CLARICE APARECIDA MEGGUER

ENZYMATIC ACTIVITIES OF THE GLYCOLYTIC PATHWAY AND GLYCOLYTIC FLUX IN SUGAR BEET ROOTS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fisiologia Vegetal, para obtenção do título de *Doctor Scientiae.*

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Aprovada: 05 de março de 2010.

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To my lovely parents, Maria and Antonio.

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BIOGRAPHY

CLARICE APARECIDA MEGGUER, daughter of Maria Dudar Megguer and Antonio Pascoal Megguer, was born on June 19th, 1975, in Almirante Tamandaré, Paraná, Brazil.

In 1997, she entered Santa Catarina State University, graduating in agronomy engineering on July 2002.

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RESUMO

MEGGUER, Clarice Aparecida, D.Sc. Universidade Federal de Viçosa, março de 2010, **Atividade enzimática da via glicolítica e fluxo glicolítico em raízes de beterraba açucareira**. Orientador: Fernando Luiz Finger. Coorientadores: Raimundo Santos Barros e José Geraldo Barbosa.

A beterraba açucareira é uma espécie de clima temperado, cujas raízes são utilizadas para a produção de açúcar, portanto é essencial evitar a degradação da sacarose tanto durante o cultivo como na pós-colheita. A sacarose é quebrada por ação das enzimas sucrolíticas – sacarose sintase, invertase ácida ou invertase alcalina – e os produtos da atividade destas enzimas são catabolizados pela glicólise para fornecer substratos para o ciclo do TCA, consequentemente para a cadeia de transporte de elétrons. Enquanto as enzimas sucrolíticas tem sido bem estudadas, poucos estudos tem avaliado a atividade das enzimas glicolíticas. Desta forma, a determinação da atividade de todas as enzimas glicolíticas e o fluxo de carbono através da glicólise é uma etapa necessária para melhor entender os processos envolvidos na perda e degradação da sacarose em raízes de beterraba açucareira. Para o experimento da determinação da atividade enzimática, raízes de beterraba açucareira crescidas em casa-de-vegetação por 16 semanas foram colhidas, lavadas e armazenadas por 0, 1, 2, 3, 4, 7, 10, 30, 60 e 100 dias a 10 ^{o}C por 10 dias, de forma que as raízes armazenadas por período superior a 10 dias foram transferidas para a temperatura de 4 ^{o}C permanecendo nesta temperatura até o fim do armazenamento. Em cada data as raízes foram avaliadas quanto a atividade respiratória e então coletado material para a determinação da atividade enzimática. Para as análises do fluxo de carbono as raízes foram colhidas e armazenadas a 10 ^{o}C para o experimento 1, a 4, 10 e 20 ^{o}C para o experimento 2. As raízes foram armazenadas nas respectivas temperaturas por um período de 10 dias. A falta de correlação entre qualquer enzima glicolítica individual e a taxa respiratória durante o armazenamento sugere que a glicólise não controla a disponibilidade de substrato respiratório ou que não existe uma única enzima envolvida no controle da taxa respiratória na via glicolítica. A

análise canônica (PCA) identificou quatro grupos de enzimas que compartilham similaridades na maneira em que suas atividades mudam similarmente durante o armazenamento. No experimento para determinar o fluxo de carbono observou-se que a maioria do material radioativo incorporado pelas raízes permaneceu como sacarose e não foi metabolizado. A concentração de glucose e fructose foi maior para discos de raízes incubados a 4^{o}C do que aqueles incubados a 10 or 20^{o}C. O aumento da temperatura de incubação promoveu mudanças na quantidade relativa de material radioativo na glicose, frutose e fração fosfoester, sugerindo que a elevação da temperatura favorece o fluxo glicolítico.

ABSTRACT

MEGGUER, Clarice Aparecida, D.Sc. Universidade Federal de Viçosa, March, 2010, **Enzymatic activities of the glycolytic pathway and glycolytic flux in sugar beet roots.** Adviser: Fernando Luiz Finger. Co-advisers: Raimundo Santos Barros and José Geraldo Barbosa.

Sugarbeet is a temperate species whose taproots are used for sugar production. For maximum yield, however, it is essential to avoid sucrose degradation during cultivation and postharvest. Sucrose is cleaved by sucrolytic enzymes – sucrose synthase, acid invertase or alkaline invertase – and the products of these enzyme activities are catabolized by glycolysis to provide substrates for the TCA cycle, which provides substrates for the electron transfer chain. While sucrolytic enzymes have been studied in considerable detail, few studies have examined the activity of glycolytic enzymes. Determination of activities of glycolytic enzymes and measurement of flux of carbon through glycolysis is a necessary step to better understand the glycolytic pathway in sugarbeet roots and its contribution to sucrose loss. For determining enzymatic activities, sugarbeet roots grown in green house for 16 weeks were harvested, washed and stored for 0, 1, 2, 3, 4, 7, 10, 30, 60 and 100 days, with the first 10 days of storage at 10^{o}C and subsequent storage at 4^{o}C. Sugarbeet roots were evaluated for respiratory rates and then material was collected for the determination of enzyme activities. For carbon flux, roots were harvested and stored at 10^{o}C in Experiment 1, and at 4, 10 and 20^{o}C for Experiment 2 for 10 days. The lack of correlation between any individual glycolytic enzyme and root respiration rate during storage suggests that no single enzyme in the glycolytic pathway controls respiration rate. Canonic analysis identified four groups of enzymes that shared similarities in the manner in which their activities changed during storage. In experiments for carbon flux most of the radiolabel incorporated by root tissue remained as sucrose and was unmetabolized. Glucose and fructose concentrations were greater in roots disc incubated at 4^{o}C than at 10 or 20^{o}C. The increase of the incubation temperature provided relative changes in amount of labelled glucose, fructose and phosphoester glycolytic

intermediates, suggesting that increased temperature increases the flux of carbon through early glycolytic enzymes to a greater extent than sucrolytic enzymes or late glycolytic enzymes.

GENERAL INTRODUCTION

Sugarbeet (*Beta vulgaris* L.) is produced throughout the world, especially in the USA, Russia, the European Union and Ukraine. Europe is the major producer sugar of sugarbeet and responsible for 75% of total beet sugar produced. The USA and Asia make up 19% of world production; South America and Africa account for the remainder (Draycott, 2006).

In 2007, the USA produced about 32 million tons of sugarbeet in 504 mil hectares (FAOSTAT, 2007). Production for the 2009/2101 crop has yet to be quantified, but the National Agricultural Statistics Service (NASS) forecasts that for 2009/10, sugarbeet will be harvested from 470 mil hectares, a 15.3 percent increase over that of 2008/09, and 31 million tons of sugarbeet will be produced. In contrast, in the USA sugar cane production is estimated at approximately 303 mil hectares and 4 million tons (Haley & Dohlman, 2009). These numbers demonstrate the importance of sugarbeet for sugar production in the USA.

In the USA, sugarbeet is cultivated in different regions including the Upper Midwest (Minnesota and North Dakota), the Far West (California, Idaho, Oregon, and Washington State), the Great Plains (Colorado, Nebraska, Montana, and Wyoming) and the Great Lakes (Michigan) (Harveson et al., 2009). The sugarbeet crop can be grown commercially in a wide variety of temperate climates, but is mostly grown at latitudes between 30 and 60oN (Draycott, 2006). In recent years, the development of tropical sugarbeet varieties has created an interest in cultivation in regions of tropical climates (Joshi et al., 2005).

Beta vulgaris L. is a member of the Chenopodiaceae family and is agriculturally important because of its ability to accumulate a large quantity of sugar in its storage root (Milford, 2006). The sugarbeet is a biennial plant, requiring vernalization (temperature near 6oC) and long-day conditions (photo-thermal induction) to induce flowering and seed production (Lewellen et al., 2009; Milford, 2006). In commercial beet production, the root is harvested after the first growing season. The crop needs a relatively long growing period, normally from 140-160 up to 200 days to produce a commercially viable root, which typically weighs 1-2 kg and contains 15-20% sucrose based on fresh weight (Jaggard & Qi, 2006). However, the growing period and consequently

root yield and sucrose content are directly dependent on the production region (Went, 1954).

After harvest, sugarbeet roots are typically stored in large outdoor piles. Postharvest storage conditions and practices, however, vary substantially between production regions. In Mediterranean regions, including Southern Europe and North Africa, and in California, roots are processed shortly after harvest, usually within a few days, with harvest extending over two to five months to provide a constant supply of roots to processing factories. In Western Europe, sugarbeet roots are harvested until the first frost and roots are stored on-farm in piles called clamps for 3-6 weeks. In regions with short growing seasons and cold winters, e.g. those in the Northern USA, and parts of Russia, harvesting is delayed until just before the first frost, and roots are stored for up to 200 days prior to processing (Tungland et al., 1998). In the USA, roots are delivered to central piling stations where they are stored in piles 6-10 m high and 55-70 m wide and 400 m long. The piles are cooled by ambient winter air by natural or forced air ventilation. In these regions, roots may be stored up to 120 days prior to freezing or processing.

A major challenge for the sugarbeet industry is to preserve the sugar accumulated during postharvest storage. Sugarbeet roots lose, on average, 0.1% of their sucrose content per day in storage at 3^{o}C, although losses can reach 1.8% of their sugar content per day at temperatures higher than 30^{o}C (Wyse & Dexter, 1971). Given the duration of storage, it is common for 10 to 15% of the sucrose present at harvest to be lost during storage. Sucrose loss in storage is due to respiration, disease and conversion of sucrose to other carbohydrates including glucose, fructose, and raffinose. Respiration, however, is the principal cause of sucrose loss with estimates that 60 to 80% of the sucrose lost during storage is due to this process (Wyse & Dexter, 1971).

Respiration is the process by which compounds normally present in plant cells, such as starch, sugars, and organic acids are oxidatively degraded to carbon dioxide and water (Salveit, 2004; Siedow & Day, 2000). Concomitant with this catabolic reaction is the production of energy and intermediate molecules that are required to maintain the physiological integrity of living cells (Wyse, 1973; Salveit, 2004). In sugarbeet root, respiration utilizes sucrose as its

principal substrate (Barbour & Wang, 1961) and generates the energy and substrates needed to maintain healthy tissue, heal wounds incurred during harvest and piling, and defend against pathogens (Wyse & Dexter, 1971; Wyse, 1973). Respiration, therefore, is essential for biological process, but economically detrimental, and limiting its impact on sucrose loss is a major goal for the sugarbeet industry.

Respiration involves a complex series of reactions and involves sucrolysis, glycolysis, the citric acid cycle (TCA), and electron transfer/oxidative phosphorylation (Campbell & Klotz, 2006). In sugarbeet root, respiration begins with cleavage of sucrose by sucrolytic enzymes which cleave sucrose to hexose sugars. Glycolytic enzymes degrade the hexose sugars to the three carbon organic acid, pyruvate and with a concomitant release of energy. Pyruvate enters the TCA cycle which degrades the pyruvate to carbon dioxide and generates NADH and FADH2 which are substrates for the electron transfer chain (Klotz et al., 2006; Salveit, 2004).

Many factors are known to affect postharvest sugarbeet respiration, including storage temperature, the extent of mechanical damage incurred during harvest and piling, and disease (Fugate & Campbell, 2009). The most important factor affecting the rate of respiration, however, is temperature. Respiration rates generally decline with decreasing temperature, with rates decreasing by approximately one half for each 10oC reduction in temperature (Wyse, 1973 and 1978). The optimum temperature range for stored sugarbeet is thought to be between 1.5 and 5oC. Injury caused by harvest and piling operations is substantial, with typical root injuries including root breakage, splitting, surface abrasions, cuts, loss of small fragments, and bruising. Root injury increases respiration rate within 24 hours and causes root respiration rate to be elevated throughout the duration of storage (Wyse & Peterson, 1979; Wiltshire & Cobb, 2000). Moreover, injury increases the incidence of storage disease since the two major storage diseases of sugarbeet roots, Botrytis and Penicillium, require a wound in the epidermis and exposure of internal tissues to establish infection (Mumford & Wyse, 1976). Storage diseases also increase root respiration rate, with the increase in root respiration rate proportional to the surface area of the infection (Mumford & Wyse, 1976).

While the environmental factors that affect sugarbeet root respiration rate are known, the internal factors regulating root respiration rate are unknown. In plants, respiration rate is regulated by substrate availability, total respiratory activity, or energy status of the cell (Klotz et al., 2008; Shugaev & Bukhov, 1997). In sugarbeet root, the mechanism by which respiration rate is controlled has not been established. Studies, however, have demonstrated that root respiration rate is not associated with total respiratory activity or energy status of the cell. The lack of association between root respiration rate and total respiratory activity and cellular energy status suggests that respiration is regulated by the availability of respiratory substrates (Klotz et al., 2008).

Understanding the processes involved in the generation of substrates for respiration is, therefore, extremely important to understanding postharvest sucrose loss in sugar beet roots. The metabolic reactions involved in sucrose catabolism are well known. Sucrose is cleaved by one of three sucrolytic enzymes – sucrose synthase, acid invertase or alkaline invertase – and the products of these enzyme activities are catabolized by glycolysis to provide substrates for the TCA cycle, which provides substrates for the electron tranfer chain (Siedow & Day, 2000; Klotz & Finger, 2004). While sucrolytic enzymes have been studied in considerable detail, few studies have examined the activity of glycolytic enzymes and the flux of carbon substrates through this pathway. The paucity of studies dealing with the glycolytic pathway is surprising considering its central role in plant metabolism and its ability to limit respiration and anabolic pathways by restricting the availability of substrates for these processes.

In sugarbeet root, little information of glycolytic enzyme activities and their changes during storage is known. To date, hexokinase, fructokinase, phosphofructokinase and pyruvate kinase activities have been determined in response to wounding (Klotz et al., 2006), hexokinase, phosphofructokinase and pyruvate kinase were determined in dormant and sliced and aged roots (Moorhead & Plaxton, 1988), and hexokinase, fructokinase, and glucose 6-phosphate isomerase activities have been determined after storage at unusually high temperatures (Sakalo & Tyltu, 1997). These studies determined the activities of the glycolytic enzymes that have traditionally been considered

regulatory of the pathway. Current research, however, has questioned the regulatory importance of those reactions and suggested that other enzymes in the pathway may contribute to the regulation of glycolysis. Thus, determination of activity of all glycolytic enzymes and measurement of the flux of carbon through glycolysis is a necessary step to better understand the glycolytic pathway in sugarbeet roots.

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CHAPTER 1

GLYCOLYTIC ENZYME ACTIVITY IN ROOTS OF SUGARBEET

ABSTRACT

Glycolysis is ubiquituous in nearly all organisms and serves as the primary pathway for carbohydrate catabolism. The oxidative pentose phosphate pathway (OPPP) provides an alternative pathway for carbohydrate catabolism. Glycolysis and the OPPP operate somewhat independently in plant cells, but interact through common intermediates. In most plants and plant organs, however, and in sugarbeet root in particular, glycolysis, is the predominant pathway for hexose catabolism. Control of glycolysis in plants is not well understood. Little is known about the activities of glycolytic enzymes or the regulation of glycolysis in sugarbeet root. The present study determined the activity of glycolytic enzymes in sugarbeet roots throughout 100 days of storage. The purpose of this work was to provide basic information for understanding the activities of the enzymes involved in glycolysis, their relative activities, and the effect of storage on them. Sugarbeet hybrid VDH66156 was greenhouse grown for 16 weeks, when roots were harvested, gently hand washed, and kept at 10^{o}C and 90 ± 5% relative humidity for 10 days, then incubated at 4^{o}C, 90 ± 5% relative humidity for up to 100 days. Respiration rate of individual roots was determined at 10^{o}C after 0, 1, 2, 3, 4, 7, 10, 30, 60 and 100 days of storage, then tissue samples were flash frozen in liquid nitrogen, lyophilized, ground to a fine powder, and stored at −80^{o}C until analysis of enzymatic activity. Respiration rate was nearly 23 mg CO_2 kg^{-1} h^{-1} after harvest, but declined 65% after one day of storage. A second decline of 55%, occurred during the first week in storage to a rate of 3.6 mg CO_2 kg^{-1} h^{-1}, after which respiration rate was practically. The high activities observed for TPI, UDPase, G6PI and PGM suggest that the reactions catalyzed by these enzymes occur readily. The high activities observed for G6PI and PGM suggest that the hexose phosphates, fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate, are readily

interconverted. Low relative activities were found for PFK, ALD, GAPDH, PGK, PGlyM. Interestingly, these enzymes catalyze the sequential reactions catalyzing the conversion of fructose 6-phosphate to 2-phosphoglycerate. Very low activities were observed for HK, FK and PFP, enzymes catalyzing the first reaction in the glycolytic pathway (HK and FK) and a bypass of the reaction catalyzed by PFK. Low PFP activity is perhaps not surprising, since little glycolytic flux typically occurs through this enzyme (Dennis & Blakely, 2000).

