THE CHARACTERIZATION OF JERUSALEM ARTICHOKE (*HELIANTHUS TUBEROSUS* L.) INULASES AND THEIR EFFECTS ON THE TUBER CARBOHYDRATES DURING STORAGE AND PROCESSING

by

REGINALD E. BACCHUS

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ABSTRACT

The inulases (A and B) of Jerusalem Artichoke (Helianthus tuberosus L.) are part of the enzyme system involved with sugar transformation in this plant. During storage of the Jerusalem Artichoke tuber, the inulases break down inulin (Degree of Polymerization - D.P. = 35) and other high D.P. polyfructans causing an increase in lower D.P. polyfructans and a subsequent decrease in the fructose/glucose ratio. This effect of the inulases on the tuber polyfructans during storage is undesirable in view of producing high fructose syrup from stored tubers. In order to produce high fructose syrup with the highest possible fructose content, polyfructans of the highest possible D.P. must be maintained during storage, consequently maintaining the highest possible fructose/glucose ratio in the tuber.

Investigation of factors which affect the inulases as they relate to storage and processing of the Jerusalem Artichoke tuber was undertaken by first, characterization of the enzymes, which showed that when inulin and Substrate M (D.P. = 17) (a standard mixture of oligosaccharides prepared from the tubers) were used as substrates for the inulases, activity was much higher with Substrate M. Inulases A and B differed slightly in pH optima. The optimum for inulase A was pH 5.0 while that for inulase B was pH 5.5 . The enzymes also differed slightly in temperature optima. Inulases A and B had temperature optima at 30°C. and 35°C. respectively. However, these enzymes gave indications of also having high activity at low temperatures. The Michaelis constants (K_ms) for the enzymes were similar; 7.31 mM Substrate M and 7.99 mM Substrate M for inulases A and B respectively. The inhibition constants (K_is) for the enzymes, with sucrose as inhibitor, were 10.4 mM sucrose and 3.56 mM sucrose for inulases A and B

respectively, indicating that inulase B was more susceptible to sucrose inhibition than was inulase A. Several metal ions investigated had neither inhibitory nor activation effects on the inulases. Energies of inactivation for the enzymes were calculated from Arrhenius plots derived from temperature stability curves. The energies of inactivation for both enzymes were low. Inulase A required 158 kJ/mole to be inactivated while inulase B required 148 kJ/mole.

Second, "practical" implications regarding the enzymes' activity during storage and processing were considered. The effects of drying and freezing on the inulases were examined. Drying the tubers resulted in very low residual activity while most of the activity of the inulases was present after freezing and thawing of the tubers. Six strains of Jerusalem Artichoke tubers were screened for inulase activity. It was found that significant differences in inulase activity existed between different strains grown, harvested and stored under the same conditions.

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CHAPTER 1 INTRODUCTION

In recent years, fructose in the form of high fructose corn syrups has gained increased application as a sweetener in the food industry in Canada and the United States of America. The most recent prediction of USDA in 1980 indicated that the use of high fructose corn syrups was to increase continuously and that it would capture about one-third of the sweetener market by the mid 1980's. At the present time, the beverage industry is the largest user of high fructose syrups, however, many other sectors of the food industry (bakery, confectionery, processed foods, dairy products, etc.) are showing increased demands for this product. One major reason for this is that fructose is the sweetest naturally occurring sugar. It is 1.5 - 1.7 times sweeter than sucrose. This allows production of "light" or reduced-calorie foods (jams, salad dressing, canned fruit, etc.) since the required sweetness level can be achieved with less sugar and therefore less calories.

High fructose corn syrups are derived from corn starch by a high technology process which includes, among others, two enzymatic steps, namely, enzymatic hydrolysis of starch and isomerization of glucose to fructose. This process yields a 42% fructose/58% glucose stream. This represents the 42% syrup from which the 55% and 90% high fructose syrups are derived by fractionation and enrichment.

