acid invertase levels decreased sucrose concentrations (Komor 2000).

The magnitude of metabolite flux through any metabolic pathway depends on the activities of the individual enzymes that are involved. Coarse control is achieved through varying the total population of enzyme molecules by altering the rates of enzyme biosynthesis or proteolysis. Fine control is concerned with modulating the activity of pre-existing enzymes to respond to momentary metabolic requirements and

adjusting the rate of metabolite flux through the various pathways accordingly (Plaxton 1996).

Fine control of plant glycolysis is primarily exerted by those enzymes that catalyse reactions involved in the conversion of hexose to hexose phosphates, F6P to fructose-1,6-bisphosphate, and phosphoenolpyruvate to pyruvate (Plaxton 1996). Relatively little is known about the existence, functions of, or genetic basis for tissue or developmental specific isoforms of plant glycolytic enzymes. Such tissue or development specific isozymes could contribute to cell specific metabolism in higher plants (Plaxton 1996). Organ and development specific changes in the proportion of several hexose kinase isozymes have been proposed to contribute to the regulation of hexose metabolism in the potato plant (Renz *et al.* 1993).

CHAPTER 3

PURIFICATION AND CHARACTERISATION OF FRUCTOKINASE FROM THE CULM OF SUGARCANE

(Submitted to Physiologia Plantarum)

ABSTRACT

Five hexose kinases, two fructokinases and three hexokinases, were identified in sugarcane culm. The two fructokinase isoforms, FRK1 and FRK2, were purified to homogeneity. Although both isoforms have pH optima of 8.0, the kinetic properties of the FRK isoforms differ largely. FRK2 is subject to substrate inhibition by fructose in excess of 0.1 mM. The isoform is not NTP specific and is not inhibited by fructose when assayed with UTP. In the presence of both ATP and UTP, the inhibition is restored. FRK1 is not fructose sensitive and shows ATP specificity. Both isoforms appear to be homodimers of 33 kDa subunits, with a native molecular weight of 69 kDa.

3.1 INTRODUCTION

The phosphorylation of free hexoses is not only the initial step of glycolysis, but is also required for the mobilisation of all hexoses taken up by the cell. Fructose phosphorylation by fructokinase (FRK, EC 2.7.1.4) is an irreversible and near rate-limiting reaction *in vivo* (Viola 1996), and could thus be an important site for the regulation of carbohydrate metabolism.

Sucrose can be converted to hexose sugars via two pathways. Invertase (EC 3.2.1.26) cleavage of sucrose generates glucose and fructose, and sucrose synthase (EC 2.4.1.13) generates UDP-glucose and fructose, consuming UDP. The produced hexoses are phosphorylated by two enzymes, hexokinase (HK, EC 2.7.1.1) and FRK, using a nucleotide triphosphate as the phosphoryl donor. HK preferentially phosphorylates glucose, whereas FRK specifically phosphorylates fructose.

Sugarcane stores carbohydrate principally as sucrose, and contains little or no starch. Both sucrose and hexose are taken up by the storage parenchyma cells (Komor 2000). However, during sucrose accumulation there is significant cycling of carbon between the hexoses and sucrose (Sacher *et al.* 1963; Batta and Singh 1986; Wendler *et al.* 1990; Whittaker and Botha 1997) with both sucrose synthase and invertase contributing to sucrose degradation. Despite the functioning of two breakdown pathways, the concentrations of the reducing sugars are low in mature tissue (Gayler and Glasziou 1972; Lingle and Smith 1991; Whittaker and Botha 1997; Rose and Botha 2000). Maintenance of low hexose concentrations would require high hexokinase and fructokinase activity. Despite their obvious importance, little is known about the hexose kinases of sugarcane.

Hexose kinases from potato (Gardner *et al.* 1992; Renz *et al.* 1993), tomato (Schaffer and Petreikov 1997a; Kanayama *et al.* 1998), spinach leaves (Schnarrenberger 1990), maize (Doehlert 1989) and barley leaves (Baysdorfer *et al.* 1989) have been characterised, and these studies indicate that plant tissues typically contain three to five hexose kinases. These differ in their physical and kinetic properties. It has been reported that some FRK isozymes may be inhibited by high concentrations of the substrate, fructose (Turner *et al.* 1977; Baysdorfer *et al.* 1989; Doehlert 1989; Gardner *et al.* 1992; Renz and Stitt 1993; Chaubron *et al.* 1995; Martinez-Barajas and Randall 1996; Shaffer and Petreikov 1997).

Here we report that at least five hexose phosphorylating enzymes are present in the sugarcane culm. In addition, the purification of the FRK isoforms is described and the contrasting kinetic properties of the FRK isozymes are presented.

3.2 MATERIAL AND METHODS

3.2.1 Chemicals

All chemicals, enzymes and substrates were supplied by either Sigma-Aldrich SA or Roche Diagnostics SA. Prepacked 5 ml HiTrap Q anion exchange columns, a Superose 6 FPLC gel filtration column and the PlusOne silver staining kit were obtained from Amersham Pharmacia Biotech (England). All other resins were from Sigma-Aldrich SA. The Mini Whole Gel Eluter was obtained from Bio-Rad Laboratories (California, USA).

3.2.2 Plant material

Mature, non-flowering, field-grown (Stellenbosch, South Africa) sugarcane plants (*Saccharum* spp. N19 var.) were sampled. Internode one is defined as the internode attached to the leaf with the first visible dewlap (Van Dillewijn 1952).

3.2.3 Enzyme extraction

The rind was removed from excised internodes and the tissue ground to a fine powder in liquid nitrogen. Protein was extracted at 4 °C using approximately 2 mL extraction buffer per gram tissue. The extraction buffer contained 100 mM HEPES-KOH (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT and 10 % (v/v) ethanediol (buffer A), to which 2 % (m/v) polyvinylpolypyrrolidone was added. The extract was filtered through muslin and centrifuged (20 000 *g*, 15 min) to remove coarse material. The pellet was discarded and the supernatant fractionated with $(NH_4)_2SO_4$. The fraction precipitating between 45 % and 70 % saturation was collected after centrifugation (20 000 *g*, 15 min) and resuspended in a small amount of 10 mM HEPES-KOH (pH 8.0), 1 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT and 10 % (v/v) ethanediol (buffer B). 2.5 mL aliquots of the extract was applied to a prepacked PD-10 desalting column equilibrated with buffer B and eluted with 3.5 mL of the same buffer. The volume of the eluted protein was adjusted to 30 mL with buffer B to ensure adequate dilution of salt before ion exchange chromatography.

3.2.4 Enzyme purification

The extract was applied to a DEAE-Sephacel anion exchange column (5.6 x 2.6 cm) equilibrated with buffer B at a flow rate of 1 mL min^{-1}. Unbound protein was eluted with the same buffer, followed by a linear gradient of 0 to 0.25 M KCl. Fractions of 2 mL were collected and assayed. All fractions showing FRK activity were pooled, saturated to 70 % $(NH_4)_2SO_4$ and then precipitated by centrifugation. The pellet was dissolved in approximately 1.5 mL and applied to a Sephacryl S-300-HR gel filtration column (109 x 1.5 cm) equilibrated with buffer A. Protein was eluted at a flow rate of 0.34 mL min^{-1} and fractions of 4 mL were collected and assayed. Fractions containing FRK activity were pooled and applied at a flow rate of 1 mL min^{-1} to a prepacked 5 mL HiTrap Q anion exchange column preequilibrated with buffer A. Unbound protein was eluted with the same buffer, followed by a linear gradient of 0 to 0.25 M KCl. Fractions of 1 mL were collected and assayed. FRK1 and FRK2 fractions were pooled separately and each then applied at a flow rate of 0.5 mL min^{-1} to an ATP-agarose affinity column equilibrated with buffer A. Unbound protein was eluted with the same buffer, followed

by a linear gradient of 0 to 1 mM ATP. Active fractions were pooled, rapidly frozen in 1 mL aliquots in liquid N_2 and stored at -80°C.

Throughout the purification procedure all steps were carried out at 4 °C and fractions stored on ice or at -80 °C.

3.2.5 Enzyme assays

Fructokinase activity was routinely measured in a final volume of 250 µL containing 50 mM HEPES-KOH (pH 8.0), 4mM $MgCl_2$, 2.5 mM ATP, 0.33 mM $NADP^+$, 0.1 mM fructose, 0.25 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 0.25 units of phosphoglucose isomerase (EC 5.3.1.9). To assay hexokinase, 2 mM glucose was used instead of fructose, and phosphoglucose isomerase was omitted. One unit of activity (U) is defined as 1 µmol product produced per minute.

3.2.6 Determination of pH optimum

Fructokinase activity was determined between pH 6.5 and 11.0. When assaying between pH 6.5 and 9.5, 50 mM Tris and 50 mM MES were added to the standard assay, and the pH adjusted to the appropriate value. A 200 mM carbonate-bicarbonate buffer was used when assaying between pH 9.0 and 11.0.

3.2.7 Electrophoresis

The purity of the purified proteins was analysed on a discontinuous 12 % (m/v) polyacrylamide gel (37.5 acrylamide : 1 bis-acrylamide) with a 4 % (m/v) stacking gel using one-dimensional SDS-PAGE by developing at 120 V as described by Laemmli (Laemmli 1970), followed by silver staining using the PlusOne silver staining kit (Amersham International).

3.2.8 Enzyme assay in gel fragments

Enzyme was loaded in duplicate on a 7 % (m/v) non-denaturing PAGE gel and the gel was developed. Both lanes were excised and cut into 5 mm segments. The segments of one lane were stored in 30 % (v/v) methanol at 4 °C and then incubated for 30 minutes in a denaturing loading buffer solution containing 62.5 mM Tris-HCl (pH 6.8), 10 % (v/v) glycerol, 2.3 % (m/v) SDS and 5 % (v/v) 2-mercapto-ethanol. The segments were inserted into the wells of a 12 % SDS-PAGE gel. The gel was developed and then silver stained.

The segments of the other lane were each immersed in 200 µL reaction mixture (50 mM HEPES-KOH pH 8.0, 4 mM $MgCl_2$, 2.5 mM ATP, 0.1 mM fructose) and incubated for 4 h at room temperature. The production of fructose-6-phosphate was measured by adding 25 µL of the reaction mixture to 225 µL of 50 mM HEPES-KOH pH 8.0, 4 mM $MgCl_2$, 0.33 mM $NADP^+$, 0.25 U glucose-6-phosphate dehydrogenase and 0.25 U phosphoglucose isomerase.

The protein profile obtained after silver staining was compared to the activity profile obtained after assaying.

3.2.9 Gel purification of fructokinase

To obtain pure FRK, FRK1 and FRK2 were electro-eluted from SDS-PAGE gels. Each polypeptide was applied to a 12 % (m/v) denaturing PAGE gel and the gels were developed. The gels were equilibrated in transfer buffer (25 mM Tris and 192 mM glycine) and eluted using the Mini Whole Gel Eluter according to the manufacturer's instructions. Elution was performed at 100 mA for 30 min followed by 10 seconds of reversed current to loosen protein sticking to the dialysis membrane at the bottom of the wells.

Equal volumes of the eluted fractions were applied to a 12 % (m/v) denaturing PAGE gel. The gel was developed and silver stained to identify the fractions containing the desired polypeptide.

3.2.10 Determination of native molecular weight

Native molecular weight was determined using a Superose 6 FPLC gel filtration column equilibrated with 100 mM HEPES-KOH (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT and 100 mM KCl. Native purified FRK1 and FRK2 were each applied to the column at a flow rate of 0.4 mL min^{-1} and elution was continuously monitored at 280 nm.

Ferritin (440 kDa), catalase (232 kDa), BSA (67 kDa), egg albumin (47 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (13.7 kDa) were used as molecular weight standards.

3.2.11 Calculation of kinetic constants

For the kinetic analysis, the concentration of fructose and NTP were varied and the data was analysed by linear and non-linear regression. Linear regression analysis was used in preliminary work to establish reaction kinetics (Cornish-Bowden 1981). This was followed by non-linear regression analysis using equation 1 when no inhibition was evident and equation 2 when inhibition was apparent.

$$v = V_{max} * [A] * [B] / (K_b * [A] + K_a * [B] + [A] * [B]) \quad (1)$$

$$v = V_{max} * [A] * [B] / (K_b * [A] + K_a * [B] * (1 + [B] / K_{bi}) + [A] * [B]) \quad (2)$$

where v is the experimentally determined initial velocity, V_{max} is the maximum velocity when the substrate concentration is optimal, K_a and K_b are the limiting Michaelis constants for the varied substrates A and B, respectively, and K_{bi} is the Michaelis constant for the inhibition by substrate B.