1. INTRODUCTION

Glycolysis is the metabolic pathway that converts hexose sugars to pyruvate, releasing energy and generating substrates for the tricarboxylic acid cyle, respiration and the biosynthesis of biological compounds including amino acids, fatty acids, nucleic acids, phenolic compounds, and alkaloids. Overall, glycolysis catalyzes the following reaction:

$$\text{glucose} + 2\ \text{ADP} + 2\ P_i + 2\ NAD^+ \rightarrow 2\ \text{pyruvate} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ H^+$$

Glycolysis is ubiquituous in nearly all organisms and serves as the primary pathway for carbohydrate catabolism. The oxidative pentose phosphate pathway (OPPP) provides an alternative pathway for carbohydrate catabolism. Glycolysis and the OPPP operate somewhat independently in plant cells, but interact through the common intermediates, glucose 6-phosphate, fructose 6-phosphate, and glyceraldehyde 3-phosphate (Bowsher et al., 2008). In most plants and plant organs, however, and in sugarbeet root in particular, glycolysis, is the predominant pathway for hexose catabolism (ap Rees, 1980; Wang & Barbour, 1961).

Fourteen enzymes catalyze the 10 chemical reactions of glycolysis with 9 distinct intermediates (Table 1, Fig. 1). In plants, the number of participating enzymes is greater than the number of reactions since glycolysis can utilize two substrates, glucose and fructose, and three of the pathway's reactions are catalyzed by two enzymes. Glycolysis requires 5 cofactors, ATP, ADP, NAD+, phosphate, and pyrophosphate. The pathway consumes energy in the form of ATP in its initial two steps, and produces 4 ATP in later reactions for an overall net energy yield of two ATP and two NADH molecules per hexose oxidized (Nelson & Cox, 2008).

Sucrose catabolism provides the substrates for glycolysis in sugarbeet root (Barbour & Wang, 1961). Sucrose catabolism is catalzyed by three enzymes in plants (Fig. 1): acid invertase (E.C. 3.2.1.26), alkaline invertase (E.C. 3.2.1.53), and sucrose synthase (E.C. 2.4.1.13, SuSy). In sugarbeet roots, all the three enzymes are found, although sucrose catabolism is thought to be largely catalyzed by SuSy (Echeverria & Gonzalez, 2003; Klotz & Finger, 2004). SuSy is a cytoplasmatic enzyme that catalyses the reaction of uridine 5'-

diphosphate (UDP) with sucrose, generating fructose and UDP-glucose. The fructose formed in this reaction can readily be used as a substrate for glycolysis. UDP-glucose can also be utilized as a glycolytic substrate after its conversion to glucose 6-phosphate by the combined activities of UDP-glucose pyrophosphorylase (UDPase) and phosphoglucomutase (PGM) (Leigh et al., 1979). UDPase is a cytoplasmic enzyme that catalyzes the reversible reaction of UDP-glucose with pyrophosphate to form glucose 1-phosphate and UTP. PGM catalyzes the conversion of glucose 1-phosphate to glucose 6-phosphate. Although reversible, the equilibrium constant of PGM favors glucose 6-phosphate formation (Dennis et al., 1997).

Table 1. Glycolytic enzymes, Enzyme Commission (E.C.) numbers and abbreviations used.

Enzyme	**E.C.**	**abbreviation**
Aldolase	4.1.2.13	ALD
ATP-dependent phosphofructokinase	2.1.7.11	PFK
Enolase	4.2.1.11	ENO
Fructokinase	2.7.1.4	FK
Glucose 6-phosphate isomerase	5.3.1.9	G6PI
Glyceraldehyde 3-phosphate dehydrogenase	1.2.1.12	GAPDH
Nonphosphorylating glyceraldehyde 3-phosphate dehydrogenase	1.2.1.13	NGAPDH
Hexokinase	2.7.1.1	HK
Phosphoglucose mutase	5.4.2.2	PGM
Phosphoglycerate kinase	2.7.2.3	PGK
Phosphoglycerate mutase	2.7.5.3	PGlyM
Phosphoenolpyruvate phosphatase	3.1.3.60	PEPase
Pyrophosphate-dependent phosphofructokinase	2.7.1.90	PFP
Pyruvate kinase	2.7.1.40	PK
Triose phosphate isomerase	5.3.1.1	TPI
UDP-glucose pyrophosphorylase	2.7.7.9	UDPase

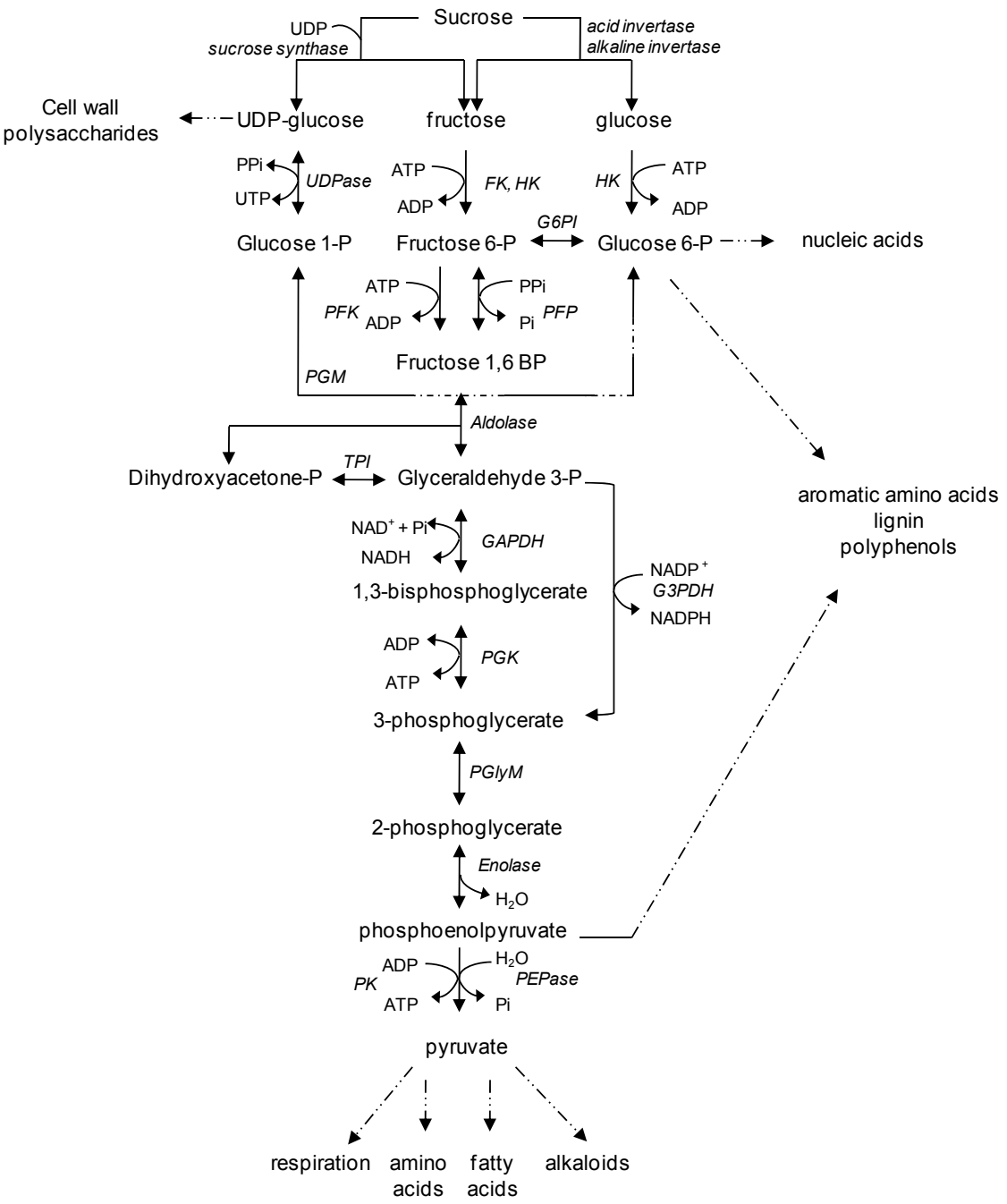

Fig. 1. Schematic representation of glycolysis, including the enzymes that provide substrates for the pathway and the biological compounds synthesized from the pathway.

Glycolysis starts with the phosphorylation of the hexose sugars, fructose and glucose, to glucose 6-phosphate and fructose 6-phosphate by the action of hexokinase (HK) and fructokinase (FK), respectively. Both enzymes catalyze irreversible reactions and consume ATP. HK and FK activities exhibit substrate specificities that allow independent regulation of glucose and fructose utilization

(Renz & Stitt, 1993). Hexokinases are highly reactive with glucose and limitedly reactive with fructose; fructokinases react specifically with fructose (Claeyssen & Rivoal, 2007). In addition, HK can act as a hexose sensor and mediate changes in gene expression in response to carbohydrate status (Rolland et al., 2002). The glucose 6-phosphate formed by HK reaction is converted to fructose 6-phosphate by the action of glucose 6-phosphate isomerase (G6PI), a reversible enzyme with an equilibrium constant that slightly favors glucose 6-phosphate formation (Dennis et al., 1997).

Fructose 6-phosphate is converted to fructose 1,6-bisphosphate by the activities of ATP-dependent phosphofructokinase (PFK) and pyrophosphate-dependent phosphofructokinase (PFP). PFK, however, is primarily responsible for fructose 1,6-bisphosphate formation in most plant cells (Dennis & Blakely, 2000). PFK catalyses an irreversible reaction and is an important control point in the glycolytic pathway (Bowsher et al., 2008). In contrast, PFP, located exclusively in the cytosol, catalyzes a readily reversible reaction with an equilibrium constant that favors fructose 1,6 bisphosphate formation and operates near equilibrium in vivo (Plaxton, 1996; Dennis et al., 1997).

Aldolase (ALD) catalyzes an aldol cleavage of fructose 1,6-bisphosphate (F1,6BP) to form glyceraldehyde 3-phosphate and dyhydroxyacetone, two compounds that are interconverted by the action of triose phosphate isomerase (TPI). Both ALD and TPI catalyze reversible reactions with equilibrium constants that strongly favor F1,6BP and dihydroxyacetone formation, respectively (Dennis et al., 1997). Since the aldolase reaction equilibrium favors the reverse reaction, TPI has the important role of pulling the ALD reaction forward by keeping glyceraldehyde 3-phosphate concentrations low. Reactions in the lower part of the glycolytic pathway also consume glyceraldehyde 3-phosphate, and function to drive the ALD reaction forward (Bowsher et al., 2008).

Glyceraldehyde 3-phosphate is converted to 1,3-bisphosphoglycerate by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The reaction links the oxidation of glyceraldehyde 3-phosphate to the reduction of NAD and requires inorganic phosphate (Bowsher et al., 2008). The GAPDH reaction is readily reversible with an equilibrium that favors glyceraldehyde 3-phosphate by about 10:1 over 1,3-bisphosphoglycerate (Dennis et al., 1997). The enzyme

phosphoglycerate kinase (PGK) transfers a phosphate group from 1,3-bisphosphoglycerate to ADP. The reaction produces ATP and 3-phosphoglycerate and is the first of two ATP-generating reactions in glycolysis (Dennis et al., 1997; Nelson & Cox, 2008). While reversible, PGK reaction favors 3-phosphoglycerate formation. A bypass of the ATP-forming PGK reaction is catalyzed by nonphosphorylating glyceraldehyde 3-phosphate dehydrogenase (NGAPDH). NGAPDH catalyzes the oxidation of glyceraldehyde 3-phosphate to 3-phosphoglycerate with concommittal reduction of NADP+ to NADPH (Dennis et al., 1997).

Phosphoglycerate mutase (PGlyM) catalyzes the interconversion of 3-phosphoglycerate and 2-phosphoglycerate (Nelson & Cox, 2008). The 2-phosphoglycerate formed by PGlyM reaction is dehydrated by enolase (ENO) to produce the high-energy compound phosphoenolpyruvate (PEP) (Bowsher et al., 2008). Both PGlyM and ENO catalyze readily reversible reactions.

In the final reaction of glycolysis, PEP is converted to pyruvate by pyruvate kinase (PK). The phosphate attached to the 2-position of PEP has a high negative free energy of hydrolysis and is transferred to ADP with the resulting formation of ATP and pyruvate in an irreversible reaction (Bowsher et al., 2008). Conversion of PEP to pyruvate can also be catalyzed by phosphoenolpyruvate phosphatase (PEPase). PEPase cleaves the phosphate group from PEP without ATP formation. PEPase, however, is located in the vacuole and inhibited by inorganic phosphate and is thought to be inactive under normal cellular conditions (Dennis et al., 1997).

Control of glycolysis in plants is not well understood. In plants, PFK is thought to have a central role in the regulation of the pathway, with its activity regulated by glycolytic downstream products and intermediates. PFK activity is inhibited by low concentrations of PEP, moderate concentrations of 3-phosphoglycerate and 2-phosphoglycerate, and ATP. It is activated by inorganic phosphate and Mg+2. The major factor controlling PFK activity, however, is thought to be the ratio of PEP: Pi (Givan, 1999). The central role of PFK in regulating glycolytic flux, however, is uncertain. Several studies have demonstrated that alterations in PFK activitiy has little or no effect on glycolytic flux (Burrell et al., 1994, Thomas et al., 1997). Other studies have suggested

that other glycolytic enzymes contribute to glycolytic control (Hatzfeld et al., 1989), and a growing body of evidence suggests that all enzymes of a pathway incrementally contribute to pathway regulation (Geigenberger et al., 2004).

Little is known about the activities of glycolytic enzymes or the regulation of glycolysis in sugarbeet root. In sugarbeet roots, hexokinase, fructokinase, phosphofructokinase, pyrophosphate dependent phosphofructokinase, aldolase, glucose 6-phosphate isomerase and pyruvate kinase activities have been determined in response to wounding and temperature (Klotz et al., 2006; Moorhead & Plaxton, 1988; Sakalo & Tyltu, 1997). The present study intends to determine the activity of glycolytic enzymes in sugarbeet roots throughout 100 days of storage. The purpose of this work is to provide basic information for understanding the activities of the enzymes involved in glycolysis, their relative activities, and the effect of storage on them.

2. MATERIAL AND METHODS

2.1. Plant material and postharvest treatments

Sugarbeet hybrid VDH66156 (VanDerHave, Rillands, Netherlands) was greenhouse grown in 15 L pots with supplemental light under a 16 h light/8 h dark regime, in United States Department of Agriculture (USDA). Roots were harvested 16 weeks after planting, gently hand washed, and kept at 10^{o}C and 90 ± 5% relative humidity for 10 days, then incubated at 4^{o}C, 90 ± 5% relative humidity for up to 100 days. Samples were collected after 0, 1, 2, 3, 4, 7, 10, 30, 60 and 100 days of storage. Longitudinal root sections, comprising approximately one quarter of the root and containing crown and root tissue that was representative of the whole root, were collected at each time point, with 7 replicate roots collected at each time point. Tissue samples were flash frozen in liquid nitrogen, lyophilized, ground to a fine powder, and stored at −80^{o}C until analysis. The experiment was conducted twice.