Alternate potential sources for the production of these high fructose syrups are plants which contain fructofuranosides. Jerusalem Artichoke (*Helianthus tuberosus* L.), chicory and dandelion are such plants. Jerusalem Artichoke tubers contain inulin and related polyfructans which upon hydrolysis could yield high fructose syrups. Fructans are

called inulin when their degree of polymerization (D.P.) reaches 30-35. This represents a fructose/glucose ratio of 29/1 - 34/1. Hydrolysis of inulin would therefore result in a more than 90% high fructose syrup which would make the Jerusalem Artichoke potentially superior to corn for high fructose syrup production. This has stimulated renewed interest in Jerusalem Artichoke as a source of fructose. Research in recent years has included investigations on agronomic aspects, storage and processing.

An important aspect in view of fructose syrup production was that changes in the carbohydrate fraction of Jerusalem Artichoke tubers occurred during storage and processing of the tubers. Jerusalem Artichoke tubers contain a wide range of fructans from sucrose to inulin. During storage, a decrease of the high molecular fraction (inulin) and a concurrent increase of the low molecular fraction has been observed. This resulted in a decrease of the fructose/glucose ratio. Changes during storage and also during processing were attributable to activity of enzymes, in particular to enzymes which hydrolyse inulin. For maximal fructose production it appears necessary to control enzymatic activities during storage and processing of the tubers. For example, the aim of storage of tubers is to obtain or maintain tubers with a high fructose/glucose ratio because such tubers would yield syrups of a high fructose level. Information about the tuber enzymes involved with inulin metabolism is therefore required.

Reports in the literature indicated that there were two classes of enzymes of major importance. These were the transferases which are involved in inulin synthesis and the hydrolases, comprising the inulases and invertase, which are involved in inulin breakdown. It has also been

reported (Edelman and Jefford, 1967) that the role of invertase in inulin metabolism was not significant. It is the inulases therefore, which are important in inulin breakdown during storage and processing of Jerusalem Artichoke tubers. The goal of this study was to generate information on these enzymes and to estimate their role in determining the fructose levels in high fructose syrup produced from Jerusalem Artichoke tubers. This was achieved through the following objectives:

(1) the isolation of inulases A and B in Manitoba grown Jerusalem Artichoke tubers;

(2) the characterization of these enzymes;

(3) the investigation of the stability of these enzymes during processing of the tubers; and

(4) the determination of the variability of inulase activity in new varieties generated from the breeding program at the Canada Department of Agriculture Research Station, Morden, Manitoba.

CHAPTER 2 REVIEW OF LITERATURE

2.1 JERUSALEM ARTICHOKE (HELIANTHUS TUBEROSUS L.)

The Jerusalem Artichoke is native to North America and grows well in the Prairie Provinces of Canada. The plant grows to about six feet tall, is upright and branches to varying degrees, depending on the variety. Tuber shapes may vary from round, knobby clusters to long, smooth single tubers. The sizes also vary considerably. Chubey and Dorrel (1974) found a range of tuber weights from 12g in a Manitoba strain to 438g in a Russian strain. The tuber may be either white or purple.

The growing period for Jerusalem Artichoke extends from early Spring (May) to late Fall (October). Tuber formation is initiated in the early Spring and the tuber is mature by late Fall. Inulin and its related polyfructans are synthesized from sucrose and fructose during this period. The tubers mature and can, under Prairie conditions, be harvested by the end of September or left in the ground. Once harvested and in storage, or if left in the ground, the tubers enter a state of dormancy which lasts until the end of April. In early spring, sprouting is initiated and a new plant results.

The yield potential of Jerusalem Artichoke is very encouraging when compared to corn and sugar beet, as is evident from the data in Table 1. Jerusalem Artichoke has a mean yield of 4650 lb/acre (Table 1) compared with 3317 lb/acre and 4330 lb/acre for corn and sugar beets respectively. There are many different strains of Jerusalem Artichokes and they differ in yield as well as fructose/glucose ratio. Chubey and Dorrel (1974) found, using various strains of Jerusalem Artichokes, a wide range of yields from 1600 lb/acre to 13,600 lb/acre. Therefore, Jerusalem Artichoke,

TABLE 1 CARBOHYDRATE YIELD DATA FOR JERUSALEM ARITCHOKE, CORN AND SUGAR BEET

Country	Carbohydrate Yield (lbs/acre)
JERUSALEM ARTICHOKE TUBERS	
Canada	1600 - 13600
USA	3430 - 6400
Netherlands	3200 - 5400; 6480
France	2180 - 6800
Germany	1080 - 2870
USSR	1425 - 2140; 8500*
Mean Value - 4650	
CORN KERNELS	
Canada	3240 - 3700
USA	3010
Mean Value - 3317	
SUGAR BEETS	
Canada	4390 - 5900
USSR	2700
Mean Value - 4330	

* Sunflower - Artichoke hybrid

Taken From: Fleming and Grootwassink, 1978

having the highest yield of carbohydrate, is the best potential source of fructose and high fructose syrup when compared to corn and sugar beets.