3.3 RESULTS

3.3.1 Separation of three hexokinases and two fructokinases from sugarcane culm

Five different activity peaks were identified following extraction and ion exchange chromatography (Fig. 3.1). FRK activity peaks were named according to the nomenclature proposed by Pego and Smeekens (2000). Two peaks, termed FRK2 and FRK1, were eluted at 120 and 190 mM KCl, respectively. Together they accounted for more than 95 % of the fructokinase activity while showing very little activity with glucose, and as a result they were classified as fructokinases. Three more peaks of activity were found, and since glucose appeared to be the preferred substrate they were named HK1, HK2 and HK3. These eluted at 200, 220 and 300 mM KCl, respectively.

The possibility that the peak termed HK1 was merely a result of glucose phosphorylating activity of FRK1 was addressed after purification of FRK1.

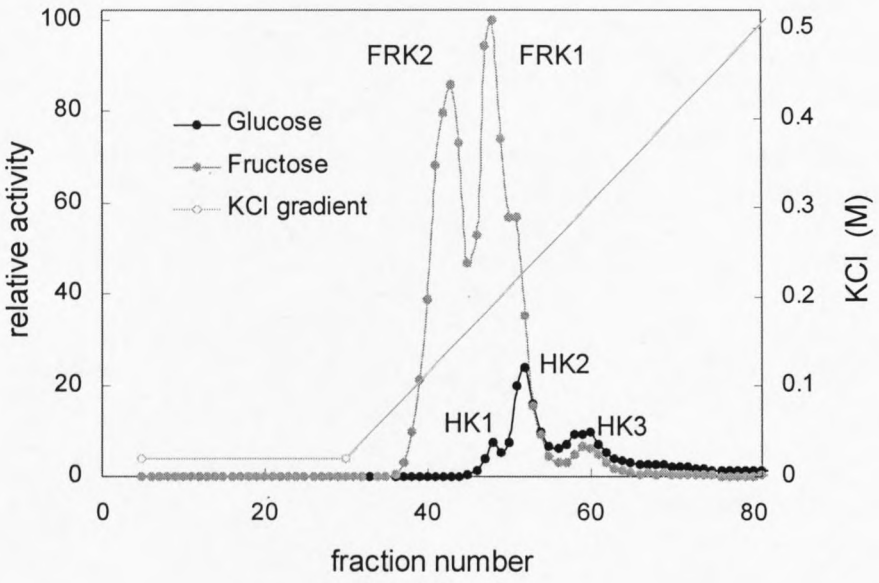


Figure 3.1 Elution profile of HK and FRK activities (relative to maximum activity) from a HiTrap Q ion exchange column (assayed with 2 mM glucose and 0.1 mM fructose). The two FRK peaks were named FRK1 and FRK2 according to the system proposed by Pego and Smeekens (2000), and the HK peaks were named HK1, HK2 and HK3.

3.3.2 Purification of fructokinase isozymes

FRK1 was purified to a specific activity of 44 U mg^{-1} protein (Table 3.1) according to the protocol described earlier and FRK2 to 18.8 U mg^{-1} protein (Table 3.2). Recovery of active enzyme was low (4 % for FRK1 and 0.4 % for FRK2) and could not be improved by the addition of fructose to the buffer or by adding protease inhibitors to the extraction buffer.

The purified FRK1 was assayed with fructose and with glucose. When the assays were done on the semi-purified preparation (Fig. 3.1) FRK1's fructose phosphorylation activity was 10-fold higher than its glucose phosphorylation activity. However, purified FRK1 exhibits 100-fold higher hexose phosphorylation activity with fructose than with glucose. We therefore conclude that FRK1 and HK1 are two distinct enzymes that are separated from each other during subsequent purification steps and that the FRK1 preparation assayed following anion exchange chromatography (Fig. 3.1) consisted of a mixture of FRK1 and HK1.

Table 3.1 Purification of FRK1. Activity was measured in an assay containing 1.0 mM fructose.

Fraction	Protein (mg)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification (-fold)
crude	57.12	0.123	100	1
DEAE-Sephacel ion exchange	18.61	0.177	72.52	2.43
gel filtration	4.57	0.311	14.72	6.17
FRK1 from HiTrap Q ion exchange	2.12	0.395	8.65	7.82
FRK1 from ATP-agarose	0.01	44.054	4.17	872.80

Table 3.2 Purification of FRK2. Activity was measured in an assay containing 0.1 mM fructose.

Fraction	Protein (mg)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification (-fold)
crude	57.12	0.128	100	1
DEAE-Sephacel ion exchange	18.61	0.147	37.22	1.14
gel filtration	4.57	0.199	12.45	1.56
FRK2 from HiTrap Q ion exchange	0.97	0.084	1.11	0.65
FRK2 from ATP-agarose	0.0015	18.832	0.39	146.77

3.3.3 SDS-PAGE analysis of purity of protein

A single polypeptide with a molecular mass of approximately 33 kDa was observed for FRK2, indicating that the enzyme was purified to homogeneity (Fig. 3.2). Three polypeptides were present in the FRK1 sample. To determine which one was FRK1 the activity of each was measured following non-denaturing PAGE. The 33 kDa polypeptide was the only one corresponding to fructokinase activity (Fig. 3.3).

3.3.4 Native size determination

Linear regression analysis of the elution pattern of the molecular weight standards was done. Fructokinase activity eluted as a single peak from the Sepharose column and the calculated molecular weight of both isoforms was 69 kDa. Since both FRK2 and FRK1 have a subunit of 33 kDa, the enzymes are homodimers in their active form.

3.3.5 pH optimum

A pH activity profile was obtained for each FRK isoform (Fig. 3.4). Both FRK1 and FRK2 showed a broad pH optimum with maximum activity between pH 7.5 and 9.0. In both cases 40 % of activity is lost at pH 6.0 and 10.0.

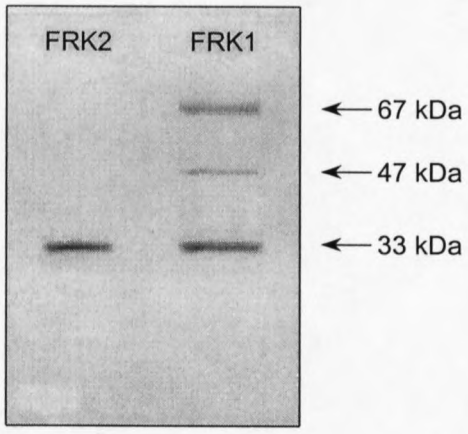


Figure 3.2 SDS-PAGE analysis of protein purity, visualised by silver staining. A single polypeptide was visualised for the FRK2 preparation. Three polypeptides were present in the FRK1 preparation.

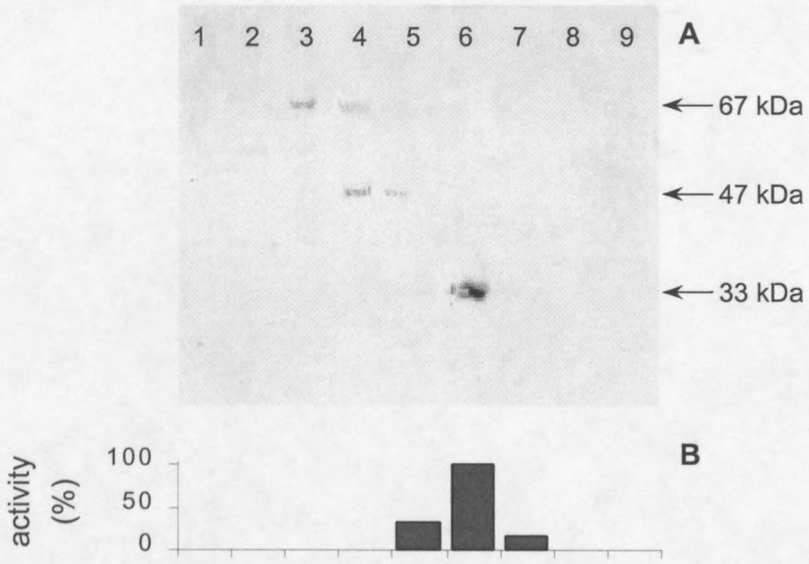


Figure 3.3 PAGE, silver stain and activity measurement of purified FRK1. Lanes 1, 2, 7, 8 and 9 contains gel pieces where no proteins were visible (see Fig. 3.2). Panel B indicates the fructose phosphorylating activity in each gel segment.

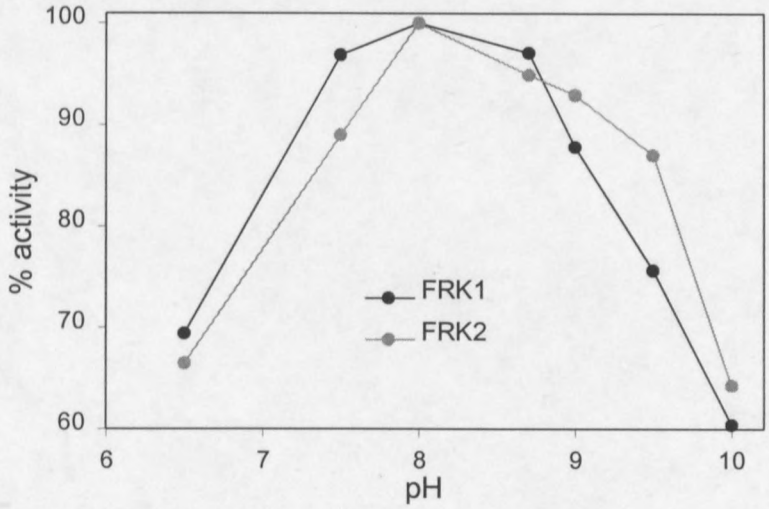


Figure 3.4 pH optimum of FRK1 and FRK2 (relative to maximum activity), assayed with 0.1 mM fructose.

3.3.6 Kinetic properties of the fructokinases

FRK1 and FRK2 were assayed at 2.5 mM ATP and varying fructose concentrations. FRK1 exhibit normal hyperbolic kinetics (Fig. 3.5) and reached saturation at fructose concentrations exceeding 0.2 mM. No inhibition was observed when the fructose concentration was increased to 1 mM (data not shown). FRK2 reached fructose saturation at 0.1 mM fructose and was inhibited by higher concentrations of substrate. FRK2 activity was inhibited more than 50 % by 0.4 mM fructose.

FRK1 and FRK2 were then assayed at 0.2 and 0.08 mM fructose respectively, and varying ATP concentrations. Both enzymes exhibit normal hyperbolic kinetics (Fig. 3.6) and reached maximum activity at ATP concentrations exceeding 2.5 mM.

It is apparent from the data that FRK1 is not inhibited by any one of its substrates (Fig. 3.7 A and B). In contrast FRK2 is strongly inhibited by its substrate fructose (Fig. 3.7 C and D). This was confirmed by non-linear regression analysis when a significant Ki value was obtained for FRK2 (Table 3.3) and no significant value for FRK1.

Apparent K_m values for fructose were 0.028 mM for FRK1 and 0.074 mM for FRK2 (Table 3.3). K_m values for ATP were 0.140 mM for FRK1 and 0.180 mM for FRK2, and the K_i value for substrate inhibition of FRK2 by fructose was 0.016 mM.