2.2. Respiration rate determination

Respiration rate of individual roots was determined at 10^{o}C by infrared CO_2 analysis using an open system with a continuous airflow of 1000 μmol s^{-1}. Respiration rates were determined using a LICOR 6400 gas analyzer (Lincoln, NE, USA) modified for use with a 7 L sample chamber (Haagenson et al., 2006). For respiration rate determinations at 30, 60 and 100 days, roots were equilibrated at 10^{o}C for 2 days prior to measurement.

2.3. Protein extraction and quantification

Soluble proteins were extracted using 0.1 g of lyophilized tissue and 1 mL extraction buffer, with all extraction steps conducted at 4^{o}C. The extraction buffer contained 100 mM HEPES-NaOH, pH 7.5, 5 mM DTT, 1 mM EDTA, 0.5% Triton X-100, and 0.5% BSA when extracts were assayed for phosphoenolpyruvate phosphatase activity. For all other assays, the extraction buffer contained 100 mM HEPES-NaOH, pH 7.5, 2 mM MgCl2, 5 mM

dithiothreitol (DTT), and 20 mM Na_2SO_3. Tissue and extraction buffer were mixed by vortexing and homogenized by sonication for 10 min at 4°C. Extracts were clarified by centrifuging for 20 min at 17,000 x *g* and the supernatants were desalted by passage over a Sephadex G-25 (GE-Health Care Bio Sciences AB, Uppsala, Sweden) which was pre-equilibrated with 10 mM Hepes-NaOH (pH 7.2). Desalted extracts were centrifuged for 1 min at 2000 rpm at 4°C.

Total protein concentration was determined using the Bio-Rad Protein Assay Kit (Hercules, CA, USA) with bovine serum albumin as a standard. Protein assays for each replicate were performed in triplicate.

2.4. Enzyme activity assays

Enzyme activity assays were performed using modifications of the protocols of Moorhead & Plaxton, 1988 (HK, FK, PFK, and PK), Davies et al., 2003 & Manjunath et al., 1998 (PGM), Burrell et al., 1994 (G6PI, ALD, TPI, PGlyM, PGK, ENO, PEPase, PFP, and UDPase), and Plaxton, 1990, (GAPDH). All enzyme assays had a final volume of 180 µL and were performed in triplicate at 25°C by monitoring NADH oxidation or NAD^+ reduction at 340 nm using a SpectraMax Plus microplate spectrophotometer (Molecular Devices Corp., Union City, CA). Assay components are as follows. HK: 125 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 7 mM glucose, 1.5 mM NAD, 2 U glucose 6-phosphate dehydrogenase, and 0.25 mM ATP; FK: 125 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 3 mM fructose, 1.5 mM NAD, 2U glucose 6-phosphate dehydrogenase, 6U phosphoglucose isomerase, and 0.25 mM ATP; PFK: 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 2 mM EDTA, 2 mM fructose 6-phosphate, 0.1 mM NADH, 2U aldolase, 2U triose phosphate isomerase, 5 U glycerol 3-phosphate dehydrogenase, and 0.12 mM ATP; PK: 50 mM HEPES-NaOH (pH 7.0), 50 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 0.4 mg mL^{-1} BSA, 1 mM phosphoenolpyruvate, 0.075 mM NADH, 26 U lactate dehydrogenase, and 1 mM ADP; PGM: 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 30 uM glucose 1,6-bisphosphate, 0.5 mM NAD, 2U glucose 6-phosphate dehydrogenase, and 0.9 mM glucose 1-phosphate; G6PI: 75 mM Gly-Gly (pH 8.5), 10 mM $MgCl_2$, 1 mM NAD, 1 mM fructose 6-phosphate, 0.5U glucose 6-phosphate dehydrogenase; ALD: 40 mM HEPES-NaOH (pH 7.7), 0.1 mM NADH, 5 mM fructose 1,6-bisphosphate, 1.7U glycerol 3-phosphate dehydrogenase, 17 U triose phosphate

isomerase; TPI: 100 mM HEPES-NaOH (pH 8.0), 5 mM EDTA, 0.2 mM NADH, 1.5 mM DL-glyceraldehyde 3-phosphate, and 1 U glycerol 3-phosphate dehydrogenase; PGK: 100 mM HEPES-NaOH (pH 7.6), 1 mM EDTA, 2 mM $MgSO_4$, 0.2 mM NADH, 6.5 mM 3-phosphoglycerate, 1 mM ATP, and 3.3 U glycerol 3-phosphate dehydrogenase; PGlyM: 100 mM Tris-HCl (pH 7.6), 10 mM $MgSO_4$, 2.7 mM ADP, 0.2 mM NADH, 3 mM 3-phosphoglycerate, 1 U enolase, 5 U pyruvate kinase, 6 U lactate dehydrogenase, and 50 mM 3-phosphoglycerate; ENO: 100 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM NADH, 2.7 mM ADP, 0.5 mM 2-phosphoglycerate, 5 U pyruvate kinase, 6 U lactate dehydrogenase, and 10 mM 2-phosphoglycerate; PEPase: 50 mM Tris-HCl (pH 7.5), 1 mM phosphoenolpyruvate, 4 mM $MgCl_2$, 0.2 mM NADH, and 3 U lactate dehydrogenase; PFP: 100 mM Tris-HCl (pH 8.0), 5 mM fructose 6-phosphate, 2 mM sodium pyrophosphate, 5 mM $MgCl_2$, 0.20 mM NADH, 1U aldolase, 1.3 U glycerol 3-phosphate dehydrogenase, and 10 U triose phosphate isomerase; UDPase: 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1.6 mM NAD, 0.8 mM UDP-glucose, 4 U phosphoglucomutase, 4 U glucose 6-phosphate dehydrogenase, and 0.4 mM sodium pyrophosphate; GAPDH: 100 mM Tris-HCl (pH 7.8), 4.5 mM 3-phosphoglycerate, 8 mM $MgSO_4$, 0.32 mM NADH, 2 mM ATP, 1 mM EDTA, 2 mM DTT, and 1.8 U 3-phosphoglycerate kinase. Assay reactions were initiated by the addition of ATP for HK, FK and PFK; ADP for PK assay; glucose 1-phosphate for PGM; 3-PGA for PGlyM; 2-PGA for ENO; sodium pyrophosphate for UDPase; and protein extract for G6PI, ALD, TPI, PGK, PEPase, PFP, and GAPDH. See appendix 1, 2 and 3 for more details.

2.5. Statistical analysis

Experiment was conducted in a completely randomized design. Results were analyzed by ANOVA and the means compared with the Scott-Knott test at significance degree α = 0.05 using SAS statistical software (SAS 9.1, SAS Institute, Inc., Cary, NC, USA). Principal component of analysis (PCA) was performed using standardized data and SAS statistical software.

3. RESULTS AND DISCUSSION

3.1. Storage period effects on root respiration rate

Respiration rate was nearly 23 mg CO_2 kg^{-1} h^{-1} after harvest, but declined 65% after one day of storage (Fig. 2). Respiration rate declined another 55% during the first week in storage to a rate of 3.6 mg CO_2 kg^{-1} h^{-1}. Between 7 and 60 days in storage, respiration rate was practically constant. After 100 days storage, respiration rate increased by 48%.

The high respiration rate observed after harvest is likely due to both temperature and injury effects on respiration rate. Although the temperature of roots after harvest was not determined, root temperature was greater than 10 ^{o}C since roots did not have sufficient time to equilibrate to their storage temperature, and respiration rate is known to be affected by temperature (Wyse, 1978). Injury may also have contributed to the high respiration rate after harvest as well as during the first week of storage. Although roots were gently hand harvested, injury was inevitable, as roots were scalped to remove all leaves and vegetative buds from the root crown, lateral roots were removed during harvest and the lower portion of the root tail was excised. Many studies have demonstrated that root injury increases root respiration rate, with the greatest impact of injury on root respiration rate occurring in the first three days after injury (Dilley et al., 1970; Akeson, et al., 1974; Cole, 1977; Wyse & Peterson, 1979).

That respiration rate declined during the first week of storage is probably due to healing of harvest-related injuries during the first week. Ibrahim et al. (2001) have shown that most wound-healing processes occur within the first 9 days after injury. Wound healing in sugarbeet root involves the deposition of polymers including lignin and suberin to seal off wound sites. Biosynthesis of these compounds prevents dehydration and restricts entry by microorganisms, but requires substrates and energy which are provided by the respiratory pathway.

The cause for the increase in respiration rate at 100 days in storage is unknown, but may be the result of dehydration. Dehydration is common during

postharvest storage and is known to increase root respiration rate (Lafta & Fugate, 2009). The increase in respiration is not thought to be related to the development of storage diseases since no visual signs of fungal or bacterial contamination were evident.

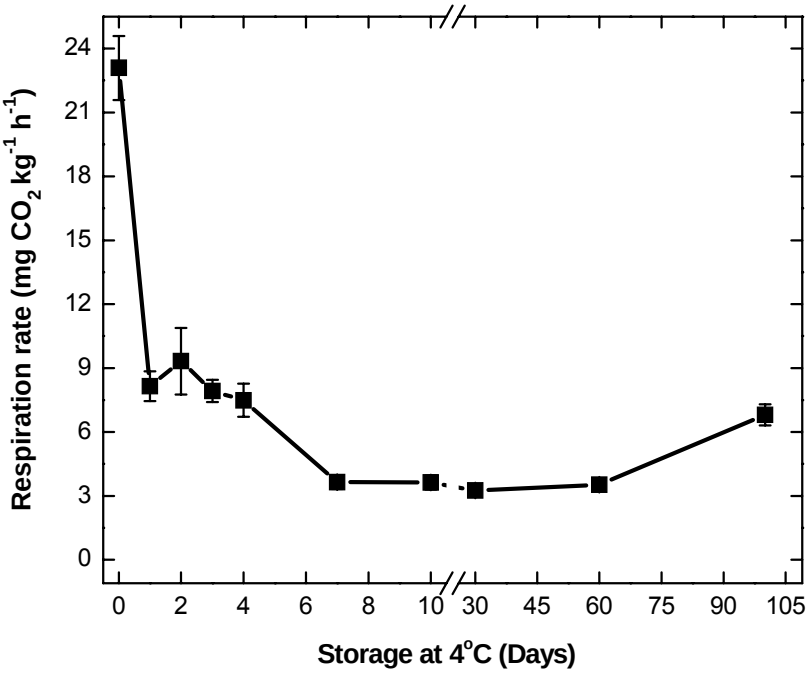

Fig. 2. Respiration rate of sugarbeet roots (VDH66156) storage for 10 days at 10°C, followed by 4°C until 100 days. Respiration rate were determined after equilibrium of temperature at 10°C. Vertical bars represent the standard error of the mean (*n*=7).

3.2. Enzymatic activity

Activities of the enzymes of the glycolytic pathway and UDP-glucose pyrophosphorylase were determined throughout 100 days storage. Prior to determining activities, all enzyme activity assays were optimized for substrate concentrations and checked for linearity.

Considerable variations were observed between the activities of individual enzymes (Table 2). Mean activities varied by more than 7000-fold and ranged from 0.9 µmol min^{-1} g^{-1} protein for PFP to 6608 µmol min^{-1} g^{-1} protein for TPI. Enzymes involved in glycolysis of sugarbeet roots were separated into five

groups by activity level: 1) very high (higher than 500 μmol min^{-1} g^{-1} protein); 2) high (101 - 500 μmol min^{-1} g^{-1} protein); 3) moderate (26 - 100 μmol min^{-1} g^{-1} protein); 4) low (2.1 - 25 μmol min^{-1} g^{-1} protein); 5) very low (≤ 2.0 μmol min^{-1} g^{-1} protein).

Table 2. Clustering of glycolytic enzymes by catalytic activity in sugarbeet roots

Classification	Enzyme	Activity (μmol min^{-1} g^{-1} protein)	
		Mean	Range
Very High	TPI	6608	5680 – 7271
High	UDPase	362	287 – 507
	G6PI	245	168 – 334
	PGM	103	93 – 113
Moderate	PK	71	61 – 84
	ENO	30	27 – 34
Low	PGK	16	12 – 21
	ALD	15	14 – 16
	PGlyM	13	10 – 16
	PFK	4.9	4.2 - 5.5
	GAPDH	3.9	3.4 - 4.3
	PEPase	3.0	2.6 – 3.4
Very low	HK	2.0	1.4 - 2.3
	FK	1.0	0.9 - 1.2
	PFP	0.9	0.6 - 1.0

The high activities observed for TPI, UDPase, G6PI and PGM suggest that the reactions catalyzed by these enzymes occur readily. The high activities observed for G6PI and PGM suggest that the hexose phosphates, fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate, are readily interconverted, as has previously been suggested by Kruger (1997). Low relative activities were found for PFK, ALD, GAPDH, PGK, PGlyM. Interestingly, these enzymes catalyze the sequential reactions catalyzing the conversion of fructose 6-phosphate to 2-phosphoglycerate. Very low activities were observed for HK, FK and PFP, enzymes catalyzing the first reaction in the glycolytic

pathway (HK and FK) and a bypass of the reaction catalyzed by PFK. Low PFP activity is perhaps not surprising, since little glycolytic flux typically occurs through this enzyme (Dennis & Blakely, 2000).

The enzymatic activities of sugarbeet roots, presented in Fig. 3, 4, 5 and appendix 4, are represented individually for UDPase and glycolytic enzymes. UDPase activity generally declined between 0 – 4 days, and was reduced nearly 40% by 4 days in storage. For the remaining 100 days of the storage period, UDPase activity was practically constant. The activity ranged from 508 μmol min^{-1} g^{-1} protein at harvest to 290 μmol min^{-1} g^{-1} protein at 100 days of storage (Fig. 3).

Hexokinase activity was elevated from levels at harvest from day 2 - 10 of the storage period (Fig. 3). Highest HK activity occurred on the third day of storage increasing nearly 16% over its activity at harvest. With subsequent storage, HK activity declined by 20%. HK activity was, on average 2-fold greater than FK. Fructokinase activity exhibited a similar reaction to storage duration as that observed for HK (Fig. 3). FK activity was increased from harvest levels between days 2 - 30 of the storage period. Maximal FK activity occurred on the third day after storage reaching 1.30 μmol min^{-1} g^{-1} protein, i.e., 26% higher than that at harvest. After 30 days storage, FK activity declined and was 12% lower than levels at harvest. The end-products of the HK and FK reactions, glucose 6-phosphate and fructose 6-phosphate, were interconverted by glucose 6-phosphate isomerase at relatively constant enzymatic activity throughout the first 10 days of storage (Fig. 3). With subsequent storage, G6PI activity, relative to that at harvest, declined by 20% at 30 days, increased by 26% at 60 days and declined by 36% at 100 days storage. Phosphoglucomutase activity, catalyzing the interconversion of glucose 1-phosphate and glucose 6-phosphate, was elevated from harvest levels by approximately 14% between 2 – 60 days of storage (Fig. 3). By 100 days, however, PGM activity returned to levels similar to those at harvest.

Fig. 3. Enzymatic activities (μmol min^{-1} g^{-1} protein) of UDP-glucose pyrophosphorylase (UDPase), hexokinase (HK), fructokinase (FK), phosphoglucose isomerase (G6PI), phosphoglucose mutase (PGM), ATP-dependent phosphofructokinase (PFK), pyrophosphate-dependent phosphofructokinase (PFP) in sugarbeet roots stored for 10 days at 10°C, followed by storage at 4°C for up to 100 days. Vertical bars represent the standard error of the mean (*n* = 7).