Jerusalem Artichoke does not require a high level of management to realize a high yield of carbohydrate (Dorrel and Chubey, 1977). High yielding strains are readily available and fertilizer has little effect on carbohydrate yield in non-stress situations. Harvesting of the tubers is similar to that of potatoes and should be simple to implement using slightly modified potato equipment. Dorrel and Chubey (1977), however, found that harvest date was the only management aspect which should be given special attention if the highest possible fructose content is to be realized. They found that delaying the harvest date between 17th September and 29th October caused a reduction in the fructose concentration.

2.2 COMPOSITION OF JERUSALEM ARTICHOKE TUBERS

The composition of the Jerusalem Artichoke tuber has not been thoroughly elucidated. Except for the proximate analyses shown in Table 2, little detailed information is available. The tuber is about 80% water with a carbohydrate content ranging from 68.4 - 83.1% of the tuber dry weight (Fleming and Grootwassink, 1978), most of the carbohydrate being inulin and its related polyfructans. Hemicellulose and cellulose make up 31.1% of the tuber dry weight (Fleming and Grootwassink, 1978), while nitrogen constitutes 1.45 - 1.55%, only 50% of which is protein related. The ash component in the tuber makes up 4.7% of the dry weight (Fleming and Grootwassink, 1978). This includes the mineral nutrients; phosphorus, 0.39%; calcium, 0.07% and iron, 0.017% on a dry weight basis. The iron content of the tuber (0.017%) is unusually high for a vegetable, when compared with the iron content of raw potato (.003%) and raw green

TABLE 2

PROXIMATE COMPOSITION OF JERUSALEM ARTICHOKE TUBERS

	Composition	
	Fresh Wt. %	Dry Wt. %
Water	80	-
Carbohydrate	15.2 - 28.8	68.4 - 83.1
Lipid	.1	.5
Nitrogen	.29 - .31	1.45 - 1.55
Ash	1.1	4.7
Cellulose and Hemicellulose	2.62	13.1

peas (.0095% dry weight) (Watt and Merrill, 1975). The Jerusalem Artichoke tuber could be of some nutritional importance in terms of its high iron content. The lipid constituent in the tuber is 0.5% of the dry weight (Watt and Merrill, 1975). This fraction has not been studied much however, and little is known about it. Several phenolic compounds have been reported present in the Jerusalem Artichoke tuber. Paupardin (1965)identified p-hydroxybenzoic, chlorogenic, vanillic, genteric, p-coumaric, caffeic and ferulic acids in the tuber. These compounds may be of concern in view of processing of the tubers for high fructose syrup since phenolics are often involved in the formation of undesirable dark-coloured pigments.

2.3 CARBOHYDRATES, INULIN AND RELATED POLYFRUCTANS

2.3.1 NATURE OF INULIN AND THE POLYFRUCTANS IN JERUSALEM ARTICHOKE TUBERS

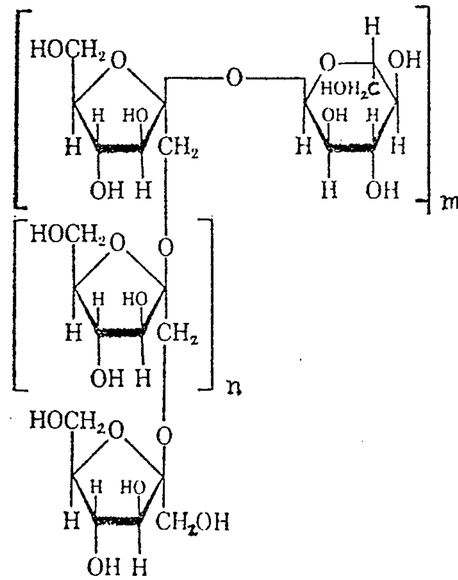
The carbohydrate fraction of the Jerusalem Artichoke tubers is of major commercial importance and is also best investigated. Inulin and its related polyfructans make up most of this fraction. The nature of this fraction determines the fructose/glucose ratio which is of particular importance in the production of high fructose syrup.