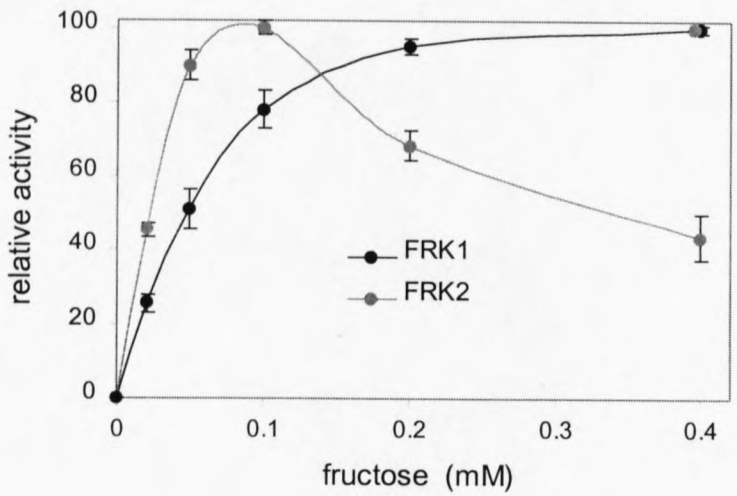


Figure 3.5 Fructose dependence of FRK1 and FRK2 (relative to maximum activity). All assays were done with 2.5 mM ATP. Error bars indicate standard error of three repetitions.

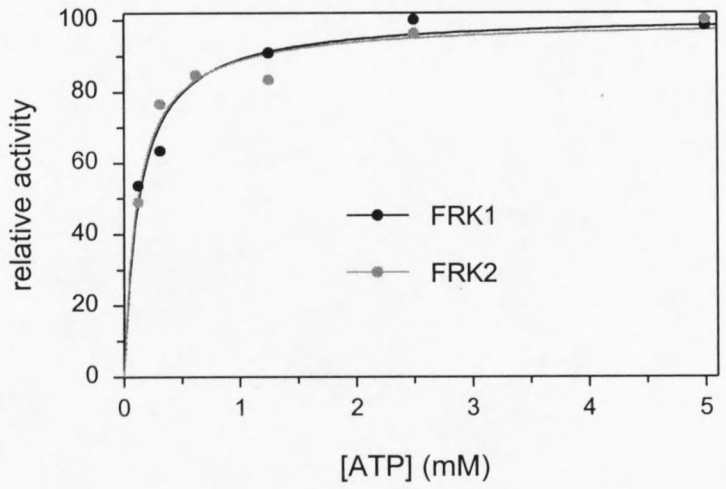


Figure 3.6 ATP dependence of FRK1 and FRK2 (relative to maximum activity). FRK1 was assayed with 0.2 mM fructose and FRK2 with 0.08 mM fructose.

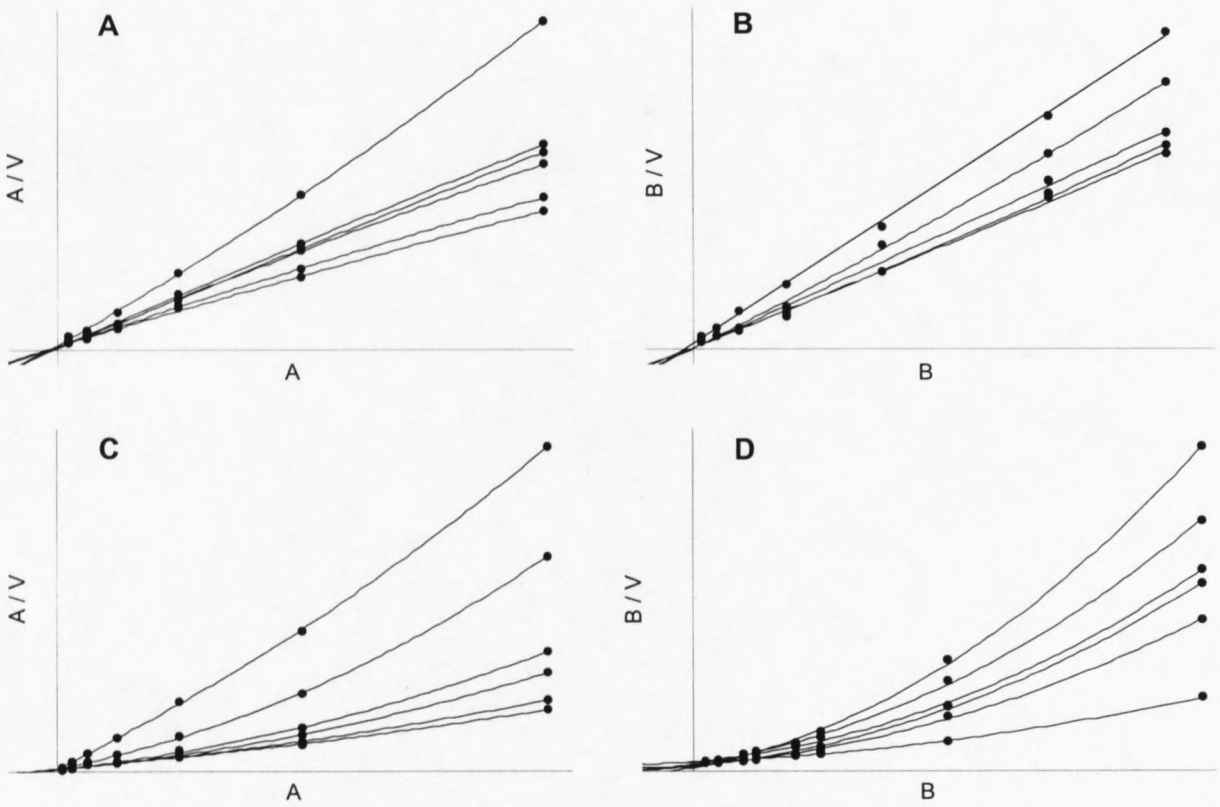


Figure 3.7 Regression analysis to determine reaction kinetics. The substrate concentration to reaction velocity ratio was plotted against substrate concentration. Substrate A is ATP and substrate B fructose. (A) and (B) are FRK1. (C) and (D) are FRK2.

Table 3.3 Kinetic constants of FRK1 and FRK2.

	V_{max} (U mg^{-1} protein)	K_{mATP} (mM)	K_{mFru} (mM)	K_{iFru} (mM)
FRK1	47.36 ± 0.34	0.140 ± 0.014	0.028 ± 0.003	*
FRK2	16.92 ± 0.19	0.180 ± 0.042	0.074 ± 0.011	0.0164 ± 0.008

± Standard error of three repetitions

* No significant inhibition constant (P = 0.05)

Nucleotide triphosphates other than ATP were also evaluated. The V_{max} of FRK1 was up to 80 % lower with CTP, GTP and UTP than with ATP (Table 3.4). FRK2 does not show preference for ATP. When assayed with CTP and UTP more than 80 % of the activity observed with ATP was found. When FRK2 was assayed with GTP, higher activity than with ATP was observed. The inhibitory effect of increasing fructose concentrations on FRK2 activity was not observed when UTP was used as substrate (Table 3.5).

When FRK2 was assayed with only ATP as phosphoryl donor the expected inhibition by fructose was observed (Table 3.6). Adding UTP to the assay did not reverse the inhibitory effect. When the assay was done with UTP as substrate, no inhibition by increasing fructose concentrations was found. However, when ATP was added to the assay the inhibition again became apparent. The inhibition occurred when as little as 0.5 mM ATP was added.

Table 3.4 Influence of NTP on activity of FRK1 and FRK2.

	FRK1 assayed with 1.0 mM fructose (%)	FRK2 assayed with 0.1 mM fructose (%)
ATP	100	100
GTP	23 ± 0.8	135 ± 2.6
CTP	35 ± 1.1	83 ± 2.1
UTP	39 ± 0.9	84 ± 1.7

± Standard error of three repetitions

Table 3.5 Effect of NTP on the kinetic properties of FRK2.

	K_{mNTP} in presence of		Effect of increasing fructose concentration		
	0.1 mM Fru	0.4 mM Fru	$V_{0.1\ mM\ fructose}$	$V_{0.4\ mM\ fructose}$	% inhibition
ATP	0.19 ± 0.06	1.74 ± 0.47	3.58	1.02	71
GTP	0.35 ± 0.06	0.81 ± 0.16	4.85	2.30	53
CTP	0.68 ± 0.04	1.01 ± 0.42	2.74	2.01	27
UTP	1 ± 0.05	0.95 ± 0.06	2.97	2.92	0

± Standard error of three repetitions

Table 3.6 Effect of NTP on fructose inhibition of FRK2.

[fructose]	Activity in the presence of 2.5 mM ATP and		
	0 mM UTP	1.25 mM UTP	2.5 mM UTP
0.1 mM	1	1.06	1.29
1.0 mM	0.121	0.171	0.216

[fructose]	Activity in the presence of 2.5 mM UTP and		
	0 mM ATP	1.25 mM ATP	2.5 mM ATP
0.1 mM	1	1.08	1.12
1.0 mM	1	0.203	0.248

3.4 DISCUSSION

It is evident that at least five forms of hexose phosphorylating enzymes are present in the sugarcane culm. Two of these preferentially utilised fructose and are therefore classified as fructokinases (EC 2.7.1.4). The remaining three forms are classical hexokinases (EC 2.7.1.1) based on their ability to phosphorylate both fructose and glucose. Despite the similarity in their molecular weights the proteins could be

separated from each other on ion exchange chromatography. This indicates major differences in their charges, possibly as a result of substantial differences in their amino acid compositions.

Pego and Smeekens (2000) proposed a new naming system for the fructokinases based on the assumption that all FRK activities studied in higher plants to date could be a attributed to two different enzymes, FRK1 and FRK2. FRK2 is usually sensitive to substrate inhibition by fructose and FRK1 not. One of the FRK isoforms in the sugarcane culm is inhibited by fructose, the other not. We therefore conclude that there is one FRK1 and one FRK2 expressed in the culm of sugarcane.

Both sugarcane fructokinases appear to be homodimers of 69 kDa, consisting of subunits of approximately 33 kDa. This is consistent with findings in other plant species (Gardner *et al.* 1992; Chaubron *et al.* 1995; Martinez-Barajas and Randall 1996; Kanayama *et al.* 1998). However, it differs largely from the 102 - 118 kDa active proteins reported for potato (Renz *et al.* 1993).

Taylor *et al.* (1995) found one FRK encoding gene in potato, while two divergent genes were identified in tomato (Kanayama *et al.* 1997; Martinez-Barajas *et al.* 1997). *Frk1* codes for a protein with calculated molecular weight of 37 kDa and *Frk2* for 34.8 kDa. This is close to the size of the subunits of FRK1 and FRK2 found here.

Both FRK isoforms have a broad pH optimum (both approximately pH 8.0) consistent with values previously observed for plant fructokinases (Chaubron *et al.* 1995; Martinez-Barajas and Randall 1996).

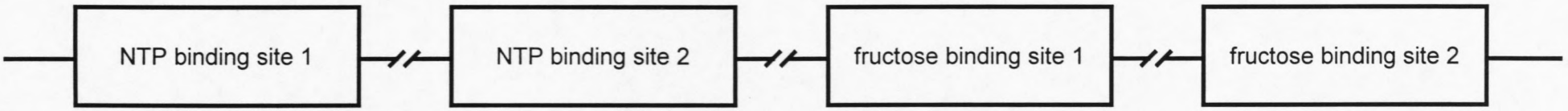
The K_{mFru} values of both fructokinase isoforms indicate a very high affinity for fructose (0.074 mM for FRK2 and 0.045 mM for FRK1). FRK activity accounts for over 80 % of the total hexose phosphorylation activity in young sugarcane tissue (Fig. 3.1) and maximum activity of the FRK isoforms is several times higher than that of the HK with highest maximum activity. This could emphasise the importance of the operation of two pathways that produce fructose from sucrose (SuSy and invertase). An alternative explanation is that SuSy is the main sucrose degrading enzyme, producing fructose and UDP-glucose (Kruger 1997; Schaffer and Petreikov 1997). This would mean that only the glucose transported into the cell would need to be phosphorylated, while there is a continuous intracellular production of fructose by SuSy.

The occurrence of multiple fructokinase isoforms and substrate inhibition of at least one fructokinase isoform by fructose has been extensively documented in a variety of tissues including barley leaves (Baysdorfer *et al.* 1989), spinach leaves (Schnarrenberger 1990), sugar beet (Chaubron *et al.* 1995), pea seeds (Turner *et al.* 1977), tomato (Martinez-Barajas and Randall 1996, Schaffer and Petreikov 1997), maize kernels (Doehlert 1989) and potato (Gardner *et al.* 1992; Renz *et al.* 1993; Renz and Stitt 1993). However, the concentration of fructose that is required to induce inhibition is usually in the 0.5 mM to 2 mM range. This is substantially higher than the 0.1 mM that we report here.