Phosphofructokinase activity increased during 2 - 10 days of storage (Fig. 3). Maximum PFK activity, 5.4 μmol min^{-1} g^{-1} protein, occurred after 3 days in storage, when activity increased by 13% relative to the activity at harvest. With continued storage, PFK activity returned to levels similar to those occurring after harvest. Pyrophosphate-dependent phosphofructokinase activity, which produces the same glycolytic intermediate as PFK, was statistically unchanged during the first four days in storage (Fig. 3). With continued storage, PFP activity declined stepwise, declining, on average, 11% from harvest levels in roots stored for 7 - 30 days, and declining, on average, 27% from harvest levels in roots stored 60 and 100 days. On average, PFK activity was 5.4 fold greater than PFP activity.

Aldolase activity was unchanged during storage, with activity at harvest and after 100 days of storarge equal to 15.6 μmol min^{-1} g^{-1} protein (Fig. 4). While some minor fluctuations in ALD activity occurred during the storage period, these were all statistically non-significant. Triose phosphate isomerase activity was generally increased throughout storage with TPI activity between 1 and 100 days elevated by an average of 11% (Fig. 4). At 60 days storage, a transient decrease in TPI activity was observed, although TPI activity was elevated after 30 and 100 days storage. Maximum TPI activity, 7271 μmol min^{-1} g^{-1} protein, occurred after 100 days of storage, activity was increasing by 21% relative to the activity at harvest. Glyceraldehyde 3-phosphate dehydrogenase activity did not change during storage. Minor fluctuations in GAPDH activity were observed but these were not significant. Phosphoglycerate kinase activity was constant for the first 4 days in storage, but increased 56% at day 7, relative to activity at harvest. PGK activity remained elevated for the remainder of the storage period (Fig. 4). Maximum PGK activity occurred at 7 days, when activity was 21.5 μmol min^{-1} g^{-1} protein.

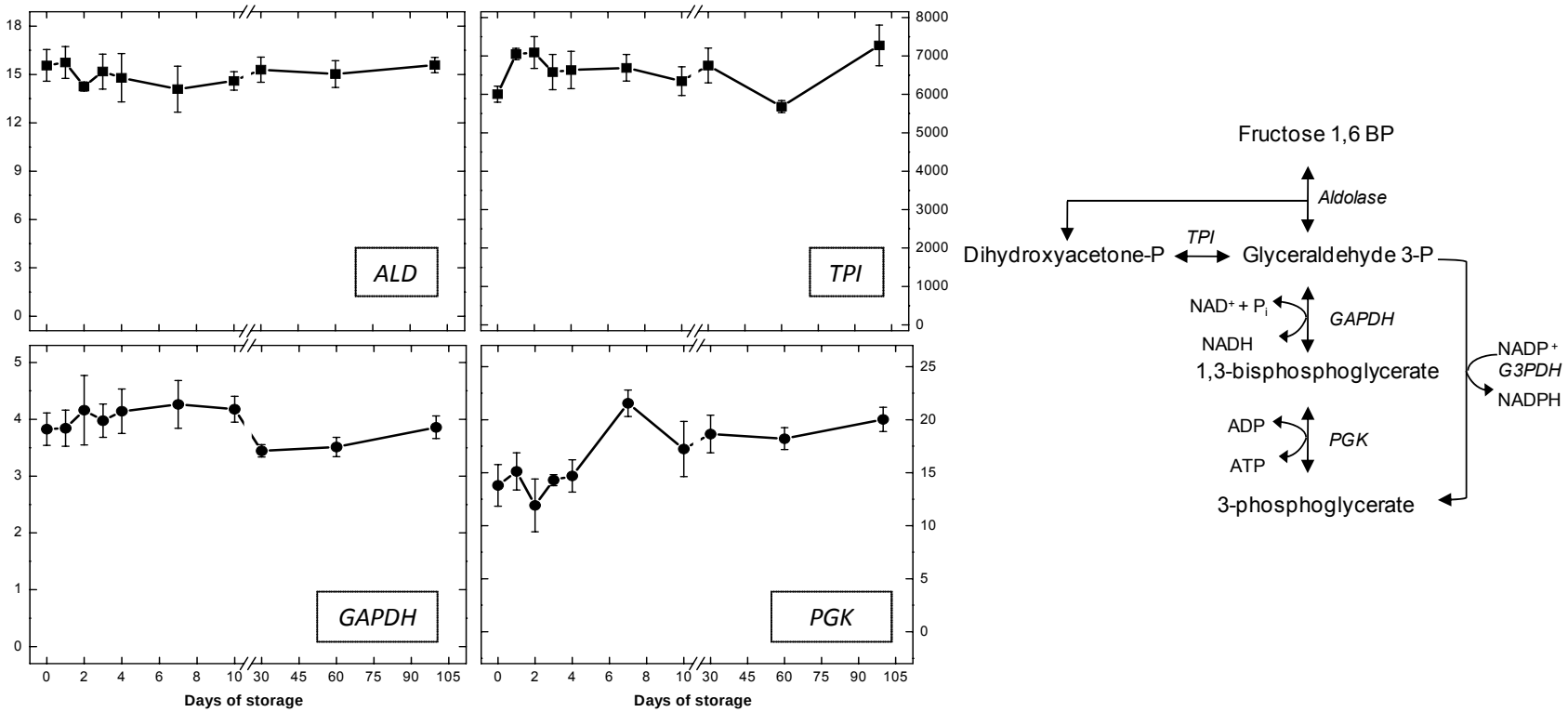

Fig. 4. Enzymatic activities (μmol min^{-1} g^{-1} protein) of aldolase (ALD), triose phosphate isomerase (TPI), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) in sugarbeet roots stored for 10 days at 10°C, followed by storage at 4°C for up to 100 days. Vertical bars represent the standard error of the mean (*n* = 7).

Phosphoglycerate mutase activity, which catalyzes the reversible conversion of 3-phosphoglycerate to 2-phosphoglycerate, was elevated throughout 100 days in storage, with the increase occurring in two steps. PGlyM activity increased 23% after one day in storage and generally remained elevated through day 30 (Fig. 5). At 60 days, PGlyM activity increased an additional 30% and remaining at this level through 100 days storage.

Enolase activity also exhibited a two-step increase during storage (Fig. 5). Enolase activity was constant between 0 - 2 days of storage, increased 12% in the third day in storage, and generally remaining at this elevated level through day 30 in the storage period. At 60 days, ENO activity increased an additional 11% and remained at this level through 100 days storage.

The irreversible conversion of phosphoenolpyruvate to pyruvate can occur by PK and PEPase activity. In the present study, PK activity transiently increased during days 1 to 4 of the storage period, with an average increase in activity of 31%, relative to activity at harvest (Fig. 5). A second, transient elevation in PK activity occurred between days 10 and 60 in the storage period when activity increased, on average, 8% relative to the activity at harvest. Maximum PK activity, 84.44 μmol min^{-1} g^{-1} protein, was observed 2 days after harvest. Phosphoenolpyruvate phosphatase activity also exhibited a transient increase in activity during days 1 to 4 of the storage period. The increase in PEPase activity, however, was much smaller than that observed for PK activity with an average 9% increase in PEPase activity during this period. With prolonged storage, PEPase activity increased. The increase in activity was first evident in roots stored for 60 days and continued until 100 days when activity was elevated by 30% relative to activity at harvest. PEPase activity, on average was 4% the activity of PK.

Similar changes in activity with respect to storage duration were noted for many glycolytic enzymes. ALD and GAPDH exhibited no statistic differences during the 100 days of storage. Several enzymes were elevated throughout most of the storage period, including PGM (2 - 60 days), TPI (1 -100 days, except for day 60), PGlyM (1 – 100 days), PGK (7 – 100 days), and ENO (3 -100 days).

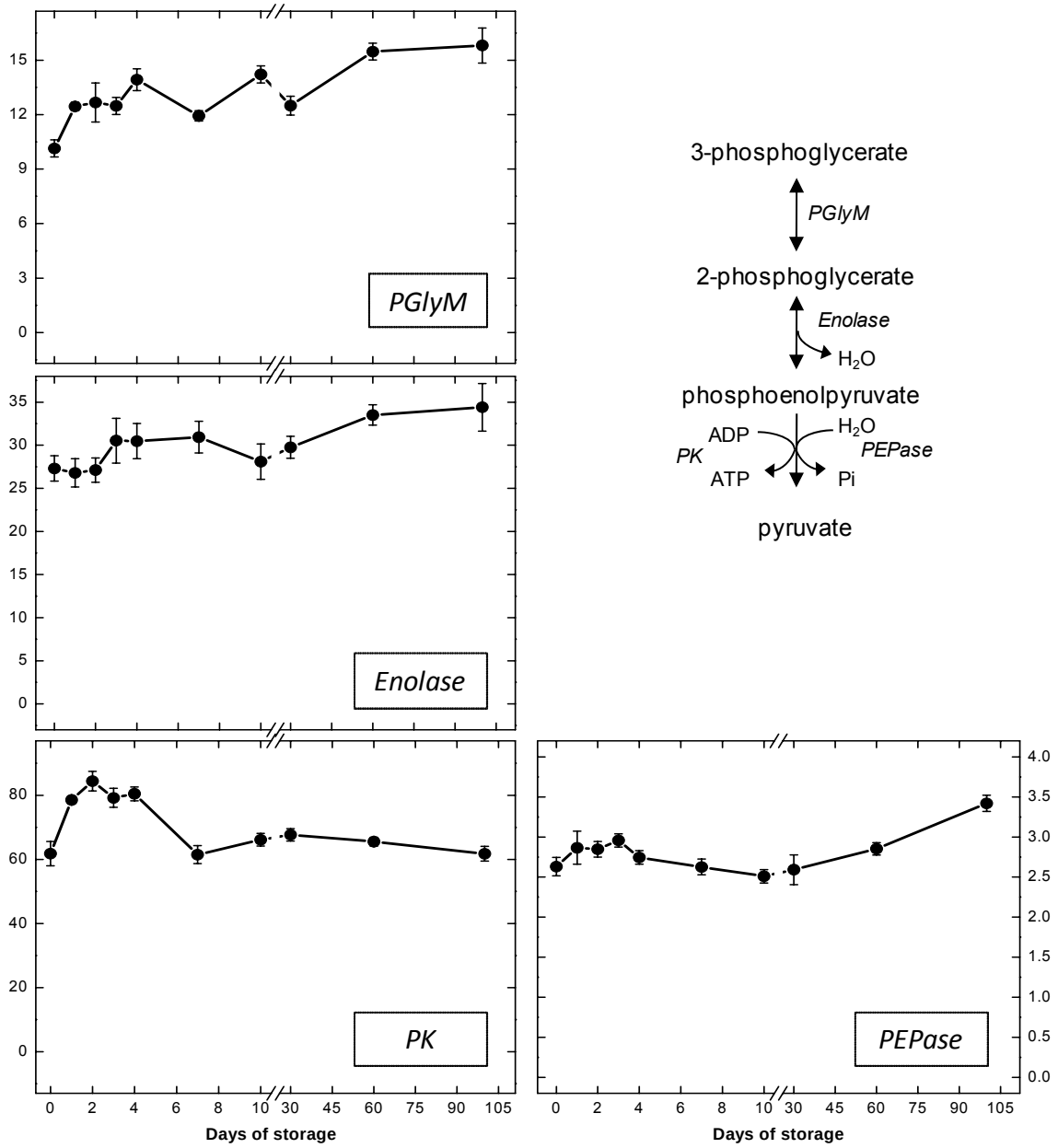

Fig. 5. Enzymatic activities (μmol min^{-1} g^{-1} protein) of phosphoglycerate mutase (PGlyM), enolase, pyruvate kinase (PK) and phosphoenolpyruvate phosphatase (PEPase) in sugarbeet roots stored for 10 days at 10°C, followed by storage at 4°C for up to 100 days. Vertical bars represent the standard error of the mean (*n* = 7).

HK, FK, and PFK activities all exhibited a transient increase in activity for days 2 through 10 of the storage period, with maximum activity on day 3. The increase in

activity was greatest for FK. FK activity increased as much as 26% relative to its activity at harvest; HK and PFK activities peaked at levels that were 16% and 13% of their activities at harvest. The elevation in FK activity also endured longer than was observed for HK and PFK activities, and FK activity remained elevated at 30 days storage. The activity of all three enzymes declined with prolonged storage, and by 100 days, HK and FK activities had decreased to levels below those occurring at harvest and PFK activity decreased to levels similar to those occurring at harvest.

Transient increases in HK, FK and PFK have previously been observed in severely wounded roots (Klotz et al., 2006). The increases in severely wounded roots exceeded those found in this study, which was conducted with minimally wounded roots. In severely wounded roots, HK, FK and PFK activities increased maximally by 60, 150, and 70%, respectively. The differences in magnitude of the elevation between the two studies may reflect differences in the intensity of wounding. In the previous study, it was postulated that HK, FK and PFK activities in wounded roots were elevated to meet the demand for glycolytic intermediates and products for wound-healing processes. Interestingly, in both studies, FK activity was elevated to a greater extent and for a longer portion of the storage period than HK or PFK activities. HK, FK and PFK were all present in stored sugarbeet roots at low concentrations, indicating that their activities may be limiting. All three enzymes catalyze irreversible reactions and have been suggested to have a role in the regulation of glycolysis, with PFK attributed a central role in the regulation (Plaxton, 1996).

PK and PEPase, both of which catalyze the conversion of phosphoenolpyruvate to pyruvate, exhibited similar changes in activity during the first week of storage. The activities of both enzymes increased during the first four days in storage and returned to levels similar to those at harvest by day 7, with PK activity increasing an average of 31% and PEPase increasing an average of 11%. A similar transient increase in PK activity has been previously observed in wounded roots during storage (Klotz et al., 2006). Formation of pyruvate is expected to proceed primarily by the action of PK. In this study, PK activity was, on average, 24-fold greater than PEPase activity. Moreover, PEPase is located in the vacuole, inhibited by inorganic phosphate at typical cellular concentrations, and thought to be inactive under normal cellular conditions (Dennis et al., 1997). Pyruvate kinase exhibited

moderate activity and was more than 30-fold, 60-fold and 15-fold greater than HK, FK, and PFK, respectively (Appendix 4). The high activity of PK in relation to these early glycolytic enzymes suggests this enzyme is not limiting in sugarbeet root. A similar conclusion was drawn in an earlier study with wounded roots (Klotz et al., 2006). PK, however, may have a key role in regulating glycolysis by affecting cellular PEP concentrations, as suggested by Plaxton (1996). PEP is a potent inhibitor of PFK activity and a reduction in PEP levels, brought about by an enhancement of PK activity, will stimulate PFK activity (Plaxton, 1996, Dennis et al., 1997).

While many similarities were observed between enzymes in the glycolytic pathway, no relationship was observed between root respiration rate and any glycolytic enzyme activity. Respiration in sugarbeet root is thought to be limited by the availability of respiratory substrates which are provided by the combined actions of sucrolytic, glycolytic, and TCA cycle pathway enzymes. Previous research has demonstrated that respiration rate is not associated with any sucrolytic enzymes (Klotz et al., 2006). The lack of association between respiration rate and any glycolytic enzymes suggests that glycolysis does not control the availability of respiratory substrate or that no single enzyme in the glycolytic pathway controls respiration rate. This is perhaps not surprising, since a growing body of evidence suggests that all enzymes of a pathway incrementally contribute to pathway regulation (Geigenberger et al., 2004).

3.3. Principal component analysis (PCA)

To better understand relationships between glycolytic enzymes, glycolytic enzyme activities were standardized to unit variance and subjected to principal component analysis. Principal component analysis (PCA) is a statistical tool that is often used to compare data sets and identify similar trends between them. The procedure is based on the assumption that correlations exist between data sets and attempts to reduce the number of variables in the data into a smaller number of principal components. The principal components are artificial variables that are obtained mathematically to best fit the data and have no real meaning. The procedure often can reveal the internal structure of the data and assist in explaining the variance in the data. Principal components were generated that explained 57% of the variation in the data. Principal component 1 accounted for 31% of the

variability in the data; principal component 2 accounted for 26% of the variability. The procedure used to calculate the principle components is shown in Appendix 5.