Inulin was first described, from studies on its structure (McDonald, 1946), as the polyfructan having a degree of polymerization (D.P.) of 35, a rather arbitrary number. Since then, inulin has been generally described as the polyfructans having D.P.s between 30 - 35. However, common usage now allows reference to all higher D.P. fractions as inulin since Edelman and Jefford (1964), using paper chromatography, showed that 95% of the content of commercial inulin had D.P.s between 20 - 30. Edelman and Jefford (1967) also demonstrated, using extended development techniques, that the higher limits of this series may considerably exceed D.P. 35.

In Jerusalem Artichoke tubers there is a homologous series of polyfructans ranging from D.P. 2 to about D.P. 35. As the structure in Figure 1 shows, each polyfructan has the general formula, Glc-Fru-Fru_n, where Glc-Fru (m) represents a terminal sucrosyl group and (n) can be any number from 1 up to about 35 (Edelman and Jefford, 1963). Average chain length for a population can be determined by observing the fructose/glucose ratio. A molecule population with a high fructose/glucose ratio would have a high average chain length (high inulin content) and vice versa. A high fructose/glucose ratio is very important in the production of high fructose syrup; the higher the ratio, the higher the percentage of fructose in the product.

The conformation of the inulin molecule was first investigated in 1964 by Phelps. Using sedimentation, viscometry and gel filtration, among other techniques, he determined that the molecule was in the form of a helix with four residues per turn of the helix. Each turn was 12 A° in both length and diameter. Later, Middleton (1976), calculated the molecular dimensions of inulin from physiochemical data obtained from the literature. He suggested that it was not possible to have a helix with exactly four units per turn. Such a situation, he observed, would result in steric repulsion of the end units in the turn. Instead, he suggested that there were 3.9 units per turn with a length of 22.5 A° and diameter of 15 A°. Middleton (1976) also observed that a slight decrease in bond angle from the centre of the helix to its extremities could bring the last two adjacent turns into contact with each other, thereby limiting the helix to a certain number of fructofuranose units (30 - 35). Marchessault _et al_ (1980), using X-ray diffraction techniques, confirmed the existence of inulin in helical form, as suggested by Phelps (1964) and Middleton (1976).

FIGURE 1 INULIN MOLECULE



m represents the terminal sucrosyl group.

n represents the fructofuranose moiety

[n = 1 —> (approx.) 35]

The content of inulin and related polyfructans in Jerusalem Artichokes changes during the life cycle of the tuber. These changes affect the fructose content in the tuber and, consequently, its potential as a source of high fructose syrup. During storage (dormant tubers), inulin and related polyfructans are degraded to lower D.P. olygosaccarides, thereby reducing the fructose/glucose ratio and the fructose content of the tuber. Therefore, it is of great importance to elucidate the role of agronomic practices and storage on these changes. In the following paragraphs these two aspects will be discussed.

2.3.2 EFFECTS OF AGRONOMIC FACTORS ON INULIN AND THE POLYFRUCTANS OF JERUSALEM ARTICHOKE

In paragraph 2.1 it was pointed out that varieties differ in terms of total sugar content as well as in fructose/glucose ratio (Chubey and Dorrel, 1974). Furthermore, Dorrel and Chubey (1977) observed that regardless of cultivar, the total fructose concentration declined with delay in harvest. The decrease was accompanied by an increase in the proportion of glucose; probably due to the interconversion of fructose to glucose and synthesis of sucrose that occurs during the growth and maturation processes. These workers also found that supplemental irrigation lowered the reducing sugar concentration. The reduction was previously observed by Kiesselbach and Anderson (1929), who stated that increased tuber moisture played a part in fructose concentration reduction. However, Dorrel and Chubey (1977) pointed out that supplemental irrigation reduced stress during critical periods, thereby delaying the need for the formation of carbohydrate reserve.