In the sugarcane culm the fructose concentrations greatly exceed what is required to saturate FRK2. Even if it were assumed that only 10 % of the reducing sugars are in the cytosol it would still imply fructose concentrations of between 7 mM and 27 mM (Whittaker and Botha 1997) that would strongly inhibit FRK2.

Many authors have noted that total FRK and SuSy showed similar developmental patterns (Ross *et al.* 1994; Botha *et al.* 1996; Schaffer and Petreikov 1997). Like FRK2, SuSy is inhibited by high fructose (Doehlert 1987; Schaffer and Petreikov 1997). Under conditions of high fructose production FRK would need to phosphorylate fructose efficiently to prevent build-up of fructose and subsequent inhibition of both FRK2 and SuSy. Sucrose use could thus be controlled through high fructose concentrations by a "double brake" mechanism (Pego and Smeekens 2000). However, only FRK2 is inhibited by high fructose concentrations. FRK1 is not affected and on its own accounts for most of the fructose phosphorylating activity required (Chapter 4; Whittaker and Botha 1999).

Following the study by Huber and Akazawa (1986) showing FRK activity with UTP, the NTP specificity of FRK has been the subject of many studies. Some isoforms showed specificity for ATP (Baysdorfer *et al.* 1989; Doehlert 1989; Schnarrenberger 1990; Gardner *et al.* 1992), some not (Schnarrenberger 1990; Gardner *et al.* 1992; Chaubron *et al.* 1995; Martinez-Barajas and Randall 1996). In the cases where ATP specificity was absent, the K_{mATP} values usually were at least 5 times lower than the K_{mUTP} values (Huber and Akazawa 1986; Copeland and Tanner 1988; Gardner *et al.* 1992; Renz and Stitt 1993; Chaubron *et al.* 1995; Martinez-Barajas and Randall 1996) and the authors concluded that activity with UTP would not be significant *in vivo*.



- binds ATP
- no catalytic activity
- functional in FRK2, inactivated in FRK1

- binds ATP, UTP, GTP and CTP
- affinity for ATP is highest at low fructose concentrations; affinity for UTP is not affected by fructose
- catalytically active
- present and functional in FRK1 and FRK2

- specific for fructose
- catalytic site

- specific for fructose
- allosteric regulatory site; binding only possible after binding of ATP to NTP binding site 1

Figure 3.8 Schematic model of NTP and fructose binding sites on FRK protein.

Sugarcane FRK1 is highly specific for ATP. FRK2 does not show NTP specificity but K_{mATP} is lower than K_{mUTP}. However, when FRK2 is assayed with UTP instead of ATP the substrate inhibition by fructose is not observed. To our knowledge it is the first time that an NTP-related effect on substrate inhibition of FRK by fructose has been shown in plants. When ATP is added to this assay the inhibitory effect returns. This leads to the conclusion that more than one NTP binding site exist.

We propose the existence of four binding sites on the FRK protein (Fig. 3.8). Two of these are NTP-binding sites. One is the catalytic site and the other one is a regulatory site affecting substrate inhibition by fructose. The inhibitory effect of high fructose would be the result of ATP binding to the regulatory site. This site is very specific for ATP and has a higher affinity for ATP than UTP, since the inhibition is seen at low ATP concentrations and is not reversed by increasing the UTP concentration. The NTP binding site that catalyses phosphorylation is not ATP specific since any of the tested NTPs can be used as phosphoryl donor. This site does not have regulatory properties. The two remaining sites bind fructose. One is a catalytic site and the other an allosteric regulatory site. Binding of fructose in the allosteric site is only possible if ATP is present in the NTP-binding regulatory site. Such a mechanism could serve as a regulator of metabolism, acting in response to the energy status of the cell. If ATP levels were high, fructose phosphorylation would be slowed down.

Alignment of the deduced amino acid sequences of FRK1 and FRK2 from a variety of plants (Kanayama *et al.* 1997; Pego and Smeekens 2000) revealed several areas that are conserved. A fructose substrate recognition site and two ATP-binding sites were proposed. One of the ATP-binding sites was perfectly preserved between the FRK2 proteins of the four plants that were investigated: *Arabidopsis*, tomato, sugarbeet and potato. The presence of two sites implicated in ATP binding strengthens our hypothesis.

Preliminary results indicate that FRK2 is primarily expressed in young tissue where tight control of metabolism is necessary. This aspect is reported on in chapter 4.

CHAPTER 4

EXPRESSION OF FRUCTOKINASE IN SUGARCANE CULM

(Submitted to Australian Journal of Plant Physiology)

ABSTRACT

Two isoforms of fructokinase, FRK1 and FRK2, are present in sugarcane internodal tissue. FRK2 is inhibited by fructose concentrations exceeding 0.1 mM. FRK1 activity is not negatively affected even at 1.0 mM fructose. The ratio of FRK2 to FRK1 activity is dependent on the developmental stage (ripening) of the tissue. FRK1 appears to be the isoform that is preferentially expressed in mature tissue. Total fructokinase activity decreases during tissue maturation. This is the result of changes in expression (concentration) of the isoforms and not inactivation of existing protein. A mathematical method for determining the activities of the two isoforms of fructokinase in crude extracts is presented.

4.1 INTRODUCTION

Although it could be argued that uptake of sugars into the cell is the initial step in its utilisation, the phosphorylation of hexoses is the first dedicated reaction towards glycolysis (Cárdenas *et al.* 1998; Schnarrenberger 1990). In plants two enzyme groups, the hexokinases (HK, EC 2.7.1.1) and the fructokinases (FRK, EC 2.7.1.4) can facilitate this irreversible reaction using a nucleotide triphosphate as a phosphoryl donor. HK can phosphorylate both glucose and fructose, whereas FRK specifically phosphorylates fructose (Doehlert 1989; Kanayama *et al.* 1998; Pego and Smeekens 2000).

In sugarcane both sucrose, the primary storage product of sugarcane (Kruger 1997), and its hexoses are taken up by the storage parenchyma cells (Komor 2000). During sucrose accumulation there is significant cycling of carbon between the hexoses and sucrose (Sacher *et al.* 1963; Batta and Singh 1986; Wendler *et al.* 1990; Whittaker and

Botha 1997). Thus the intracellular hexose pool consists of a mixture of imported hexoses and the products of sucrose breakdown.

In the cell, sucrose is degraded to hexoses for mobilisation via two pathways: the invertase (EC 3.2.1.26) pathway produces fructose and glucose, while the sucrose synthase (EC 2.4.1.13) pathway produces fructose and UDP-glucose. Both invertase and sucrose synthase contributes to sucrose degradation in this cycle and both produce fructose. The maintenance of the low fructose concentrations that is found in mature tissue (Gayler and Glasziou 1972; Lingle and Smith 1991) would therefore require high fructose phosphorylating activity.

FRK has been studied in various plant tissues (Copeland and Tanner 1988; Baysdorfer *et al.* 1989; Doehlert 1989; Schnarrenberger 1990; Gardner *et al.* 1992; Chaubron *et al.* 1995; Martinez-Barajas and Randall 1996; Renz and Stitt 1993; Guglielminetti *et al.* 2000). However, only a few of these studies investigated the influence of tissue maturation on the presence and distribution of FRK isoforms (Renz *et al.* 1993; Ross *et al.* 1994; Chaubron *et al.* 1995; Martinez-Barajas and Randall 1996).

Multiple isoforms of FRK were found in several plant tissues (Turner *et al.* 1977; Baysdorfer *et al.* 1989; Schnarrenberger 1990) including sugarcane (chapter 3). The substrate kinetics of the isoforms differ largely (Pego and Smeekens 2000) making it necessary to separate the isoforms to allow accurate measurement of total FRK activity. However, previous measurements of total activity in crude extracts did not take into account these differences when assaying. Thus, the FRK activities measured in sugarcane crude extracts (Botha *et al.* 1996) and sugarcane cell suspension cultures (Wendler *et al.* 1990; Goldner *et al.* 1991) would not have been a true reflection of enzyme activity. The same is true for measurements of FRK in crude extracts of other plants (Marrè *et al.* 1968; Doehlert *et al.* 1988; Kuo *et al.* 1990; Nakamura *et al.* 1992; Ross *et al.* 1994), should there be kinetic differences between isoforms.

In this study we investigated the relationship between tissue maturity (representing various developmental stages) and expression of the different FRK isoforms. We also address the relationship between FRK activity and FRK expression, and propose a mathematical method for approximating the activities of FRK isoforms from crude extracts of sugarcane.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

All chemicals, enzymes and substrates were supplied by either Sigma-Aldrich SA or Roche Biochemicals SA. Prepacked 5 mL HiTrap Q anion exchange columns, PlusOne silver staining kit and Hybond-C Extra nitrocellulose membranes were obtained from Amersham Pharmacia Biotech (England). All other resins were from Sigma-Aldrich SA. The Mini Whole Gel Eluter and the Trans-Blot SD system were supplied by Bio-Rad Laboratories (California, USA).

4.2.2 Plant material

Mature, non-flowering, field-grown (Stellenbosch, South Africa) sugarcane plants (*Saccharum* spp. N19 variety) were sampled. Internode one is defined as the internode attached to the leaf with the first visible dewlap (Van Dillewijn 1952).

4.2.3 Enzyme extraction

The rind was removed from excised internodes and the tissue ground to a fine powder in liquid nitrogen. Protein was extracted at 4 °C using approximately 2 mL extraction buffer per gram tissue. The extraction buffer contained 100 mM HEPES-KOH (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT and 10 % (v/v) ethanediol (buffer A), to which 2 % (m/v) polyvinylpolypyrrolidone was added. The extract was filtered through muslin and centrifuged (20 000 *g*, 15 min) to remove coarse material. The pellet was discarded and the supernatant fractionated with $(NH_4)_2SO_4$. The fraction precipitating between 45 % and 70 % saturation was collected after centrifugation (20 000 *g*, 15 min) and resuspended in 2.5 mL of buffer A1 (10 mM HEPES-KOH (pH 8.0), 1 mM $MgCl_2$, 2 mM EDTA, 10 mM DTT and 10 % (v/v) ethanediol). The extract was applied to a prepacked PD-10 desalting column equilibrated with buffer A1 and eluted with 3.5 mL of the same buffer.

4.2.4 Enzyme assays

Fructokinase activity was routinely measured in an assay (total volume 250 µL) containing 50 mM HEPES-KOH (pH 8.0), 4 mM $MgCl_2$, 2.5 mM ATP, 0.33 mM $NADP^+$, 0.25 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 0.25 units of phosphoglucose isomerase (EC 5.3.1.9) and 0.1, 0.2 or 1.0 mM fructose. Activities were derived from the increase in A_{340} as $NADP^+$ was reduced. One unit of activity (U) is defined as 1 µmol product produced per minute.

4.2.5 Enzyme purification

The extract was applied to a DEAE-Sephacel anion exchange column (5.6 x 2.6 cm) equilibrated with buffer A1 at a flow rate of 1 mL min^{-1}. Unbound protein was eluted with the same buffer, followed by a linear gradient of 0 to 0.25 M KCl. Fractions of 2 mL were collected and assayed.

All fractions containing FRK activity were pooled, saturated to 70 % $(NH_4)_2SO_4$ and then precipitated by centrifugation. The pellet was dissolved in approximately 1.5 mL and applied to a Sephacryl S-300-HR gel filtration column (109 x 1.5 cm) equilibrated with buffer A. Protein was eluted at a flow rate of 0.34 mL min^{-1} and fractions of 4 mL were collected and assayed. Fractions containing FRK activity were pooled and applied at a flow rate of 1 mL min^{-1} to a prepacked 5 mL HiTrap Q anion exchange column equilibrated with buffer A. Unbound protein was eluted with the same buffer, followed by a linear gradient of 0 to 0.25 M KCl. Fractions of 1 mL were collected and assayed. FRK1 and FRK2 fractions were pooled separately and each then applied at a flow rate of 0.5 mL min^{-1} to an ATP-agarose affinity column equilibrated with buffer A. Unbound protein was eluted with the same buffer, followed by a linear gradient of 0 to 1 mM ATP. Active fractions were pooled, rapidly frozen in 1 mL aliquots in liquid N_2 and stored at -80 ºC.