The distance between data points in a PCA analysis reflects the extent that data sets vary. Therefore, data points that are in close proximity to each other reflect data sets that are similar. In the PCA of sugarbeet root glycolytic enzymes, four distinct groupings of enzymes was apparent (Fig. 6).

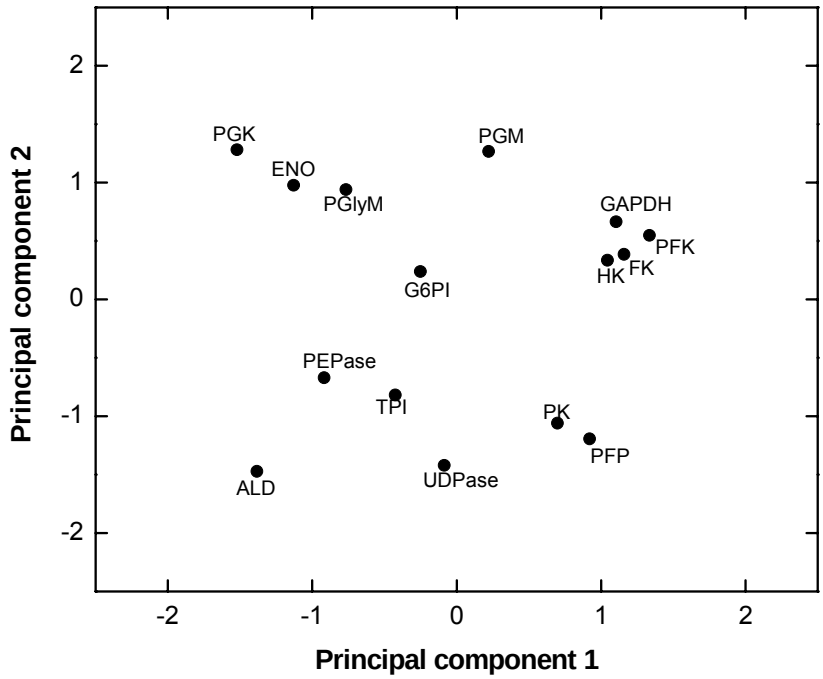

Fig. 6. Principal component analysis 1 and 2, for glycolytic enzymes and UDPase standardized as enzymatic activity of sugarbeet roots storage for 10 days at 10^{o}C, followed by 4^{o}C up to 100 days.

PCA suggested similar trends in the changes in HK, FK, PFK and GAPDH activities during storage. Similarities between HK, FK and PFK activities were apparent from Fig. 3 and those similarities were discussed above. Similarity in activity changes in these enzymes with those of GAPDH, however, are not obvious. A second cluster was comprised of PK and PFP. These two enzymes shared little similarity in their activity changes during the first 7 days of storage, but both enzyme levels declined from days 10 to 100 of the storage period. A third cluster included the enzymes, PGK, PGlyM, and ENO. Again similarities between these enzymes

were not obvious (Fig. 4 and 5), but on closer analysis, the activities of all of these enzymes were elevated from days 7 - 100 of the storage period. The last cluster contained PEPase and TPI. These enzymes had elevated activities during days 1 - 4 and 100 of the storage period. The significance of the similarities observed between the behaviors of different glycolytic enzymes during storage or revealed by PCA is unknown. However, similar changes in activity suggest that their expression may be regulated similarly.

CONCLUSIONS

- The high activities observed for UDPase, TPI, G6PI, and PGM suggest that their activities are probably not limiting.
- High activities of G6PI and PGM suggest that the hexose phosphates, fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate, are readly interconverted.
- PFK activity was 5.4 fold greater than PFP activity, indicating that most of the conversion of fructose 6-phosphate to fructose 1,6-phosphate was catalyzed by PFK.
- PK activity was, on average, 24-fold greater than PEPase activity suggesting that most of the conversion of phosphoenolpyruvate to pyruvate is catalyzed by PK.
- HK, FK and PFK were present at low activities throughout storage, and these low activities may limit the flow of carbon through the glycolytic pathway.
- HK, FK, and PFK activities exhibited similar changes during storage.
- ALD and GAPDH activity were constant during the storage period.
- PGM, TPI, PGK, PGlyM, and ENO activities generally increased during storage.
- The lack of correlation between any individual glycolytic enzyme and root respiration rate during storage suggests that glycolysis does not control the availability of respiratory substrate or that no single enzyme in the glycolytic pathway controls respiration rate.
- PCA identified 4 groups of enzymes that shared similarities in the manner in which their activities changed during storage. The similar changes in their activities suggest that their expression may be regulated similarly.

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CHAPTER 2

GLYCOLYTIC FLUX IN ROOTS OF SUGARBEET

ABSTRACT

The metabolic flux through glycolysis depends, in part, on the activity of the pathway's individual enzymes. Enzymatic activities are determined using optimum substrate concentrations, pH and temperature conditions and provide a measure of the quantities of active enzymes present. The quantity of enzyme present, measured as enzyme activity, is dependent on the rate of an enzyme's biosynthesis and degradation. Knowledge of the glycolytic enzyme activities in postharvest sugarbeet roots is useful, but may not accurately reflect the flux of carbon through the pathway. Flux through a pathway is usually determined by measuring the rate at which labeled compounds are metabolized. No studies, however, have examined the flux of carbon compounds through the glycolytic pathway in sugarbeet roots. The purpose of this work was to determine the flux of carbon through glycolysis in sugarbeet roots by following the catabolism of radiolabelled sucrose in tissue discs in response to temperature. Sugarbeet hybrid VDH66156 was greenhouse grown and roots were harvested, gently hand washed and kept at 10^{o}C and 90 ± 5% relative humidity for 10 days in Experiment 1, and at 4, 10 or 20^{o}C and 90 ± 5% relative humidity for 10 days for Experiment 2. Respiration rate was determined using whole roots. For labelling experiments, discs were collected with a cork borer. In Experiment 1, discs were incubated in 1 mL of 50 mM Mes-KOH (pH 6.5) and 70 kBq mL^{-1} [U-^{14}C] sucrose or in 50 mM Mes-KOH (pH 6.5), 70 kBq mL^{-1} [U-^{14}C] sucrose and 50 µM cycloheximide (CHI) at 20^{o}C for 3 or 5 hours. In Experiment 2, discs (14) were incubated in 1 mL of 50 mM Mes-KOH (pH 6.5), 70 kBq mL^{-1} [U-^{14}C] sucrose and 50 µM CHI for 5 hours at 4, 10 and 20 ^{o}C. Incubations were performed in petri dishs. $^{14}CO_2$ was trapped with 5 N KOH-impregnated paper filters cut to the same size as the lid. Plates in Experiment 2 were sealed with petroleum jelly. Plates were gently shaken on a rotary shaker during incubation. Soluble components were partitioned into neutral, basic, acidic and phosphate-ester

fractions. Sucrose, glucose and fructose concentrations were determined by HPLC. Most of the radiolabel incorporated by root tissue remained as sucrose and was unmetabolized. Glucose and fructose concentrations were greater in roots discs incubated at 4^{o}C than at 10 or 20^{o}C. The increase of the incubation temperature provided relative changes in the amounts of labelled glucose, fructose and phosphoester fraction, suggesting that increased temperature increases the flux of carbon through early glycolytic enzymes to a greater extent than sucrolytic enzymes or late glycolytic.

1. INTRODUCTION

The metabolic flux through glycolysis depends, in part, on the activity of the pathway's individual enzymes. Enzymatic activities are determined using optimum substrate concentrations, pH and temperature conditions and provide a measure of the quantities of active enzymes present. The quantity of enzyme present, measured as enzyme activity, is dependent on the rate of an enzyme's biosynthesis and degradation. The activities of glycolytic enzymes are believed to provide a coarse control of glycolysis in plants (Plaxton, 1996).

The optimum conditions used to determine enzyme activities, however, are not found living organisms, and enzyme activities in planta typically differ from those measured in vitro. Substrate concentrations, pH and temperature conditions deviate from those that produce maximum activity, and many glycolytic enzymes activities are affected by the concentrations of products, inhibitors, and activators, oligomerization, reversible modifications such as phosphorylation or reduction of internal disulfide bonds, and intracellular compartmentalization of sequential enzyme activities (Plaxton, 1996). For example, phosphofructokinase (PFK) activity is inhibited by phosphoenolpyruvate, 3-phosphoglycerate, 2-phosphoglycerate and ATP, and activated by inorganic phosphate and Mg+2. Phosphoenolpyruvate phosphatase (PEPase) activity is affected by intracellular compartmentalization and inhibited by inorganic phosphate. Enolase (ENO) activity is thought to be affected by phosphorylation (Miernyk & Dennis, 1992). The activity of another glycolytic enzyme, pyruvate kinase (PK) is activated by its substrate, phosphoenolpyruvate, inhibited by ATP, a product of its reaction, and affected by oligomerization (Kowallik et al., 1990; Plaxton, 1996).

Therefore, knowledge of the glycolytic enzyme activities in postharvest sugarbeet roots is useful, but may not accurately reflect the flux of carbon through the pathway. Flux through a pathway is usually determined by measuring the rate at which labeled compounds are metabolized (Dixon & ap Rees, 1980; Viola & Davies, 1994). Radiolabelled compounds have previously been used to elucidate the substrates and pathways utilized for sugarbeet respiration (Barbour & Wang, 1961; Wang & Barbour, 1961). These studies identified sucrose as the principal substrate for respiration and glycolysis as the principal pathway responsible for providing

substrates for the TCA cycle and respiration. No studies, however, have examined the flux of carbon compounds through the glycolytic pathway in sugarbeet roots. The purpose of this work was to determine the flux of carbon through glycolysis in sugarbeet roots by following the catabolism of radiolabelled sucrose in tissue discs in response to temperature.

2. MATERIAL AND METHODS

2.1. Plant material and postharvest treatments

Sugarbeet hybrid VDH66156 (VanDerHave, Rillands, Netherlands) was greenhouse grown in 15 L pots with supplemental light under a 16 h light/8 h dark regime. Roots of 1.0 to 1.5 kg were harvested, gently hand washed and kept at 10^{o}C and 90 ± 5% relative humidity for 10 days in Experiment 1, and at 4, 10 or 20^{o}C and 90 ± 5% relative humidity for 10 days for Experiment 2. Respiration rate was determined using whole roots with 3 and 6 replicates in Experiments 1 and 2, respectively. For labelling experiments, discs (5 x 3 mm, diameter x height) were collected with a cork borer, perpendicular to the roots' central axis. Tissue was collected at the widest portion of the root and contained the internal tissue between the epidermis and the central vascular cylinder. Experiments were conducted with 6 replicates comprised of 14 discs collected from a single root, with enough discs collected from each root to provide a replicate for each treatment. In Experiment 1, two replicates were collected per root; in Experiment 2, one replicate was collected per root. Discs were washed 3 times for 3 min in 3 mL of 50 mM Mes-KOH, pH 6.5, to remove debris prior to use.

2.2. Respiration rate determination

Respiration rate of individual roots was determined at the same temperature at which sugarbeet roots were stored by infrared CO_2 analysis using an open system with a continuous air flow of 1000 µmol s^{-1}. Respiration rates were determined using a LICOR 6400 gas analyzer (Lincoln, NE, USA) modified for use with a 7 L sample chamber (Haagenson et al., 2006).

2.3. Labelling experiments

Labeling experiments were performed by modification of the protocols of Viola & Davies (1994). Schematic diagram of the experiments is found in Fig. 1.

2.3.1. Tissue labelling

In Experiment 1, discs (14) were incubated in 1 mL of 50 mM Mes-KOH (pH 6.5) and 70 kBq mL^{-1} [U-^{14}C] sucrose (21.8 GBq/mmol, MP Biomedical, Solon, OH) or in 50 mM Mes-KOH (pH 6.5), 70 kBq mL^{-1} [U-^{14}C] sucrose and 50 µM cycloheximide (CHI) at 20^{o}C for 3 or 5 hours. In Experiment 2, fourteen discs were incubated in 1 mL of 50 mM Mes-KOH (pH 6.5), 70 kBq mL^{-1} [U-^{14}C] sucrose and 50 µM CHI for 5 hours at 4, 10 and 20 ^{o}C. Incubations were performed in petri dish (35 x 10 mm, Easy Grip™, Becton Dickison, Franklin Lakes, NJ). $^{14}CO_2$ was trapped with 5 N KOH-impregnated paper filters cut to the same size as the lid. Plates in Experiment 2 were sealed with petroleum jelly (Vaseline®, Unilever, Greenwich, CT). Plates were gently shaken on a rotary shaker during incubation.

2.3.2. Tissue extraction

Tissue extractions were performed using modifications of the protocols of Viola & Davies (1994).

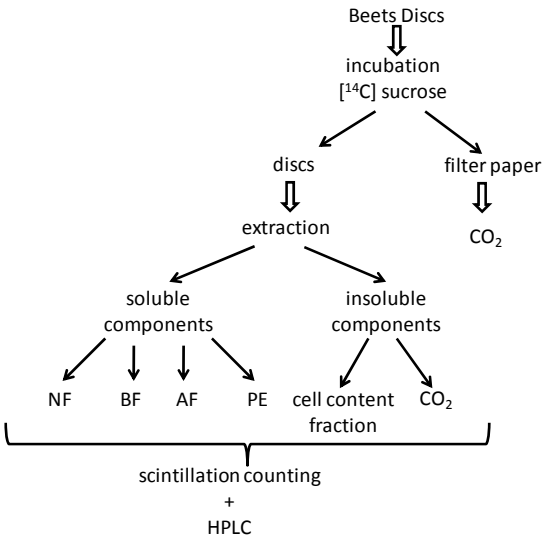

Fig. 1. Representative scheme of the separation and scintillation counting of soluble components (neutral fraction - NF; basic fraction - BF; acidic fraction - AF; and phosphoester - PE) and insoluble components (cell wall contains fraction and CO_2).

After incubations with labelled sucrose, discs were washed 3 times for 3 min in 3 mL of 50 mM Mes-KOH, pH 6.5, to remove apoplastic label, then frozen in liquid

nitrogen and lyophilized (-80^{o}C) for 24 h. Freeze-dried discs were ground to a fine powder with a glass pestle, homogenized in 10 mL of boiling 80% v/v (aq.) ethanol and sonicated for 15 min at 4^{o}C. Samples were extracted in boiling 80% ethanol (hot plate) for 20 min. Extracts were clarified by centrifuging at 17,000 x *g* for 20 min at 25^{o}C. Pellets were washed two more times with 80% v/v (aq.) ethanol and the supernatants were combined and then dried overnight on a rotary film evaporator. Samples were then resuspended in 1 mL (final volume) distilled water. The samples were separated into soluble and insoluble components (Appendix 6).

2.3.3. Analysis of ethanol-soluble components

Soluble components were partitioned into neutral, basic, acidic and phosphate-ester fractions.

2.3.3.1. Neutral fraction

Soluble extracts described in Section 2.3.2 were transferred to microcentrifuge tubes containing 500 mg of a cation-exchange resin (Dowex-50W x 81, charged with 2 M HCl, then washed with distilled water). Tubes were incubated for 30 min at 20^{o}C with gentle shaking. Supernatants were removed with a pipette, and the resin was washed 4 times with 1 mL distilled water for 10 min. Supernatants were combined and transferred to 15 mL disposable test tubes containing 250 mg of anion-exchange resin (Dowex-1 x 4200, charged with 1M sodium acetate, followed by 0.1 M acetic acid and distilled water). Tubes were incubated as describ for cation-exchange resin and supernatants were combined, forming the neutral fraction.

2.3.3.2. Phosphoester fraction

The phosphoester fraction was obtained by adding 1 mL of 5.0 mM acetate buffer, pH 5.0, containing 10 units of acid phosphatase (potato, type III, Sigma, St. Louis, MO) to tubes containing the anion-exchange resin. The enzyme was desalted on a Sephadex G-25 prepacked column (GE-Health Care Bio Sciences AB, Uppsala, Sweden) pre-equilibrated with 5 mM acetate buffer, pH 5.0 prior to use. After incubation at 37^{o}C for 12 hours, the supernatant was removed from the resin-

containing tube with a pipette. The resin was washed 4 times with 1.5 mL distilled water for 10 min. All supernatants were combined to form the phosphate-ester fraction.

2.3.3.3. Acidic fraction

The acidic fraction was obtained by washing the anion-exchange resin 4 times with 1 mL 4 M formic acid for 10 min. All supernatants were combined to form the acidic fraction.

2.3.3.4. Basic fraction

The basic fraction was obtained by washing the cation-exchange resin 5 times with 2 mL of 14.8 M NH_4OH for 10 min. All supernatants were combined to form the basic fraction.

2.3.4. Analysis of ethanol-insoluble components

The pellet obtained following extraction of ethanol soluble components was resuspended in 10 mL distilled water and incubated for 1 h at 100^{o}C. After thorough mixing, a 1 mL aliquot was rapidly removed and added to 1 mL of 200 mM acetate buffer, pH 4.5, containing 20 units amyloglucosidase (*Aspergillus niger,* Sigma). The samples were incubated at 37^{o}C for 12 h. Samples were then centrifuged for 30 min at 17,000 x *g* and the pellet was washed 4 times with 1 mL of distilled water for 10 min each time. Supernatants were combined and analyzed by HPLC. Pellets were transferred to paper thimbles which were oxidized using a Packard Model 307 Oxidizer (Packard Chemical, Meridan, CT). Radioactivity released as CO_2 was trapped and determined by liquid scintillation counting.

2.4. HPLC analysis of sucrose, glucose and fructose

Sucrose, glucose and fructose concentrations were determined by injection (25 µL) of diluted samples onto a 250 x 4 mm Dionex CarboPac PA1 column (Sunnyvale, CA, USA) equipped with a 50 x 4 mm PA1 guard column and eluted

isocraticly with 62 mM NaOH eluent at 1 mL min^{-1}. Compounds were identified and quantified using external standards.

2.5. TLC separation of sucrose, glucose and fructose

2.5.1. Sample preparation

Aliquots (3.5 mL) of the neutral fractions were dryed overnight on a rotary film evaporator and resuspended in 1 mL distilled water. Resuspended samples were passed over a 3 mL charcoal:Celite (1:1, w/w) column which had previously been washed sequentially with water, methanol and water (Tarpley & Vietor, 1997). Glucose and fructose were removed from the column by eluting three times with 1 mL distilled water and the eluates were combined (Fig. 2).

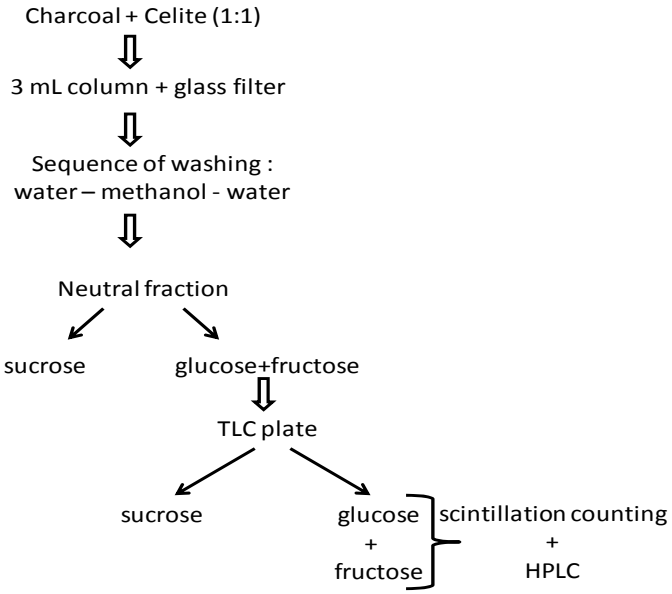

Fig. 2. Separation of sucrose, glucose + fructose from neutral fraction by charcoal column.

Sucrose was removed from the column by eluting 5 times with 1 mL 80% methanol and the eluates were combined. The two fractions were dryed overnight on a rotary film evaporator and resuspended in 500 µL distilled water. The charcoal column allowed most of the sucrose to be separated from the glucose and fructose. This was necessary to allow subsequent separation of glucose and fructose by TLC without overloading the plate with sample.

2.5.2. TLC separation

The glucose and fructose-containing fraction (3 µL) was loaded onto a preparative silica gel TLC plate (50 µM silica gel G , 20 x 20 cm; Analtech, Newark, DE), as shown in Fig. 3.

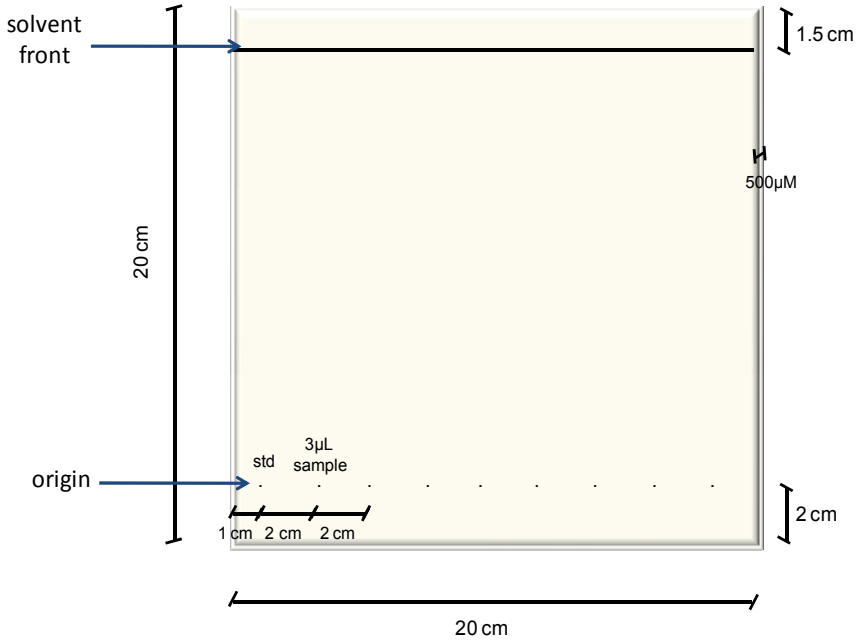

Fig. 3. Neutral fraction was loading in the origin of TLC plate. The identification of compounds was by standard comparison (sucrose, glucose and fructose).

TLC plates were developed 3 times for 4 hours with chloroform: acetic acid: water, (3:3.5:0.5), a solvent system that was determined previously to provide the best separation for sucrose, glucose and fructose. TLC plates were allowed to air dry between developments. To visualize spots, a solution containing 1 g diphenylamine and 1 mL aniline in 100 mL acetone was mixed with concentrated phosphoric acid (10:1 v/v), immediately sprayed onto plates. After sprayed TLC plates were allowed to air dry then heated on a hot plate for 2 min (Fig.4; Farag, 1979). Preliminary tests demonstrated that the best separation of sugars occurred when TLC plates were developed 3 times for 4 hours each time (Fig. 5). Individual spots for sucrose, glucose and fructose were collected using a 1 mL cotton plugged-pipette tip connected to vacuum (Fig. 6). Two cotton plugs were inserted into the large end of the pipette tip. Sample-containing silica gel collected on the cotton plugs which were transferred to vials for scintillation counting. Recovery of sample from TLC plates was determined using radiolabelled sucrose. Recovery using the

vacuum system was approximately 80%. When TLC spots were removed by conventional methods, i.e., scraping spots with a metal spatula or a razor blade, recoveries were approximately 40%.

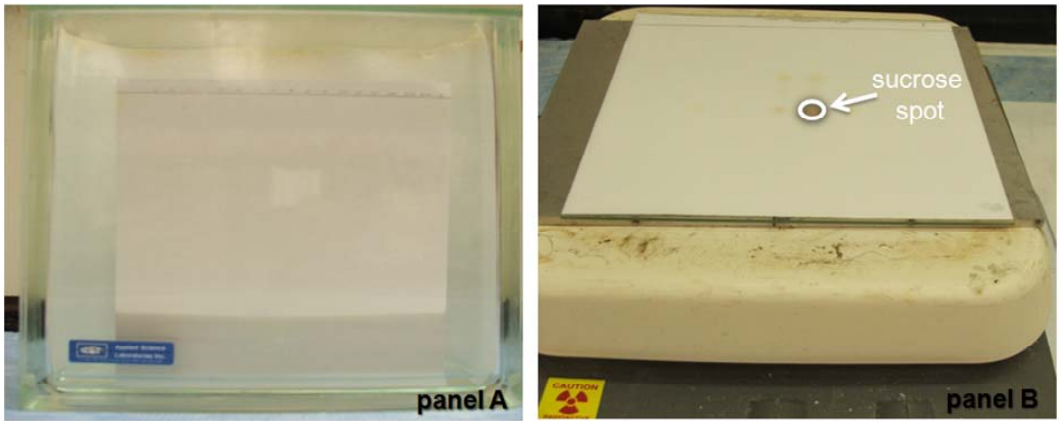

Fig. 4. Development of TLC plates (panel A) to separate sucrose, glucose and fructose and visualization of carbohydrates (panel B) using a hot plate at maximum temperature for 2 min.

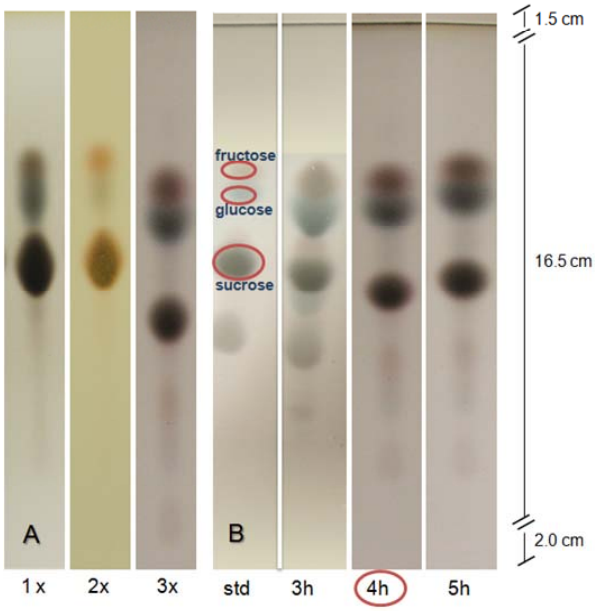

Fig. 5. Comparison of development number (A) and time (B) in the separation of sugar compounds in TLC plate.

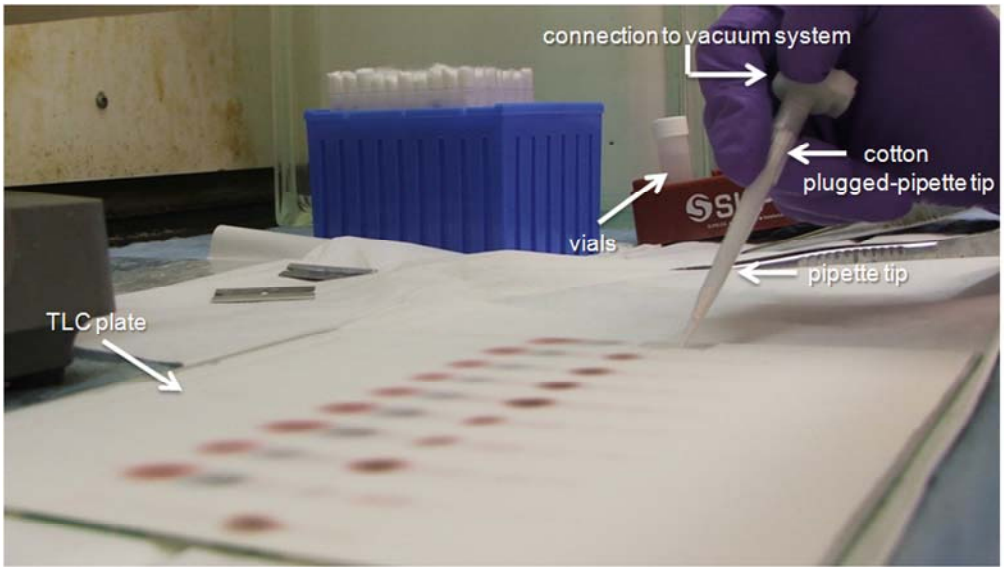

Fig. 6. Removal of spots from TLC plate and transfer to vials for scintillation counting.

2.6. Quantification of radioactivity

Radioactivity for all samples was quantified by liquid scintillation counting using a LS 6500 Multi-purpose Scintillation Counter (Beckman Coulter™, Fullerton, CA) and Bio Safe II liquid scintillation fluid (Research Products International Corp., Mount Prospect, Illinois).

2.7. Statistical analysis

Experiment was conducted in a completely randomized design. Results were analyzed by ANOVA and the means compared with the Tukey's test at significance degree α = 0.05 using SAS statistical software (SAS 9.1, SAS Institute, Inc., Cary, NC, USA).

3. RESULTS AND DISCUSSION

3.1. Preliminary test

Since protocols for radiolabelling sugarbeet root tissue are not available, a preliminary test was conducted to determine the effect of incubation time. The discs were incubated for 3 h and 5 h, using the methodology of Viola & Davies (1994). Incubation for 5 h increased the amount of labeling. However, glucose and fructose concentrations were elevated in discs incubated for 5 h relative to discs incubated for 3 h, suggesting that sucrolytic enzymes activities were induced when discs were incubated for the longer period (data not shown). It is known that acid invertase activity is induced when tissue discs are incubated in solution, with the increase in activity due to increased protein synthesis (Leigh et al., 1979).

3.2. Experiment 1: Effects of incubation time and protein synthesis inhibition

Based on the results of the preliminary test (Section 3.1), an experiment was conducted to more thoroughly analyze the effect of incubation time and the effect of a protein synthesis inhibitor. In this experiment, discs of sugarbeet roots were incubated with radiolabelled sucrose for 3 or 5 h, in the presence or absence of cycloheximide (CHI). CHI is a widely used inhibitor of eukaryotic protein synthesis and functions by preventing translational elongation (Zucher, 1968; ap Rees & Bryant, 1971). The inclusion of a protein synthesis inhibitor was tested due to concern that artificial changes in acid invertase activity affected results. Other enzyme activities may also increase during the incubation, since hexokinase, fructokinase, and phosphofructokinase activities increase in response to wounding (Klotz et al., 2006). Metabolism of the radiolabel was determined by trapping carbon dioxide produced by the discs during the incubation and fractionating tissue after incubation into neutral, phosphoester, basic, acidic, starch and non-starch insoluble components. The neutral fraction was primarily composed of sucrose, glucose and fructose (98% of incorporated radiolabel, data not shown). The phosphoester fraction was comprised principally of phosphorylated glycolytic intermediates. The basic and acidic fractions were comprised of mostly amino acids and organic acids, respectively. The non-starch pellet contained all ethanol insoluble material after

digestion of starch with amyloglucosidase and was believed to be primarily proteins and cell walls.

Sucrose, glucose and fructose concentrations were determined by HPLC (Table 1). Sucrose concentration in root discs was unchanged between treatments, independent of time and CHI. Glucose and fructose concentrations, as analyzed by ANOVA, were unaffected by incubation time, but increased when discs were incubated with CHI. Discs incubated for 5 h with CHI had higher concentrations of glucose and fructose than discs incubated for 3h with CHI.

Table 1. Sucrose, glucose and fructose concentrations in the neutral fraction of sugarbeet discs incubated at 20°C for 3 or 5 h, in the presence or absence of 50 µM cycloheximide (CHI).