Throughout the purification procedure all steps were carried out at 4 °C and fractions stored on ice or at -80 °C.

4.2.6 Sugar determination

Fructose, glucose and sucrose concentrations were determined according to the method described by Rose and Botha (2000).

4.2.7 Electrophoresis

The purity of the purified proteins was analysed on a discontinuous 12 % (m/v) polyacrylamide gel (37.5 acrylamide : 1 bis-acrylamide) with a 4 % (m/v) stacking gel using one-dimensional SDS-PAGE by developing at 120 V as described by Laemmli (Laemmli 1970), followed by silver staining using the PlusOne silver staining kit.

4.2.8 Gel purification of fructokinase

To obtain pure FRK, FRK1 and FRK2 were electro-eluted from SDS-PAGE gels. Each polypeptide was applied to a 12 % (m/v) denaturing PAGE gel and the gels were

developed. The gels were equilibrated in transfer buffer (25 mM Tris and 192 mM glycine) and eluted using the Mini Whole Gel Eluter according to the manufacturer's instructions. Elution was performed at 100 mA for 30 min followed by 10 seconds of reversed current to loosen protein sticking to the dialysis membrane at the bottom of the wells.

Equal volumes of the eluted fractions were applied to a 12 % (m/v) denaturing PAGE gel. The gel was developed and silver stained to identify the fractions containing the desired polypeptide.

4.2.9 Polyclonal antiserum

Rabbits were immunised against FRK1 and FRK2 by injecting eight fractions of 100 µg purified protein according to the method described by Bellstedt (1988).

4.2.10 Immunoblots

The separated polypeptides were transferred to nitrocellulose membranes using the Bio-Rad Trans-Blot SD system at 12 V for 1 h according to the manufacturer's instructions. The transfer buffer contained 48 mM Tris, 39 mM glycine, 0.0375 % (m/v) SDS and 20 % (v/v) methanol. Non-specific binding sites were blocked in 5 % (m/v) fat free milk powder in TBST (20 mM Tris-HCl pH 7.6, 137 mM NaCl and 0.1 % (v/v) Tween 20) for 2 h. Primary antibody was added to the blocking buffer to a final dilution of 1 : 1000 and incubated overnight. The membranes were washed three times for 20 min in TBST before incubation in the secondary antibody (alkaline phosphatase conjugated goat anti-rabbit IgG) diluted 1 : 2500 in TBST containing 3 % (m/v) fat free milk powder for 1 h. The blots were then washed for 10 min in TBST, rinsed in TBST containing 0.1 % (m/v) SDS and washed twice for 10 minutes in TBST. Cross-reacting polypeptides were detected by the enzymatic cleavage of the phosphate group of 5-bromo-4-chloro-3-indolyl-phosphate using nitroblue tetrazolium as a stain enhancer (Blake *et al.* 1984).

4.3 RESULTS

4.3.1 Isoforms of fructokinase exist in sugarcane

Two distinct peaks of fructokinase activity were evident following the second ion

exchange chromatography step and named FRK1 and FRK2 according to the system proposed by Pego and Smeekens (2000) (Fig. 4.1).

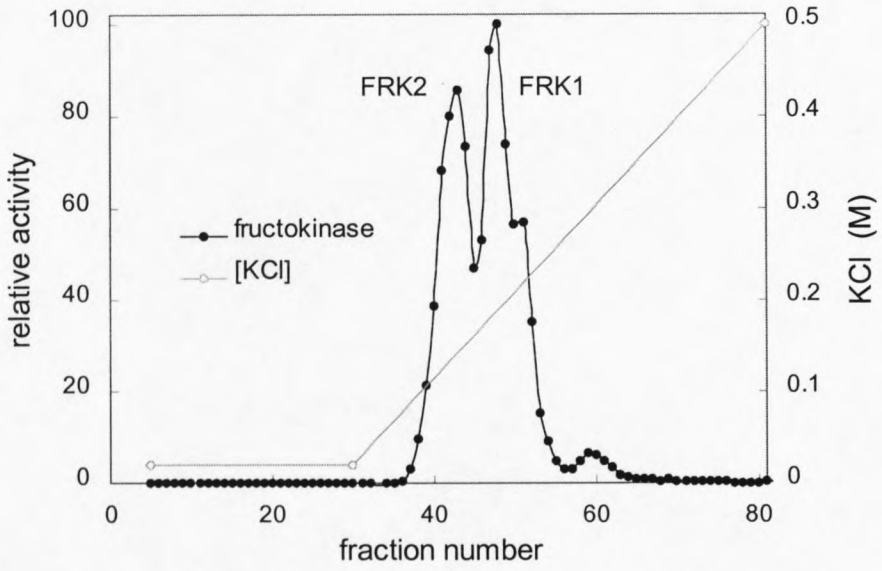


Figure 4.1 Elution profile of fructokinase isoforms from a DEAE-Sephacel column. Fructokinase activity was assayed with 0.1 mM fructose.

4.3.2 Influence of fructose on fructokinase activity

The substrate kinetics of FRK1 and FRK2 differ largely. FRK1 activity was saturated at 0.2 mM fructose and was not inhibited by 1.0 mM fructose (Table 4.1). In contrast, FRK2 reached maximum activity at 0.1 mM fructose, but was inhibited by 83 % when assayed with 1.0 mM fructose. Consequently it is not possible to accurately measure total fructokinase activity in crude extracts of sugarcane. We have therefore adopted the approach to measure at 0.1 mM fructose (100 % of FRK2 and 85 % of FRK1) and at 1.0 mM fructose (17 % of FRK2 and 100 % of FRK1).

4.3.3 Total FRK activity decrease during maturation

Total fructose phosphorylation activity decreased during tissue maturation from 41 mU mg^{-1} protein to 8 mU mg^{-1} protein when assayed with 0.1 mM fructose (Fig. 4.2). When assayed with 1.0 mM fructose the activity increased from internode 3 to internode 4, followed by a decrease in activity similar to that seen with 0.1 mM fructose.

The profiles of FRK activity (Fig. 4.2) show similarity to the developmental profile of fructose concentration (Fig. 4.3). Fructose concentration is at its highest in immature tissue, followed by a decrease down the sugarcane stem. Total FRK activity therefore seems to decrease as the fructose concentration decreases.

Table 4.1 The effect of fructose concentration on the activity of FRK.

Enzyme	Relative activity (%)		
	0.1 mM Fru	0.2 mM Fru	1 mM Fru
FRK1	85.1 ± 2.7	100	99.4 ± 1.2
FRK2	100	70.5 ± 1.5	17 ± 0.3

± Standard error of three repetitions

4.3.4 Developmental change in distribution of fructokinase isoforms

As sugarcane tissue matures, FRK1 appears to become the dominant fructokinase isoform. When assayed in the presence of 0.1 mM fructose the FRK2 to FRK1 ratio decreases from approximately 1:1 in the immature, actively growing tissue of internode 3, to approximately 1:2 in more mature, less metabolically active tissue such as internode 9 (Fig. 4.4).

In Fig. 4.2 a significant difference in the ratio of activity with 0.1 mM fructose to activity with 1.0 mM fructose can be seen as the tissue matures. This indicates a change in the relative activity of each isoform. The activity of each isoform was calculated from the data in Fig. 4.2 using equations 1 and 2.

$$A = FRK2 + 0.85\ FRK1 \qquad (1)$$

$$B = 0.17\ FRK2 + FRK1 \qquad (2)$$

(where A is the total activity measured with 0.1 mM fructose, and B is the total activity measured with 1.0 mM fructose) derived from Table 4.1. By solving these equations a ratio of FRK2 to FRK1 can be calculated (Table 4.2). The precise activity of each isoform was determined by integration of the peak areas of each isoform (Fig. 4.4).

The calculated distribution of isoforms is within 10 % of that obtained after separation of the isoforms and calculation of the peak areas.

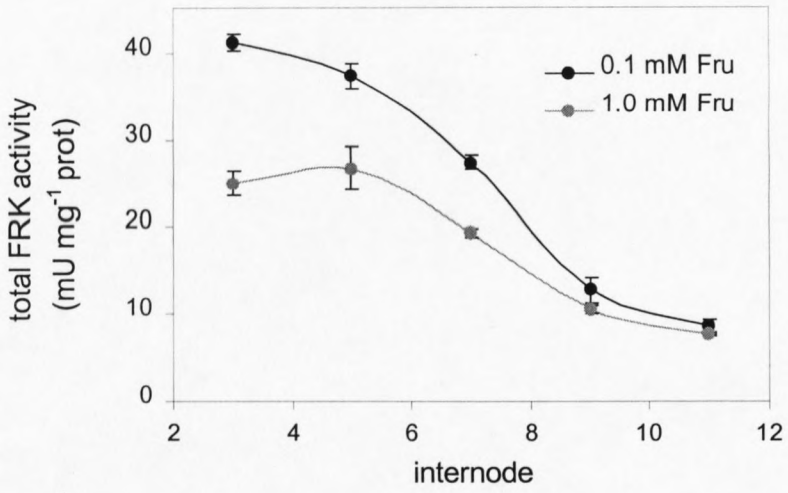


Figure 4.2 Changes in FRK activity during tissue maturation in sugarcane. FRK was assayed with 0.1 mM fructose and 1.0 mM fructose. Error bars indicate standard error of three repetitions.

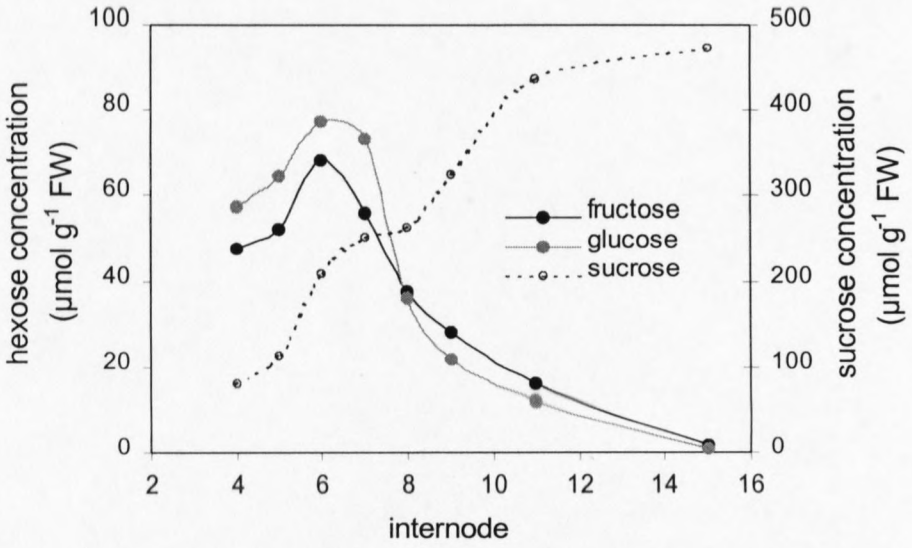


Figure 4.3 Fructose, glucose and sucrose concentrations in developing sugarcane culm.

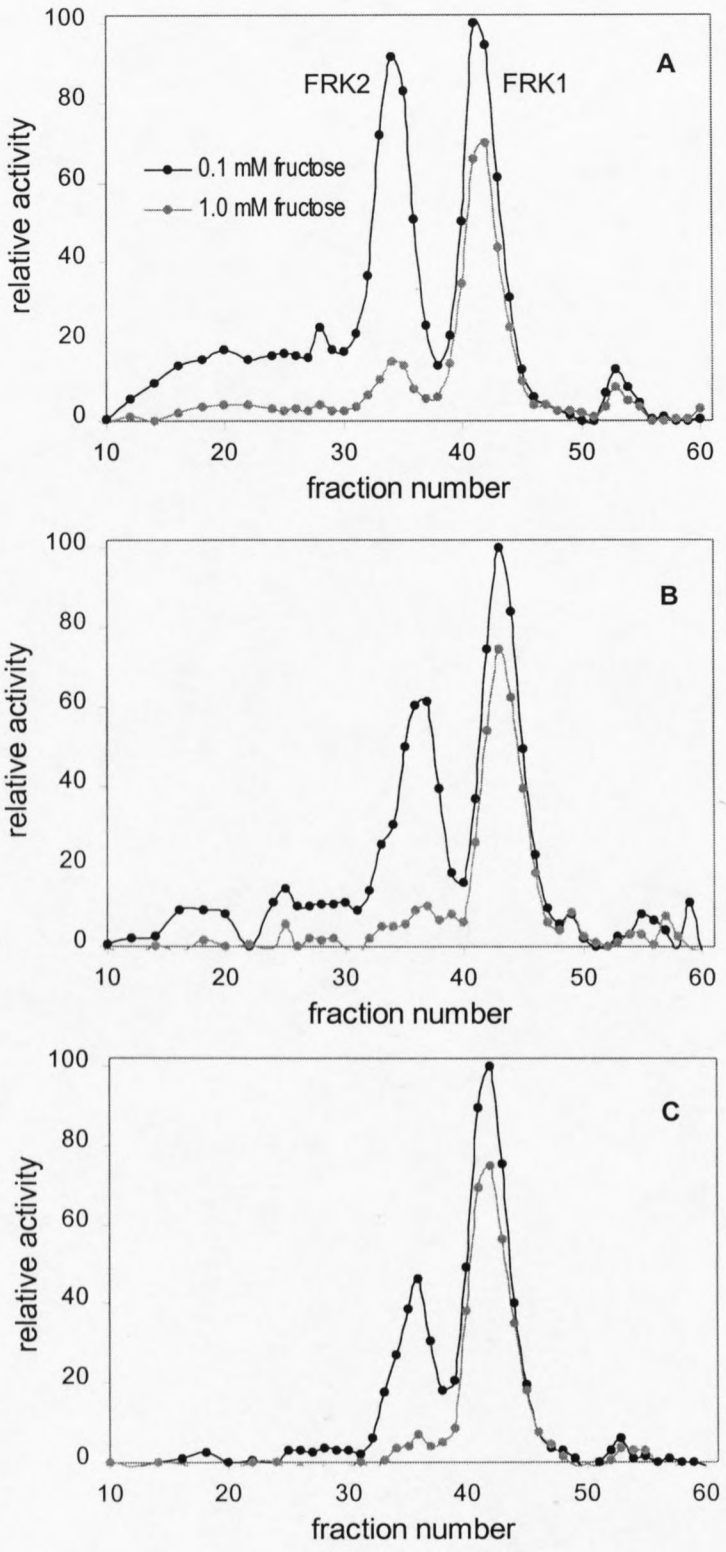


Figure 4.4 Developmental changes in the distribution pattern of FRK1 and FRK2. Sugarcane was sampled at different stages of development, namely internode 3 (A), 5 (B) and 9 (C) and fractions that eluted from an ion exchange column were assayed with 0.1 and 1.0 mM fructose to differentiate between the isoforms.

Table 4.2 Calculated and measured distribution of FRK isoforms.

	Calculated peak area (%)		Ratio	Calculated enzyme activity* (mU mg^{-1} protein)		Ratio
Internode	FRK2	FRK1	FRK2/FRK1	FRK2	FRK1	FRK2/FRK1
3	51	49	1.03	23.2 ± 1.86	21.06 ± 1.47	1.10
5	44	56	0.79	17.2 ± 1.55	23.8 ± 2.14	0.72
9	32	68	0.47	4.3 ± 0.26	9.8 ± 0.78	0.44

* Based on equation 1 and 2

± Standard error of three repetitions

4.3.5 Changes in FRK protein concentration

FRK1 and FRK2 were purified to homogeneity as described in chapter 3 and antibodies were raised against each. No cross-reacting polypeptides were evident on blots probed with pre-immune serum diluted in a ratio of 1 : 200 (results not shown). Blots were routinely performed using a 1 : 1000 dilution of the antisera although cross-reaction remained evident even when the antisera were diluted 1 : 5 000. Despite the high titre of both sera and their specificity on protein blots neither could differentiate between the two isoforms of FRK. This indicates large immunological similarity between FRK1 and FRK2.

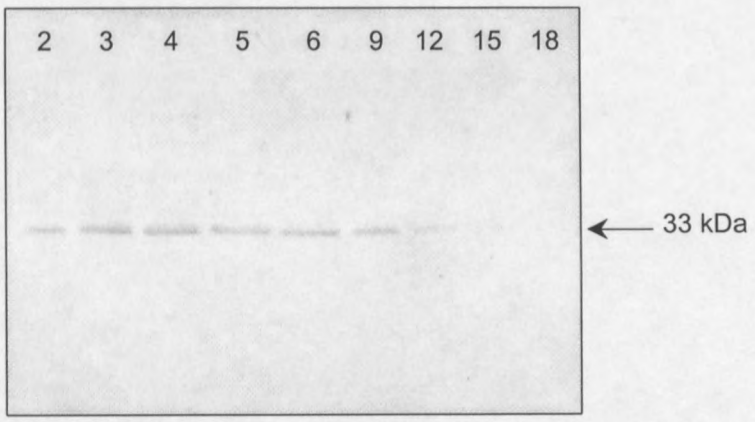


Figure 4.5 Protein blot of total FRK. 10 µg total protein present in the crude extracts were loaded in each lane. The number of each lane refers to the internode number.

Total FRK expression decreases during maturation of sugarcane internodal tissue. Crude extracts of internodal sugarcane tissue in different stages of development were probed with anti-FRK2 antiserum (Fig. 4.5). In immature tissue the signals were of equal intensity, followed by a decline in intensity in the more mature tissue.

4.4 DISCUSSION

The occurrence of multiple isoforms of FRK in plants has been shown previously, and in most cases one or more of the isoforms were sensitive to substrate inhibition by fructose (Turner *et al.* 1997; Baysdorfer *et al.* 1989; Gardner *et al.* 1992; Chaubron *et al.* 1995; Schaffer and Petreikov 1997; Pego and Smeekens 2000). This pattern was also found in sugarcane where two FRK isoforms were identified and one of these was subject to substrate inhibition by fructose.

Measuring FRK activity in extracts containing multiple isoforms is complicated by the large differences in the kinetic characters of the isoforms. This is a problem across species and by implication measurements in crude extracts would be merely an average of FRK activities under sub-optimum, possibly inhibiting conditions.

Previously FRK was measured in one of two ways, using either ATP or UTP in an assay containing a fixed fructose concentration (Marrè *et al.* 1968; Huber and Akazawa 1986; Doehlert *et al.* 1988; Xu *et al.* 1989; Kuo *et al.* 1990; Wendler *et al.* 1990; Goldner *et al.* 1991; Nakamura *et al.* 1992; Ross *et al.* 1994). If the fructose concentration was not optimal for all the isoforms, one or more could have been functioning at non-saturating substrate conditions, or could have been inhibited.

Sugarcane FRK2 is not inhibited by fructose when assayed with UTP (chapter 3). However, the method used by Botha *et al.* (1996) to assay sugarcane FRK in crude extracts would not have yielded maximum enzyme activity even though UTP was used. The 1 mM UTP that was used is insufficient to saturate the reaction (Table 3.5). Furthermore, FRK1 is highly ATP specific, showing only 40 % of its activity with ATP when assayed with UTP (Table 3.4).

We propose that total sugarcane FRK as well as the activity of each of the isoforms can be calculated from activity measurements in crude extracts if two specific substrate concentrations are used in the assays.

During sugarcane maturation total FRK activity decreases (Fig. 4.2). Similarly, the total FRK that is expressed decreases (Fig. 4.5). Since the developmental change in FRK activity correlates with change in FRK protein, we conclude that the decrease in FRK activity is the result of decreased expression of FRK, and not the result of inactivation of existing protein. A similar correlation has been shown in tuberising potato stolons (Ross *et al.* 1994).

Few studies have investigated the influence of development on the presence and distribution of FRK isoforms (Renz *et al.* 1993). In tomato there appears to be a differential pattern of expression of FRK isoforms during fruit development (Kanayama *et al.* 1997). Here we found a change in the ratio of activity of FRK2 to FRK1. As sugarcane matures FRK1 seems to become the preferentially expressed isoform (Fig. 4.4). Using the kinetic properties we could very accurately estimate the relative contribution of each isoform to the total FRK activity in crude extracts.

The role of FRK2 in sugarcane metabolism is not clear. The only way that this enzyme could play a meaningful part in fructose phosphorylation was if the fructose concentration was less than 0.2 mM. But in young sugarcane tissue, where FRK2 is primarily expressed, the concentration of fructose exceeds this limit by more than 100 times (Whittaker and Botha 1997). Even at 1 mM fructose FRK2 is severely inhibited, retaining only 17 % of its activity. However, the total FRK activity that remains at 1 mM fructose (100 % of FRK1 and 17 % of FRK2) is approximately 25 mU mg^{-1} protein in immature tissue (Fig. 4.2). This is similar to the maximum flux rate previously calculated (Whittaker and Botha 1999).

Separation of the enzyme and the bulk of its substrate into different cellular compartments could allow FRK2 to function without inhibition. In most plants that have been investigated the majority of the FRK activity is in the cytosol (Tanner *et al.* 1983; ap Rees 1985; Copeland and Morell 1985; Copeland and Tanner 1988; Schnarrenberger 1990). In sugarcane suspension cells the hexoses appear to be almost completely located in the vacuole in the early culture phases, becoming evenly distributed between cytosol and vacuole at the end of the cell culture cycle (Preisser *et*

al. 1992). Unfortunately, little is known about the intracellular distribution of sugars in intact sugarcane.

Another possibility worth considering is possible sugar sensing or signalling by FRK. In yeast both HK and galactokinase are involved in sugar sensing (Pego and Smeekens 2000). This system could be partially conserved in plants and mammals (Pego and Smeekens 2000). A null mutation in the *Frk2* gene of Arabidopsis led to sugar insensitivity of the plant. It is possible that FRK2, the substrate inhibited isoform that has been found in most of the plants studied to date, plays a greater role as a sensing rather than a metabolic enzyme. FRK2 and *Frk2* mRNA are primarily expressed in immature tissue, and levels decrease during maturation (Renz *et al.* 1993; Kanayama *et al.* 1997; Martinez-Barajas *et al.* 1997).

In young, immature, actively growing sugarcane tissue one would expect a greater need for regulation of metabolism than in mature tissue, where the whole metabolic system centres on sucrose accumulation. In potato FRK catalyses a near rate-limiting reaction (Viola 1996) that could have a regulatory effect on metabolism. FRK, and specifically FRK2, has been implicated in sugar sensing mammals. In sugarcane FRK2's regulatory influence via substrate inhibition would provide a means of imposing control over the phosphorylation of fructose. But this control would become less important as the tissue matures and the need for FRK2 would decrease, possibly resulting in the change in ratio of the isoforms that was observed.

CHAPTER 5

DISCUSSION

Current research aimed at improving sugarcane yields involves genetic manipulation of key reaction steps. But without in-depth understanding of the processes involved in sugarcane sucrose metabolism, manipulating these becomes a strictly trial and error effort that consumes both time and money with no promise of success.

Sugarcane, like other plants studied to date, contains several hexose kinase isoforms. Two of these are typical FRKs, one of which is subject to substrate inhibition by fructose. In this regard sugarcane is similar to tomato fruit (Martinez-Barajas and Randall 1996; Kanayama *et al.* 1998), potato tubers (Gardner *et al.* 1992; Renz and Stitt 1993), maize kernels (Doehlert 1989), barley leaves (Baysdorfer *et al.* 1989) and pea seeds (Turner *et al.* 1977; Copeland *et al.* 1984).

Despite this superficial similarity to other plants, sugarcane FRK2 is unique in that it is much more sensitive to fructose inhibition. In fact, the reaction is 4 to 50 times more sensitive to substrate inhibition than in other plants (Chapter 3). Some studies have proposed that substrate inhibition of FRK by fructose is not physiologically significant, because the concentration of fructose needed is not likely to occur *in vivo* (Copeland and Morell 1985). This is obviously not valid for sugarcane. In immature sugarcane tissue the fructose concentrations (Whittaker and Botha 1997) can exceed the requirement for inhibition of FRK2 by more than 100 times. However, FRK1 is not inhibited by fructose and at 1 mM fructose the total FRK activity that remains, i.e. FRK1 and the non-inhibited activity of FRK2, is enough to sustain the hexose flux previously reported for immature tissue (Whittaker and Botha 1999).