Fraction	Incubation conditions			
	3h	**3h+CHI**	**5h**	**5h+CHI**
	---------- µmol^{-1} g FW ----------			
Sucrose	206.7±23.2 a*	259.6±18.0 a	226.2±25.0 a	249.1±13.7 a
Glucose	47.7±3.35 b	56.1±4.21 ab	47.7±6.44 b	74.6±5.47 a
Fructose	43.5±4.39 b	47.7±3.56 ab	45.2±4.17 b	63.6±4.40 a

* Values are the mean ± SE (n = 6). Means followed by the same letter in a row are not significantly different by Tukey's test at $P < 0.05$.

Most ^{14}C after incubation was found in the neutral fraction (Table 2). The neutral fraction contained 63 – 74% of the total recovered radiolabel. Most of the label in the neutral fraction was found in sucrose, indicating that most of the radiolabel was unmetabolized. Sucrose contained 62 to 77% of the neutral fraction label, while the remaining radiolabel was distributed approximately equally between glucose and fructose. The fractions containing the least amount of radiolabel were the phosphoester and acidic fractions.

The partitioning of ^{14}C in root discs incubated for 3 or 5 h with or without CHI is shown in Table 2. The distribution of ^{14}C in the various fractions was calculated as the percentage of label recovered. The amount of ^{14}C incorporated increased with increasing incubation time. Incorporation of radiolabel increased by 10 and 17% as incubation time increased from 3 to 5 h in solutions with and without CHI, respectively. For incubations without CHI, the percentage of recovered ^{14}C increased in the CO_2, basic, and starch fractions and decreased in the glucose and fructose of the neutral fraction, the phosphoester fraction, and the non-starch fraction. Percentage of recovered ^{14}C was unchanged in the sucrose and the acidic fraction. For incubations with CHI, the percentage of recovered ^{14}C increased in the glucose of the neutral fraction, and the phosphoester, basic, and starch fractions. The percentage of recovered ^{14}C decreased in the carbon dioxide and the non-starch fractions and was unchanged in sucrose, fructose, and the acidic fraction.

Table 2. Partitioning of ^{14}C in sugarbeet root tissue following incubation in [U-^{14}C] sucrose (70 kBq/mL) at 20°C for 3 or 5 h, in the presence or absence of 50 µM cycloheximide (CHI).

Fraction	^{14}C per fraction as percentage of total recovered			
	3h	3h + CHI	5h	5h + CHI
CO_2	16.7	26.2	28.1	15.9
Neutral	73.3	64.2	63.2	73.6
Sucrose	45.9	49.9	49.1	51.8
Glucose	15.3	8.1	7.7	13.4
Fructose	12.1	6.2	7.2	8.4
Phosphoester	1.1	0.4	0.6	0.7
Acidic	0.8	0.5	0.9	0.5
Basic	1.7	1.1	2.1	2.1
Starch	2.5	2.3	3.7	4.0
Non-starch pellets	3.9	5.3	1.4	3.2
Total ^{14}C recovered (kBq)	8.8	10.3	12.3	7.2
^{14}C incorporated (kBq)	35.0	30.3	38.6	35.6

Incubation with radiolabel was carried out at 20°C. Values represent percentage of total recovered. Data are average values of six replicates, each consisting of 14 discs randomly selected from 6 roots.

Inhibition of protein synthesis altered the partitioning of label between fractions with respect to incubation time. For incubations without CHI, radiolabel increased with increasing incubation time in fractions containing metabolic end-products, such as carbon dioxide from respiration and starch, and decreased in early metabolic intermediates including glucose, fructose, and phosphorylated glycolytic intermediates (phosphoester fraction). For incubations that included CHI, radiolabel increased with increasing incubation time in the glucose and phosphester fractions which contained early metabolic intermediates, and decreased in the metabolic end-products, CO_2 and the non-starch pellet. Therefore, inclusion of CHI shifted the distribution of radiolabel from metabolic end-products to early metabolic intermediates and was used in the subsequent experiment.

3.3. Experiment 2: Effect of incubation temperature

The effect of temperature on respiration rate and the metabolism of sucrose were determined at 4, 10 and 20^{o}C. Respiration rate was measured on whole roots. Sucrose metabolism was analyzed using root tissue discs incubated in solutions containing ^{14}C-labelled sucrose and CHI for 5 h. A 5 h incubation time was used since more label was incorporated by the tissue after a 5 h incubation compared to a 3 h incubation (Section 3.1 and 3.2; Table 2). CHI was used to inhibit protein synthesis since metabolism of ^{14}C sucrose progressed further in its absence, as evidenced by the greater proportions of label in carbon dioxide and starch when CHI was absent and greater proportions of label in glucose and phosphorylated glycolytic intermediates (phosphoester fraction) in its presence (Section 3.2, Table 2). The increased metabolism in the absence of CHI suggested that enzyme activities responsible for sucrose catabolism increased during incubations.

Respiration rate was higher for sugarbeet roots stored at 20^{o}C than at 4 and 10^{o}C (Table 3), indicating that the increase in the temperature accelerated metabolism in sugarbeet. Respiration rate was 1.6-fold greater for roots stored at 20^{o}C compared to those stored at 10^{o}C. Previously, Wyse (1973) reported that respiration rate approximately doubled for each 10^{o}C rise in temperature. Dilley et al. (1970), however, found a temperature coefficient (Q_{10}) of 1.1 for temperatures between 0 and 10^{o}C and 2.35 for temperatures between 10 and 20^{o}C in the beginning of the storage.

Table 3. Respiration rate of sugarbeet roots at 4, 10, and 20°C after 10 days storage. Respiration rates were determined at the temperature at which roots were stored.

Treatment	Respiration rate ($mg\ kg^{-1}\ h^{-1}$)
4°C	4.0 ± 0.403 b*
10°C	4.2 ± 0.380 b
20°C	6.4 ± 0.423 a

* Values are the mean ± SE (*n* = *6*). Means followed by the same letter in a column are not significantly different by Tukey's test at $P < 0.05$.

Discs of sugarbeet roots incubated at 4, 10 and 20°C had statistically similar concentrations of sucrose (Table 4). Discs incubated at 4°C, however, had higher concentrations of glucose and fructose than discs incubated at 10°C and 20°C. No differences in glucose and fructose concentrations were found in sugarbeet discs incubated at 10°C and 20°C during 5 h with CHI (Table 4). Similarly, an increase in hexose concentrations was observed in stored potato tubers in response to low temperature (Dixon and ap Rees, 1980; Viola and Davies, 1994)

Table 4. Sucrose, glucose and fructose concentrations in the neutral fraction of sugarbeet discs incubated at 4°C, 10°C and 20°C for 5 h, in presence of 50 µM cycloheximide (CHI).

Fraction	**Incubation conditions**		
	4°C	**10 °C**	**20 °C**
	---------- μmol^{-1} g FW ----------		
Sucrose	241.9±22.98 a*	162.06±32.60 a	197.9±36.93 a
Glucose	23.29±1.07 a	16.36±1.90 b	16.77±2.13 b
Fructose	35.19±2.46 a	24.69±2.59 b	25.35±3.24 b

* Values are the mean ± SE (*n* = *6*). Means followed by the same letter in a row are not significantly different by Tukey's test at $P < 0.05$.

Discs incubated at 10 and 20°C incorporated more ^{14}C than discs incubated at 4°C (Table 5). Sucrose uptake in sugarbeet root is active and involves an ATP-dependent sucrose transporter (Buckhout, 1989). The data, therefore, suggests greater activity of this transporter at 10 and 20°C than at 4°C. Most of the radiolabel, 95 to 98%, was observed in the neutral fraction, independent of temperature treatment, with approximately 80% of the label found in sucrose. In Experiment 1, the neutral fraction contained 63 – 74% of the total recovered radiolabel, with 62 to 77% of the label found in sucrose. The differences in partitioning of label to the neutral fraction between experiments suggest that greater metabolism occurred in Experiment 1. The cause for the increased metabolism is unknown. However, tissue used in Experiment 2 was collected from roots that were significantly older than those used in Experiment 1.

Table 5. Partitioning of ^{14}C in sugarbeet root tissue following incubation in [U-^{14}C] sucrose (70 kBq/mL) at 4°C, 10°C and 20°C, in the presence of 50 µM cycloheximide (CHI).

Fraction	**^{14}C per fraction as percentage of total recovered**		
	4°C	**10 °C**	**20 °C**
CO_2	0.79	1.62	1.70
Neutral	97.5	97.0	95.4
Sucrose	77.4	78.7	80.1
Glucose	8.81	8.00	6.65
Fructose	11.3	10.3	8.63
Phosphoester	0.17	0.05	0.06
Acidic	0.23	0.13	0.11
Basic	0.18	0.06	0.09
Starch	0.50	0.58	1.21
Non-starch pellets	0.68	0.51	1.09
Total ^{14}C recovered (kBq)	13.2	30.8	17.1
^{14}C incorporated (kBq)	24.6	38.6	34.9

Incubation with radiolabel was carried out at storage temperature. Values represent percentage of total recovered. Data are average values of six replicates, each consisting of 14 discs randomly selected from 6 roots.

Partitioning of radiolabel between fractions changed with incubation temperature. The relative amount of ^{14}C increased in the collected carbon dioxide, the starch fraction and the non-starch insoluble materials as temperature increased. The relative amount of ^{14}C decreased in glucose, fructose, phosphoester, acidic and basic fractions as incubation temperature increased. Therefore, as incubation temperature increased, more label was found in the end-products of metabolism and less label was found in the intermediates of metabolism, suggesting that the rate of metabolism generally increased as temperature increased. The increase in metabolism however was not uniform for all pathways in the conversion of sucrose to CO_2. As temperature increased from 4 to 20^{o}C, the relative amount of radiolabel in glucose and fructose decreased by 25 and 24%, respectively, indicating that the effect of increasing temperature on enzymatic activity was greater for hexokinase (HK) and fructokinase (FK) than for the sucrolytic activities that form glucose and fructose. Since the relative amount of radioactivity found in the phosphoester fraction was reduced by 67% with increased temperature from 4 to 20^{o}C, glycolytic intermediates were utilized more rapidly than they were formed. This suggests that PK activity increased more than HK and FK activities in response to temperature. Organic acids also contained a smaller percentage of the radiolabel in discs incubated at 20^{o}C relative to discs incubated at 4^{o}C, and the acidic fraction from 20^{o}C incubations contained 52% less ^{14}C than the acidic fraction from 4^{o}C incubations. Label, therefore, was removed from organic acids by reaction of the TCA cycle faster than organic acids were formed by glycolysis. Reduction of label in glucose, fructose, and phosphoester fractions with increased incubation temperature, suggests an increase in glycolytic flux. The decrease of label in the acidic fraction with increased incubation temperature, suggests increased metabolism of organic acids due to an increase in flux through the TCA cycle.

The increase in label in the carbon dioxide collected during the incubation and the non-starch insoluble materials indicates an increase in respiration and cell wall synthesis with increasing temperature. While the non-starch insoluble material contained primarily cell walls and proteins, any new label incorporated in this fraction was likely due to cell wall biosynthesis since a protein synthesis inhibitor was included in the incubations. Respiration rate increased 115% with the increase in temperature from 4 to 20^{o}C. Cell wall biosynthesis increased 60%. The increase in

respiration rate of tissue discs with increasing temperature contrasts with the 60% increase in respiration rate observed for whole root when storage temperature increased from 4 to 20^{o}C. The difference in elevation in respiration rate between tissue discs and whole roots in response to temperature is likely due to differences in experimental material and conditions.

CONCLUSIONS

- Most of the radiolabel incorporated by root tissue remained as sucrose and was unmetabolized.

- Glycolytic intermediates which were contained in the phosphoester fraction had the smallest quantities of radiolabel, suggesting that label was rapidly metabolized through glycolysis.

- Comparison of incubations in the presence or absence of a protein synthesis inhibitor indicate that sucrolysis, glycolysis and respiration increased when protein synthesis was not inhibited, suggesting that one or more sucrolytic and glycolytic proteins were synthesized during the 5 h incubation period when CHI was absent.

- Respiration rate of whole roots was affected by temperature and increased 1.6-fold in roots stored at 20^{o}C compared to those stored at 4 and 10^{o}C.

- Glucose and fructose concentrations were elevated in root discs incubated at 4^{o}C relative to discs incubated at 10 or 20^{o}C, indicating that the activities of the enzymes that utilize glucose and fructose were affected by low temperature to a greater extent than the activities of the sucrolytic enzymes that produce these compounds.

- Since uptake of sucrose involves a plasmamembrane sucrose transporter, the increased incorporation of ^{14}C sucrose with increasing temperature, indicates that the activity of the sucrose transporter was sensitive to temperature.

- As temperature increased, the relative proportion of label found in the end-products of metabolism (i.e, CO_2, starch and the cell wall-containing fraction) increased, suggesting a general increase in metabolism with increased temperature.

- The increase in metabolism due to increased temperature was not uniform for all enzymes and pathways in the conversion of sucrose to CO_2. Changes in the relative amount of label found in glucose, fructose and phosphoester

fractions with increasing temperature suggest that increased temperature elevated glycolytic flux to a greater extent than sucrolytic flux, and affected pyruvate kinase to a greater extent than hexokinase and fructokinase activities which were affected to a greater extent than were the sucrolytic enzyme activities.

- The decrease of radiolabel in the acidic fraction with increased incubation temperature, suggests that metabolism of organic acids increased in response to temperature due to an increase in flux through the TCA cycle.

- The increase in radiolabel in respired CO_2 and the non-starch insoluble materials indicates an increase in respiration and cell wall synthesis with increasing temperature.

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APPENDIX

Appendix 1: ENZYME ASSAYS

Enzyme	Solution I	Solution II	Solution III	Assay solution	Assay
Hexokinase	1) 250 mM HEPES – pH 7.5 2) 20 mM MgCl2 3) 14 mM Glucose	1) 75 mM NAD	1) 2 U glucose 6-phosphate dehydrogenase	1) 1000 µL Sol'n I 2) 40 µL Sol'n II 3) 2 µL Sol'n III	1) 90 µL assay solution 2) 60 µL extract 3) 25 µL water 4) 5 µL 50 mM ATP (start reaction)
Fructokinase	1) 250 mM HEPES – pH 7.5 2) 20 mM $MgCl_2$ 3) 6 mM fructose	1) 75 mM NAD	1) 2 U glucose 6-phosphate dehydrogenase 2) 6 U phospho-glucose isomerase	1) 1000 µL Sol'n I 2) 40 µL Sol'n II 3) Sol'n III (2 µL #1, and 1.7 µL #2)	1) 90 µL assay solution 2) 60 µL extract 3) 25 µL water 4) 5 µL 50 mM ATP (start reaction)
Phosphofructokinase	1) 100 mM TRIS-HCl–pH 8.0 2) 20 mM $MgCl_2$ 3) 2 mM EDTA 4) 2 mM fructose 6-phosphate	1) 40 mM NADH	1) 2 U aldolase 2) 2 U triose phosphate isomerase 3) 5 U glycerol 3-phosphate dehydrogenase	1) 1000 µL Sol'n I 2) 5 µL Sol'n II 3) Sol'n III (6.7 µL #1, 0.1 µL #2 and 2.9 µL #3)	1) 90 µL assay solution 2) 60 µL extract (5 x dilution) 3) 25 µL water 4) 5 µL 24 mM ATP (start reaction)
Pyruvate kinase	1) 100 mM HEPES – pH 7.0 2) 100 mM KCl 3) 20 mM $MgCl_2$ 4) 4 mM DTT 5) BSA (0.4 mg/mL)	1) 15 mM NADH 2) 200 mM PEP	1) 26 U lactate dehydrogenase	1) 1000 µL Sol'n I 2) Sol'n II (10 µL#1 and 10 µL #2) 3) 5 µL Sol'n III	1) 90 µL assay solution 2) 20 µL extract (5 x dilution) 3) 50 µL water 4) 20 µL 50 mM ADP (start reaction)

* solution II and solution III were add prior use.

Enzyme	Solution I	Solution II	Solution III	Assay solution	Assay
Phosphoglucomutase	1) 100 mM TRIS-HCl–pH 7.5 2) 20 mM $MgCl_2$	1) 100 mM NAD 2) 30 µM glucose 1,6-P_2	1) 2 U glucose 6-phosphate	1) 1000 µL Sol'n I 2) Sol'n II (10 µL #1 and 0.9 µL #2) 3) 2 µL Sol'n III	1) 90 µL assay solution 2) 15 µL extract (5 x dilution) 3) 65 µL water 4) 10 µL 90 mM glucose 1-phosphate (start reaction)
Glucose 6-phosphate isomerase	1) 75 mM Gly-Gly – pH 8.5 2) 10 mM $MgCl_2$	1) 150 mM NAD 2) 150 mM fructose 6-phosphate	1) 0.5 U glucose 6-phosphate dehydrogenase	1) 1000 µL Sol'n I 2) Sol'n II (10 µL #1 and 10 µL #2) 3) 0.5 µL Sol'n III	1) 120 µL assay solution 2) 50 µL water 3) 10 µL extract (10 x dilution) (start reaction)
Aldolase	1) 40 mM HEPES – pH 7.7	1) 15 mM NADH 2) 300 mM fructose 1,6-P_2	1) 1.7 U glycerol 3-phosphate dehydrogenase 2) 17 U triose phosphate isomerase	1) 1000 µL Sol'n I 2) Sol'n II (10 µL #1 and 25 µL #2) 3) Sol'n III (1 µL #1 and 0.5 µL #2)	1) 120 µL assay solution 2) 40 µL water 3) 20 µL extract (5 x dilution) (start reaction)
Triose phosphate isomerase	1) 100 mM HEPES – pH 8.0 2) 1.5 mM DL-glyceraldehyde 3-phosphate 3) 5mM EDTA	1) 30 mM NADH	1) 1 U glycerol 3-phosphate dehydrogenase	1) 1000 µL Sol'n I 2) 10 µL Sol'n II 3) 0.6 µL Sol'n III	1) 120 µL assay solution 2) 40 µL water 3) 20 µL extract (200 x dilution) (start reaction)
Glyceraldehyde 3-phosphate dehydrogenase	1) 100 mM TRIS – pH 7.8 2) 4.5 mM 3-phosphoglycerate 3) 8 mM $MgSO_4$ 4) 1 mM EDTA 5) 2 mM DTT	1) 50 mM NADH 2) 300 mM ATP	1) 1.8 U 3-phospholycerate kinase	1) 1000 µL Sol'n I 2) Sol'n II (10 µL #1 and 10 µL #2) 3) 0.3 µL Sol'n III	1) 120 µL assay solution 2) 20 µL water 3) 40 µL extract (2 x dilution) (start reaction)

Enzyme	Solution I	Solution II	Solution III	Assay solution	Assay
Phosphoglycerate kinase	1) 100 mM HEPES – pH 7.6 2) 1 mM EDTA 3) 2 mM $MgSO_4$ 4) 6.5 mM 3-phosphoglycerate	1) 25 mM NADH 2) 110 mM ATP	1) 3.3 U glycerol 3-phosphate dehydrogenase	1) 1000 µL Sol'n I 2) Sol'n II (10 µL #1 and 10 µL #2) 3) 2 µL Sol'n III	1) 165 µL assay solution 2) 15 µL extract (start reaction)
Phosphoglycerate mutase	1) 100 mM TRIS – pH 7.6 2) 10 mM $MgSO_4$ 4) 4 mM DTT 5) BSA (0.4 mg/mL)	1) 75 mM NADH 2) 250 mM ADP	1) 1 U enolase 2) 5 U pyruvate kinase 3) 6 U lactate dehydrogenase	1) 1000 µL Sol'n I 2) Sol'n II (5 µL #1 and 20 µL #2) 3) Sol'n III (4 µL #1, 2.5 µL #2 and 1.1 µL #3)	1) 100 µL assay solution 2) 30 µL extract 3) 19 µL water 4) 11 µL 50 mM 3-phosphoglycerate (start reaction)
Enolase	1) 100 mM HEPES – pH 7.5 2) 10 mM $MgCl_2$	1) 180 mM NADH 2) 250 mM ADP	1) 5 U pyruvate kinase 2) 6 U lactate dehydrogenase	1) 1000 µL Sol'n I 2) Sol'n II (10 µL #1 and 20 µL #2) 3) Sol'n III (2.5 µL #1 and 1.1 µL #2)	1) 100 µL assay solution 2) 15 µL extract 3) 56 µL water 4) 9 µL 10 mM 2-phosphoglycerate (start reaction)
PEPase	1) 50 mM TRIS-HCl – pH 7.5 2) 4 mM $MgCl_2$	1) 30 mM NADH 2) 150 mM PEP	1) 3 U lactate dehydrogenase	1) 1000 µL Sol'n I 2) Sol'n II (10 µL #1 and 10 µL #2) 3) 1.1 µL Sol'n II	1) 120 µL assay solution 2) 40 µL water 3) 20 µL extract (start reaction)

Enzyme	Solution I	Solution II	Solution III	Assay solution	Assay
PFP	1) 100 mM TRIS – pH 8.0 2) 5 mM fructose 6-phosphate 3) 5 mM $MgCl_2$ 4) 2 mM PP_i	1) 40 mM NADH	1) 1 U aldolase 2) 1.3 U glycerol 3-phosphate dehydrogenase 3) 10 U triose phosphate isomerase	1) 1000 µL Sol'n I 2) 10 µL Sol'n II 3) Sol'n III (3.3 µL #1, 0.7 µL #2 and 0.3 µL #3)	1) 100 µL assay solution 2) 50 µL water 3) 30 µL extract (200 x dilution) (start reaction)
UDPase	1) 100 mM TRIS – pH 8.0 2) 5 mM $MgCl_2$ 3) 0.8 mM UDP-glucose	1) 85 mM NAD	1) 4 U phospho glucomutase 2) 4 U glucose 6-phosphate isomerase	1) 1000 µL Sol'n I 2) 20 µL Sol'n II 3) Sol'n III (8.2 µL #1 and 1.1 µL #2)	1) 172 µL assay solution 2) 6 µL extract 3) 2 µL 200 mM sodium pyrophosphate (start reaction)

Appendix 2: CHEMICALS USED FOR ENZYME ASSAYS AND MOLECULAR WEIGHT

Chemical	Molecular Weight
2-phosphoglycerate	252.00
3-phosphoglycerate	230.02
ADP	427.20
ATP	551.14
Cysteine	121.20
DL-Glyceraldehyde 3-phosphate	170.10
DTT	154.20
EDTA	372.20
Fructose 1,6-bisphosphate	406.06
Fructose 6-Phosphate	336.32
Glycyl-glycine	132.12
HEPES	238.30
$MgCl_2$	203.30
$MgSO_4$	246.47
NAD	663.40
NADH	763.50
NaH_2PO_4	137.99
PEP	206.13
Sodium pyrophosphate	446.10
TRIS	121.10

Appendix 3: ENZYMES USED AND RESPECTIVES UNIT

Enzyme	Supplier	Units	Units/µL
3-Phosphoglycerate kinase	Sigma-Aldrich	1,466 U/mg (4.2 mg/mg)	6.2 U
Aldolase	Sigma-Aldrich	14 U/mg (21 mg/mg)	0.3 U
Enolase	MP Biomedicals	250 U/mL	0.25 U
Glucose 6-phosphate dehydrogenase	Roche	1000 U/mL	1 U
Glycerol 3-phophate dehydrogenase	Sigma-Aldrich	170 U/mg (10 mg/mg)	1.7 U
Lactate dehydrogenase	Roche	550 U/mg (10 mg/mg)	5.5 U
Phosphooglucose isomerase	Roche	350 U/mg (10 mg/mg)	3.5 U
Phosphoglucomutase	Roche	245 U/mg (2mg/mg)	0.49U
Pyruvate kinase	Roche	200 U/mg (10 mg/mg)	2 U
Triose Phosphate isomerase	Sigma-Aldrich	5950 U/mg (5.2 mg/mL)	30.9 U

Appendix 4: SCOTT-KNOTT STATISTICAL ANALYSIS OF GLYCOLYTIC ENZYMES STORAGE UP TO 100 DAYS.

ENZYMES	DAYS OF STORAGE									
	0	1	2	3	4	7	10	30	60	100
UDPase	507.9 c	373.1 b	471.7 c	366.6 b	320.7 a	348.7 b	303.767 a	355.46 b	288.7 a	287.4 a
HK	2.013 b	1.911 b	2.201 c	2.344 c	2.117 c	2.270 c	2.191 c	1.921 b	1.660 a	1.439 a
FK	1.027 b	1.054 b	1.148 c	1.297 d	1.191 c	1.131 c	1.128 c	1.114 c	1.078 b	0.906 a
G6PI	264.4 d	241.0 d	254.4 d	253.4 d	224.2 c	247.4 d	249.2 d	211.0 b	333.6 e	168.5 a
PGM	92.84 a	94.80 a	102.8 b	113.2 b	103.8 b	104.9 b	106.4 b	103.6 b	111.0 b	93.98 a
PFK	4.823 a	4.524 a	5.150 b	5.449 b	5.348 b	5.117 b	4.970 b	4.247 a	4.621 a	4.611 a
PFP	0.903 c	0.983 c	1.013 c	1.046 c	0.991 c	0.791 b	0.813 b	0.797 b	0.646 a	0.667 a
ALD	15.56 a	15.75 a	14.25 a	15.38 a	14.80 a	14.10 a	14.60 a	15.30 a	15.03 a	15.59 a
TPI	6002 a	7047 b	7090 b	6578 b	6631 b	6688 b	6345 a	6749 b	5680 a	7271 b
PGK	13.80 a	15.11 a	11.92 a	14.29 a	14.69 a	21.55 b	17.23 b	18.64 b	18.21 b	20.03 b
GAPDH	3.837 a	3.844 a	4.160 a	3.976 a	4.143 a	4.261 a	4.177 a	3.446 a	3.513 a	3.863 a
PGlyM	10.14 a	12.46 b	12.67 b	12.48 b	13.93 c	11.94 b	14.22 c	12.50 b	15.47 d	15.81 d
ENO	27.30 a	26.80 a	27.11 a	30.53 b	30.48 b	30.94 b	28.10 a	29.76 b	33.51 c	34.40 c
PK	61.79 a	78.56 c	84.43 d	79.20 c	80.58 c	61.50 a	66.15 b	67.69 b	65.67 b	61.77 a
PEPase	2.630 a	2.867 b	2.846 b	2.960 b	2.746 b	2.627 a	2.509 a	2.590 a	2.850 b	3.420 c

For each enzyme, means followed by the same letter in lines do not differ at 5% of probability by Scott-Knott test. The results represented the enzymatic activity (μmol min^{-1} g^{-1} of protein). The colors were used for better visualization of the differences between days for each enzyme. Blue = a, grey = b, pink = c, green = d, and yellow = e.

Appendix 5: WEIGHTING FACTORS USED TO CALCULATE EIGENVECTORS FOR THE PRINCIPAL COMPONENT ANALYSIS OF THE ENZYMATIC ACTIVITY IN THE POSTHARVEST OF SUGARBEET ROOTS.

DAYS	WEIGHTING FACTOR	
	PRINCIPAL COMPONENT 1	PRINCIPAL COMPONENT 2
0	0.064711	-.360465
1	-.048703	-.550707
2	0.433277	-.259549
3	0.403331	-.399969
4	0.446516	0.068540
7	0.139502	0.434297
10	0.269862	.0444296
30	-.288780	-.051973
60	-.281893	0.322279
100	-.434183	0.023415

Appendix 6. PROTOCOL ^{14}C

1) Incubation

Beets

⇓

Slices (3 mm)

⇓

14 discs (5 mm diameter) – cork borer

⇓

Wash 3x/3 min (3 mL of 50mM MeS-KOH, pH 6.5)

⇓

Weight (fresh weight)

⇓

Transfer to plates with 1 mL of 50 mM Mes-KOH (pH 6.5) + 70 kBq mL^{-1} [^{14}C] sucrose + 50 µM CHI. Use 5 N KOH in filter paper (fixed in the lid) and use Vaseline ® for sealed plates

⇓

Plates were gently shaken for 5 h

⇓

Wash 3x/3min (3 mL of 50mM MeS-KOH, pH 6.5)

Washed liquid Discs

⇓ ⇓

Counted Frozen (liquid nitrogen)

and lyophilized (-80 ^{o}C)

2) Extraction

Lyophilized discs

⇓

Grounded with glass pestle

⇓

Homogenized in 10 mL of boiling ethanol (80%)

⇓

Sonicate in ice for 15 min at 4 °C

⇓

Boiling in ethanol (80%) for 20 min

⇓

After 5 min (when cold) centrifuge at 17,000 x *g*/10 min at 25 °C

⇓

Wash the pellet more twice in cold ethanol (80%) for 10 min each time

Pellet Combine the supernatant

⇓

Dry overnight on rotary film evaporator

3) Soluble components

After speed vacuum resuspended in 1 mL (final volume) distilled water

⇓ ⇓ ⇓ ⇓

neutral basic acid phosphoester

3.1) Neutral fraction

500 mg of a cation exchange resin (Dowex-50W x 81 charged with 2M HCl)

⇓

Wash the resin with distilled water

⇓

Transfer aqueous sample (1 mL) to tubes

⇓

Incubate at 20°C for 30 min with gentle shaking

↙ ↘

supernatant pellet

⇓

Wash 4x/10 min each time

⇓

Centrifuge at 17,000 x *g*/1 min at 25 °C

⇓

Combine the supernatants

⇓

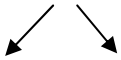
Transfer to new tube containing 250 mg of an anion-exchange

(Resin, Dowex 1 x 4, was charged with 1 M sodium acetate,

followed by 0.1 M acetic acid and distilled water)

⇓

Incubate at 20°C for 30 min with gentle shaking



supernatant pellet

⇓

Wash 4x with 1 mL of distilled water/10 min each time

⇓

Centrifuge at 17,000 x *g*/1 min at 25 °C

⇓

Combine supernatants

⇓

Neutral fraction

3.2) Phosphate-ester fraction

First desalt the enzyme on a sephadex G-25 pre-equilibrated with 5 mM acetate buffer, pH 5.0

⇓

Add 1 mL of acetate buffer (5 mM), pH 5.0 + 10 units of acid phosphatase (potato, type III, Sigma)

⇓

Tubes containing the anion-exchange resin (after extraction of neutral fraction)

⇓

Incubate at 37^{o}C/12h

⇓

Centrifuge and remove the supernatant

supernatant pellet

⇓

Wash 4x with 1.5 mL of distilled water/10 min each time

⇓

Centrifuge at 17,000 x *g*/1 min at 25 ^{o}C

⇓

Combine the supernatants

⇓

Phosphoester fraction

3.3) Acid fraction

Wash the anion-exchange resin

⇓

5x with 1 mL of formic acid (4M)/10 min each time

⇓

Acid fraction

3.4) Basic fraction

Wash the cation-exchange resin

⇓

5x with 2 mL of NH_4OH/10 min each time

⇓

Basic fraction

4) Insoluble components

Resuspended, in 10 mL, the pellet obtained following the extraction of ethanol soluble components

⇓

Incubate at 100°C (boiling water) for 1h

⇓

Transfer 1 mL after thorough mixing to microcentriguge tubes containing

[1 mL of 200 mM acetate buffer, pH 4.5 + 20 units amyloglucosidase (*Aspergilus niger*, Sigma)]

⇓

Incubate at 37°C/12h

⇓

Centrifuge at 10000 rpm/30 min and remove the supernatant

supernatant pellet

⇓

Wash 4x with 1 mL of distilled water/10 min each time

⇓

Centrifuge at 17,000 x *g*/1 min at 25 °C

⇓

Combine the supernatants

⇓

Insoluble components