Assuming that the metabolically active compartment in sugarcane suspension cultures comprises 10 to 50 % of the total cell volume, it can be estimated that the ATP concentration can vary between 0.56 and 2.8 mM (Dancer *et al.* 1990b). Both the FRK1 and FRK2 reaction would be saturated by ATP under these circumstances (Table 3.3). In immature tissue where the fructose concentration can exceed 25 mM (Whittaker and Botha 1997), FRK2 will be severely inhibited. The FRK1 reaction will be saturated but presumably not inhibited since no inhibition is observed at 1 mM

fructose. The FRK1 reaction is ATP-specific. FRK2 is not nucleotide-specific, and when assayed with UTP the inhibition by fructose is not observed (Table 3.5). As the UTP concentration in immature tissue is approximately 1.6 mM and decreased to 0.65 mM in mature internodes (Whittaker 1997) and the K_{mUTP} is 1 mM, the FRK2 reaction would not be saturated by UTP *in vivo*.

A cycling of UTP, UDP and PPi through SuSy, UTP-linked FRK, UDP-glucose pyrophosphorylase and UTP-linked phosphofructokinase was proposed (Huber and Akazawa 1986). Subsequent studies on FRK usually concluded that FRK would not utilise UTP *in vivo* since the K_{mUTP} was typically more than 5 times higher than K_{mATP}. In sugarcane the K_{mUTP} of FRK2 was 5 times higher than the K_{mATP} in the presence of non-inhibitory fructose concentrations (Table 3.5). When the fructose concentration was increased this changed dramatically. The K_{mATP} increased sharply, becoming almost double the K_{mUTP}. Under normal cellular conditions in sugarcane, where fructose concentrations will be inhibitory, it is probable that UTP utilisation could become very important.

Previous assays of FRK in crude extracts have been largely inaccurate due to the divergent kinetic characters of FRK isoforms. Optimum conditions for one isoform might be sub-optimum or inhibitory to another. Even assays that utilised UTP instead of ATP would not have been accurate. Even though FRK2 is not inhibited when assayed with UTP, FRK1 is ATP-specific and the reaction velocity would not have been a true reflection of the maximum potential catalytic activity. Furthermore, much higher concentrations of UTP than used to date are necessary to saturate the reaction in sugarcane (Chapter 3).

In chapter 4 it is convincingly shown that the application of a mathematical calculation which is based on the kinetic properties of FRK1 and FRK2 can be used to accurately determine both total and individual activity of the FRKs in crude extracts. This is a simple procedure and only requires assaying of crude extracts at two concentrations of fructose.

FRK2 is inhibited by fructose, FRK1 is not. FRK1 is very specific for ATP while FRK2 appears to be non-specific and is not inhibited by fructose when assayed with UTP. We therefore propose that two NTP-binding sites occur on the FRKs based on the diverse kinetic characters of the isoforms (Chapter 3). According to this model, an

ATP-specific regulatory site, as well as a catalytic site with a wide substrate specificity, exists. Additionally two fructose-binding sites exist. One is a catalytic site and the other an allosteric regulatory site. Binding of fructose in the allosteric site is only possible if ATP is bound in the ATP regulatory site. Such a configuration could explain the observed kinetic properties of FRK2 (Fig. 3.8).

This proposal is in agreement with the deduced amino acid sequences of FRKs from various plants, which show a striking degree of conservation in certain areas that are thought to represent substrate binding sites. Besides a fructose-binding site, two possible ATP-binding sites were identified (Kanayama *et al.* 1997; Pego and Smeekens 2000). Both potential ATP sites are conserved among both FRK1 (not substrate inhibited) and FRK2 (substrate inhibited) sequences. However, it is striking that the one site is completely conserved in the FRK2 sequences, but varies between the FRK1 and FRK2 sequences. This could well be the ATP regulatory site.

The FRK activity decreases during maturation. This is directly related to a decrease in the FRK protein levels. The decrease in activity is therefore the result of decreased expression, and not inactivation of existing protein. In addition to the ripening-related decrease in total FRK activity, there is a developmental influence on the expression of FRK isoforms. The ratio of FRK2 to FRK1 decreases from 1:1 in immature tissue to 1:2 in mature internodes. It appears as if FRK1, the isoform not regulated through substrate inhibition, becomes preferentially expressed in mature, metabolically less-active internodes. This could be as a result of a decrease in the necessity for regulation of metabolism. In immature, actively growing tissue it could be expected that there exists a greater need for regulation of metabolism than in mature tissue, where respiratory activity decreases and the whole of metabolism revolves around sucrose accumulation.

In line with recent proposals (Pego and Smeekens 2000), it is conceivable that FRK2 could, in addition to its role in fructose mobilisation, play an important part in sugar-signalling and control of gene expression. Hexose kinases such as HK and galactokinase are involved in sugar sensing in yeast and mammals, whereas FRK is involved in sugar-mediated regulatory processes in mammals. If the regulatory system is conserved across species, it is possible that FRK might play a role in sugar responses of plants too (Pego and Smeekens 2000).

Although this study has provided valuable insight into the properties and expression of the FRK isoforms in the sugarcane culm, no definite conclusion on the role of each isoform in sucrose accumulation can be drawn. Firm conclusions will only be possible once the FRK isoforms have been individually genetically manipulated.

LITERATURE CITED

ap Rees T (1985) The organization of glycolysis and the pentose phosphate pathway in plants. In Douce R and Day D (eds) *Encyclopedia of Plant Physiology*, Vol 18, pp 391 – 417, Springer-Verlag, Berlin.

ap Rees T (1988) Hexose phosphate metabolism by nonphotosynthetic tissues of higher plants. In Stumpf P K and Conn E E (eds) *The Biochemistry of Plants,* Vol 14, pp 1 – 33, Academic Press Inc, San Diego.

Avigad G (1982) Sucrose and other disaccharides. In Loewus F A and Tanner W (eds) *Encyclopedia of Plant Physiology*, pp 217 – 347, Springer-Verlag, Berlin.

Batta S K and Singh R (1986) Sucrose metabolism in sugar cane grown under varying climatic conditions: synthesis and storage of sucrose in relation to the activities of sucrose synthase, sucrose-phosphate synthase and invertase. *Phytochem* **25**: 2431 – 2437.

Baysdorfer C, Kremer D F and Sicher R C (1989) Partial purification and characterization of fructokinase activity from barley leaves. *J Plant Physiol* **134**: 156 – 161.

Bellstedt D U (1988) Ph D Thesis, University of Stellenbosch, Stellenbosch, South Africa.

Bieleski R L (1960) The physiology of sugar-cane. III. Characteristics of sugar uptake in slices of mature and immature storage tissues. *Aust J Biol Sci* **13**: 203 – 220.

Bindon K A (2000) M Sc Thesis, University of Stellenbosch, Stellenbosch, South Africa.

Blake M S, Johnston K H, Russel-Jones G J and Gotschlich E C A (1984) A rapid sensitive method for detection of alkaline phosphate-conjugated anti-body on Western blots. *Anal Biochem* **136**: 175 – 179.

Botha F C, Whittaker A, Vorster D J and Black K G (1996) Sucrose accumulation rate, carbon partitioning and expression of key enzyme activities in sugarcane stem tissue. In Wilson J R, Hogarth D M, Campbell J A and Garside A L (eds) *Sugarcane: Research towards efficient and sustainable production*, pp 98 – 101, CSIRO Division of tropical crops and pastures, Brisbane.

Bowen J E and Hunter J E (1972) Sugar transport in immature internodal tissue of sugarcane. II. Mechanism of sucrose transport. *Plant Physiol* **49**: 789 – 793.

Bull T A and Glasziou K T (1963) The evolutionary significance of sugar accumulation in *Saccharum*. *Aust J Biol Sci* **16**: 737 – 742.

Cárdenas M L, Cornish-Bowden A and Ureta T (1998) Evolution and regulatory role of the hexokinases. *Biochim Biophys Acta* **1401**: 242 – 264.

Chaubron F, Harris N, Ross H A and Davies H V (1995) Partial purification and characterization of fructokinase from developing taproots of sugar beet (*Beta vulgaris*). *Plant Sci* **110**: 181 – 186.

Copeland L and Morell M (1985) Hexose kinases from the plant cytosolic fraction of soybean nodules. *Plant Physiol* **79**: 114 – 117.

Copeland L and Tanner G J (1988) Hexose kinases of avocado. *Physiol Plant* **74**: 531 – 536.

Copeland L, Harrison D D and Turner J F (1978) Fructokinase (fraction III) of pea seeds. *Plant Physiol* **62**: 291 – 294.

Copeland L, Stone S R and Turner J F (1984) Kinetic studies of fructokinase I of pea seeds. *Arch Biochem Biophys* **233**: 748 – 760.

Cornish-Bowden A (1981) *Fundamentals of enzyme kinetics*. Butterworths & Co, Londen.

Dai N, Schaffer A, Petreikov M and Granot D (1997) Potato (*Solanum tuberosum* L.) fructokinase expressed in yeast exhibits inhibition by fructose of both in vitro enzyme activity and rate of cell proliferation. *Plant Sci* **128**: 191 – 197.

Dai N, Schaffer A, Petreikov M, Shahak Y, Giller Y, Ratner K, Levine A and Granot D (1999) Overexpression of *Arabidopsis* hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. *Plant Cell* **11**: 1253 – 1266.

Dancer J, Hatzfeld W D and Stitt M (1990a) Cytosolic cycles regulate the turnover of sucrose in heterotrophic cell-suspension cultures of *Chenopodium rubrum* L. *Planta* **182**: 223 – 231.

Dancer J, Veith R, Feil R, Komor E and Stitt M (1990b) Independent changes of inorganic pyrophosphate and the ATP / ADP or UTP / UDP ratios in plant cell suspension cultures. *Plant Sci* **66**: 59 – 63.

Doehlert D C (1987) Substrate inhibition of maize endosperm sucrose synthase by fructose and its interaction with glucose inhibition. *Plant Sci* **52**: 153 – 157.

Doehlert D C (1989) Separation and characterization of four hexose kinases from developing maize kernels. *Plant Physiol* **89**: 1042 – 1048.

Doehlert D C (1990) Fructokinases from developing maize kernels differ in their specificity for nucleoside triphosphates. *Plant Physiol* **93**: 353 – 355.

Doehlert D C, Kuo T M and Felker F C (1988) Enzymes of sucrose and hexose metabolism in developing kernels of two inbreds of maize. *Plant Physiol* **86**: 1013 – 1019.

Gardner A, Davies H V and Burch L R (1992) Purification and properties of fructokinase from developing tubers of potato (*Solanum tuberosum* L.). *Plant Physiol* **100**: 178 – 183.

Gayler K R and Glasziou K T (1972) Physiological functions of acid and neutral invertases in growth and sugar storage in sugar cane. *Physiol Plant* **27**: 25 – 31.

Geigenberger P and Stitt M (1993) Sucrose synthase catalyses a readily reversible reaction in vivo in developing potato tubers and other plant tissues. *Planta* **189**: 329 – 339.

Glasziou K T (1961) Accumulation and transformation of sugars in stalks of sugar cane. Origin of glucose and fructose in the inner space. *Plant Physiol* **36**: 175 – 179.

Glasziou K T and Gayler K R (1972a) Storage of sugars in stalks of sugar cane. *Bot Rev* **38**: 471 – 490.

Glasziou K T and Gayler K R (1972b) Sugar accumulation in sugarcane. Role of cell walls in sucrose transport. *Plant Physiol* **49**: 912 – 913.

Goldner W, Thom M and Maretzki A (1991) Sucrose metabolism in sugarcane suspension cultures. *Plant Sci* **73**: 143 – 147.

Guglielminetti L, Perata P, Morita A, Loreti E, Yamaguchi J and Alpi A (2000) Characterization of isoforms of hexose kinases in rice embryo. *Phytochem* **53**: 195 – 200.

Hartt C E, Kortschak H P, Forbes A J and Burr G O (1963) Translocation of ^{14}C in sugarcane. *Plant Physiol* **38**: 305 – 318.

Hatch M D and Glasziou K T (1964) Direct evidence for translocation of sucrose in sugar cane leaves and stems. *Plant Physiol* **39**: 180 – 184.

Hawker J S (1965) The sugar content of cell walls and intercellular spaces in sugar-cane stems and its relation to sugar transport. *Aust J Biol Sci* **18**: 959 – 969.

Hawker J S (1985) Sucrose. In Dey P M and Dixon R A (eds) *Biochemistry of storage carbohydrates in green plants,* pp 1 – 51, Academic Press, New York.

Hawker J S and Hatch M D (1965) Mechanism of sugar storage by mature stem tissue of sugar cane. *Physiol Plant* **18**: 444 – 453.

Huber S C and Akazawa T (1986) A novel sucrose synthase pathway for sucrose degradation in cultured sycamore cells. *Plant Physiol* **81**: 1008 – 1013.

Irvine J E (1975) Relations of photosynthetic rates and leaf and canopy characters to sugarcane yield. *Crop Sci* **15**: 671 – 676.

Jacobsen K R, Fisher D G, Maretzki A and Moore P H (1992) Developmental changes in the anatomy of the sugarcane stem in relation to phloem unloading and sucrose storage. *Bot Acta* **105**: 70 – 80.

Jang J-C and Sheen J (1994) Sugar sensing in higher plants. *Plant Cell* **6**: 1665 – 1679.

Jang J-C, León P, Zhou L and Sheen J (1997) Hexokinase as sugar sensor in higher plants. *Plant Cell* **9**: 5 – 19.

Kanayama Y, Dai N, Granot D, Petreikov M, Schaffer A and Bennett A B (1997) Divergent fructokinase genes are differentially expressed in tomato. *Plant Physiol* **113**: 1379 – 1384.

Kanayama Y, Granot D, Dai N, Petreikov M, Schaffer A, Powell A and Bennett A B (1998) Tomato fructokinases exhibit differential expression and substrate regulation. *Plant Physiol* **117**: 85 – 90.

Komor E (2000) The physiology of sucrose storage in sugarcane. In Gupta A K and Kaur N (eds) *Carbohydrate reserves in plants – Synthesis and regulation*, pp 35 – 53, Elsevier, Amsterdam.

Komor E, Thom M and Maretzki A (1981) The mechanism of sugar uptake by sugar cane suspension cells. *Planta* **153**: 181 – 192.

Komor E, Zingsheim O and Sprügel H (1996) Cycles of sugar transport and sucrose metabolism in sugarcane tissue: quantitative determination. In Wilson J R, Hogarth D M, Campbell J A and Garside A L (eds) *Sugarcane: Research towards efficient and sustainable production*, pp 92 – 94, CSIRO Division of tropical crops and pastures, Brisbane.

Kruger N J (1997) Carbohydrate synthesis and degradation. In Dennis D T, Turpin D H, Lefebvre D D and Layzell D B (eds) *Plant Metabolism*, 2nd edn, pp 83 – 104, Addison Wesley Longman Ltd, Harlow, England.

Kühn C, Barker L, Bürkle L and Frommer W B (1999) Update on sucrose transport in higher plants. *J Exp Bot* **50**: 935 – 953, Special issue.

Kuo T M, Doehlert D C and Crawford C G (1990) Sugar metabolism in germinating soybean seeds. *Plant Physiol* **93**: 1514 – 1520.

Laemmli U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680 – 685.

Lingle S E (1989) Evidence for the uptake of sucrose intact into sugarcane internodes. *Plant Physiol* **90**: 6 – 8.

Lingle S E (1999) Sugar metabolism during growth and development in sugarcane internodes. *Crop Sci* **39**: 480 – 486.

Lingle S E and Smith R C (1991) Sucrose metabolism related to growth and ripening in sugarcane internodes. *Crop Sci* **31**: 172 – 177.

Maretzki A and Thom M (1972) Membrane transport of sugars in cell suspensions of sugarcane. I. Evidence for sites and specificity. *Plant Physiol* **49**: 177 – 182.

Marrè E, Cornaggia M P and Bianchetti R (1968) The effects of sugars on the development of hexose phosphorylating enzymes in the castor bean cotyledons. *Phytochem* **7**: 1115 – 1123.

Martinez-Barajas E and Randall D D (1996) Purification and characterization of fructokinase from developing tomato (*Lycopersicon esculentum* Mill.) fruits. *Planta* **199**: 451 – 458.

Martinez-Barajas E, Luethy M H and Randall D D (1997) Molecular cloning and analysis of fructokinase expression in tomato (*Lycopersicon esculentum* Mill.). *Plant Sci* **125**: 13 – 20.

Moore P H (1995) Temporal and spatial regulation of sucrose metabolism in the sugarcane stem. *Aust J Plant Physiol* **22**: 661 – 679.

Moore P H and Maretzki A (1997) Sugarcane. In Zamski E and Schaffer A A (eds) *Photoassimilate distribution in plants and crops: source-sink relationships*, pp 643 – 669, Marcel-Dekker Inc, New York.

Moore P H, Botha F C, Furbank R T and Grof C P L (1997) Potential for overcoming physio-biochemical limits to sucrose accumulation. In Keating B A and Wilson J R (eds) *Intensive sugarcane production: Meeting the challenges beyond 2000*, pp 141 – 155, CAB International, Wallingford.

Nakamura N, Suzuki Y and Suzuki H (1992) Pyrophosphate-dependent phosphofructokinase from pollen: properties and possible roles in sugar metabolism. *Physiol Plant* **86**: 616 – 622.

Oparka K J and Prior D A M (1988) Movement of Lucifer Yellow CH in potato tuber storage tissues: a comparison of symplastic and apoplastic transport. *Planta* **176**: 533 – 540.

Pego J V and Smeekens S C M (2000) Plant fructokinases: a sweet family get-together. *Trends Plant Sci* **5**: 531 – 536.

Plaxton W C (1996) The organization and regulation of plant glycolysis. *Annu Rev Plant Physiol Plant Mol Biol* **47**: 185 – 214.

Preisser J and Komor E (1991) Sucrose uptake into vacuoles of sugarcane suspension cells. *Planta* **186**: 109 – 114.

Preisser J, Sprügel H and Komor E (1992) Solute distribution between vacuole and cytosol of sugarcane suspension cells: Sucrose is not accumulated in the vacuole. *Planta* **186**: 203 – 211.

Renz A and Stitt M (1993) Substrate specificity and product inhibition of different forms of fructokinases and hexokinases in developing potato tubers. *Planta* **190**: 166 – 175.

Renz A, Merlo L and Stitt M (1993) Partial purification from potato tubers of three fructokinases and three hexokinases which show differing organ and developmental specificity. *Planta* **190**: 156 – 165.

Rose S and Botha F C (2000) Distribution patterns of neutral invertase and sugar content in sugarcane internodal tissues. *Plant Physiol Biochem* **38**: 819 – 824.

Ross H A, Davies H V, Burch L R, Viola R and McRae D (1994) Developmental changes in carbohydrate content and sucrose degrading enzymes in tuberising stolons of potato (*Solanum tuberosum*). *Physiol Plant* **90**: 748 – 756.

Sacher J A, Hatch M D and Glasziou K T (1963) Sugar accumulation cycle in sugar cane. III. Physical and metabolic aspects of cycle in immature storage tissues. *Plant Physiol* **38**: 348 – 354.

Schaffer A A and Petreikov M (1997a) Inhibition of fructokinase and sucrose synthase by cytosolic levels of fructose in young tomato fruit undergoing transient starch synthesis. *Physiol Plant* **101**: 800 – 806.

Schaffer A A and Petreikov M (1997b) Sucrose-to-starch metabolism in tomato fruit undergoing transient starch accumulation. *Plant Physiol* **113**: 739 – 746.

Schnarrenberger C (1990) Characterization and compartmentation, in green leaves, of hexokinases with different specificities for glucose, fructose, and mannose and for nucleoside triphosphates. *Planta* **181**: 249 – 255.

Sheen J (1990) Metabolic repression of transcription in higher plants. *Plant Cell* **2**: 1027 – 1038.

Smeekens S and Rook F (1997) Sugar sensing and sugar-mediated signal transduction in plants. *Plant Physiol* **115**: 7 – 13.

Tanner G J, Copeland L and Turner J F (1983) Subcellular localization of hexose kinases in pea stems: mitochondrial hexokinase. *Plant Physiol* **72**: 659 – 663.

Taylor M A, Ross H A, Gardner A and Davies H V (1995) Characterisation of a cDNA encoding fructokinase from potato (*Solanum tuberosum* L.). *J Plant Physiol* **145**: 253 – 256.

Thom M and Maretzki A (1992) Evidence for direct uptake of sucrose by sugarcane stalk tissue. *J Plant Physiol* **139**: 555 – 559.

Trethewey R N, Riesmeier J W, Willmitzer L, Stitt M and Geigenberger P (1999) Tuber-specific expression of a yeast invertase and a bacterial glucokinase in potato leads to an activation of sucrose phosphate synthase and the creation of a sucrose futile cycle. *Planta* **208**: 227 – 238.

Turner J F, Harrison D D and Copeland L (1977) Fructokinase (fraction IV) of pea seeds. *Plant Physiol* **60**: 666 – 669.

Van Dillewijn C (1952) Growth. General, grand period, growth formulae. In Van Dillewijn C (ed) *Botany of sugarcane*, Vol 1, pp 97 – 162, Veenen and Zonen, Netherlands.

Viola R (1996) Hexose metabolism in discs excised from developing potato (*Solanum tuberosum* L.) tubers. II. Estimations of fluxes in vivo and evidence that fructokinase catalyses a near rate-limiting reaction. *Planta* **198**: 186 – 196.

Vorster D J and Botha F C (1999) Sugarcane internodal invertases and tissue maturity. *J Plant Physiol* **155**: 470 – 476.

Welbaum G E, Meinzer F C, Grayson R L and Thornham K T (1992) Evidence for and consequences of a barrier to solute diffusion between the apoplast and vascular bundles in sugarcane stalk tissue. *Aust J Plant Physiol* **19**: 611 – 623.

Wendler R, Veith R, Dancer J, Stitt M and Komor E (1990) Sucrose storage in cell suspension cultures of *Saccharum* sp. (sugarcane) is regulated by a cycle of synthesis and degradation. *Planta* **183**: 31 – 39.

Whittaker A (1997) Ph D Thesis, University of Natal, Durban, South Africa.

Whittaker A and Botha F C (1997) Carbon partitioning during sucrose accumulation in sugarcane internodal tissue. *Plant Physiol* **115**: 1651 – 1659.

Whittaker A and Botha F C (1999) Pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase activity patterns in relation to sucrose storage across sugarcane varieties. *Physiol Plant* **107**: 379 – 386.

Williams L, Thom M and Maretzki A (1990) Characterization of a proton translocating ATPase and sucrose uptake in a tonoplast-enriched vesicle fraction from sugarcane. *Physiol Plant* **80**: 169 – 176.

Wolosiuk R A and Pontis H G (1974) Studies on sucrose synthetase. *Arch Biochem Biophys* **165**: 140 – 145.

Xu D P, Sung S S, Loboda T, Kormanik P P and Black C C (1989) Characterization of sucrolysis via the uridine diphosphate and pyrophosphate-dependent sucrose synthase pathway. *Plant Physiol* **90**: 635 – 642.

Zhu Y J, Komor E and Moore P H (1997) Sucrose accumulation in the sugarcane stem is regulated by the difference between the activities of soluble acid invertase and sucrose phosphate synthase. *Plant Physiol* **115**: 609 – 616.

CURRICULUM VITAE

1993	Matriculated from Montagu High School, Montagu
1996	Completed B Sc degree at the University of Stellenbosch (Biochemistry and Genetics)
1997	Completed B Sc (Honours) degree at the Department of Biochemistry, University of Stellenbosch
2000	Second prize – Young scientist of the year award of the South African Association of Botanists, SAAB Conference 2000
	Accepted the position of Technical Assistant to Prof F C Botha at the Institute for Plant Biotechnology, University of Stellenbosch
2001	Completed M Sc degree at the Department of Botany, University of Stellenbosch