In terms of agronomic considerations, a tuber with high sugar content and high fructose/glucose ratio could be realized by: (i) selecting a variety with the desired qualities, (ii) by paying particular attention to harvest date, and (iii) by not employing supplemental irrigation.

2.3.3 EFFECTS OF STORAGE ON INULIN AND THE POLYFRUCTANS OF JERUSALEM ARTICHOKE

The Jerusalem Artichoke tuber must be stored if it is to be used for high fructose syrup production year round. Cold storage is the only storage format for Jerusalem Artichoke tubers that has been investigated extensively. Ideal storage conditions were found to be between October and April at 3 ± 1°C and 95% relative humidity. Under these conditions, the carbohydrate composition undergoes significant changes. In general, the average chain length of the polyfructans decrease. The polyfructans of higher D.P. are hydrolysed giving a corresponding increase in the soluble, lower D.P. sugars, without a significant net loss in the total carbohydrate content (Rutherford and Flood, 1970; Rutherford and Weston, 1967; Edelman and Jefford, 1964). Breakdown of the high D.P. polyfructans is accompanied by a marked reduction in the fructose/glucose ratio. Hoehn (1978) found a 20% reduction in the fructose/glucose ratio. Chubey and Dorrel (1972, 1974) found a decrease in fructose concentration accompanied by an increase in the proportion of glucose. Fleming and Grootwassink (1978) observed that there was a slight loss in total reducing sugar during cold storage. However, this small loss could not account for the significant change in the fructose/glucose ratio which has been observed.

Edelman and Jefford (1963), observed that storage at 2°C resulted in an accelerated decrease in the average chain length of the polyfructans in the tuber as compared to storage at 20°C where the rate of polyfructan breakdown is slower (Rutherford and Weston, 1967; Edelman and Jefford, 1963). At this temperature, however microbial breakdown of the tuber tissue occurs rapidly. It has also been observed that the degradation of the high D.P. polyfructans during cold storage is faster during the early stages of storage (5-6 weeks) after which the rate of degradation slows down considerably (Rutherford and Weston, 1967). This difference may be as a result of the disappearance of the enzyme sucrose-sucrose 1 - fructosyl transferase (SST) at the onset of dormancy, as mentioned in paragraph 2.5.2. The most satisfactory storage temperatures have been reported to be between 0° and 4°C (Pilnik and Vervelde, 1976). Hoehn (1978) found that 3 ± 1°C and 95% relative humidity provided the best conditions for storage.

The general trend towards a reduction in the fructose/glucose ratio during cold storage can be minimized by storing the tubers under ideal conditions as mentioned previously. However, other storage formats like dehydration and freezing may also minimize carbohydrate degradation in the Jerusalem Artichoke tuber.

2.4 USES OF JERUSALEM ARTICHOKE TUBERS

The Jerusalem Artichoke tuber is used as a fresh vegetable but only on a small scale. Flour derived from the tuber has been used to supplement wheat flour in the manufacture of pasta products. Industrial alcohol has been produced from Jerusalem Artichoke tubers whose high yield and high sugar content makes it ideal for this purpose. However,

a promising use of the Jerusalem Artichoke tuber may be the production of high fructose syrup. The latter has been investigated in some way as far back as 1937. Underkofler et al (1937) grew and investigated Jerusalem Artichoke in the U.S.A. In Japan, Yamazaki (1954) proposed a commercial process for producing high fructose syrup from Jerusalem Artichoke. More recently, Dorrel and Chubey (1972); Fleming and Grootwassink (1978) and Hoehn (1978) have all investigated Jerusalem Artichoke.

2.5 ENZYMES INVOLVED IN POLYFRUCTAN METABOLISM IN JERUSALEM ARTICHOKE TUBERS

2.5.1 INTRODUCTION

The chemical changes which occur during tuber formation, dormancy and sprouting in the Jerusalem Artichoke tuber are the result of activity by several enzymes present in the tuber. These enzymes catalyse the synthesis of polyfructans and inulin during tuber formation; the breakdown of high D.P. polyfructans and inulin to lower D.P. fractions during dormancy (storage) and the total degradation of the polyfructans and inulin to fructose and glucose during sprouting.

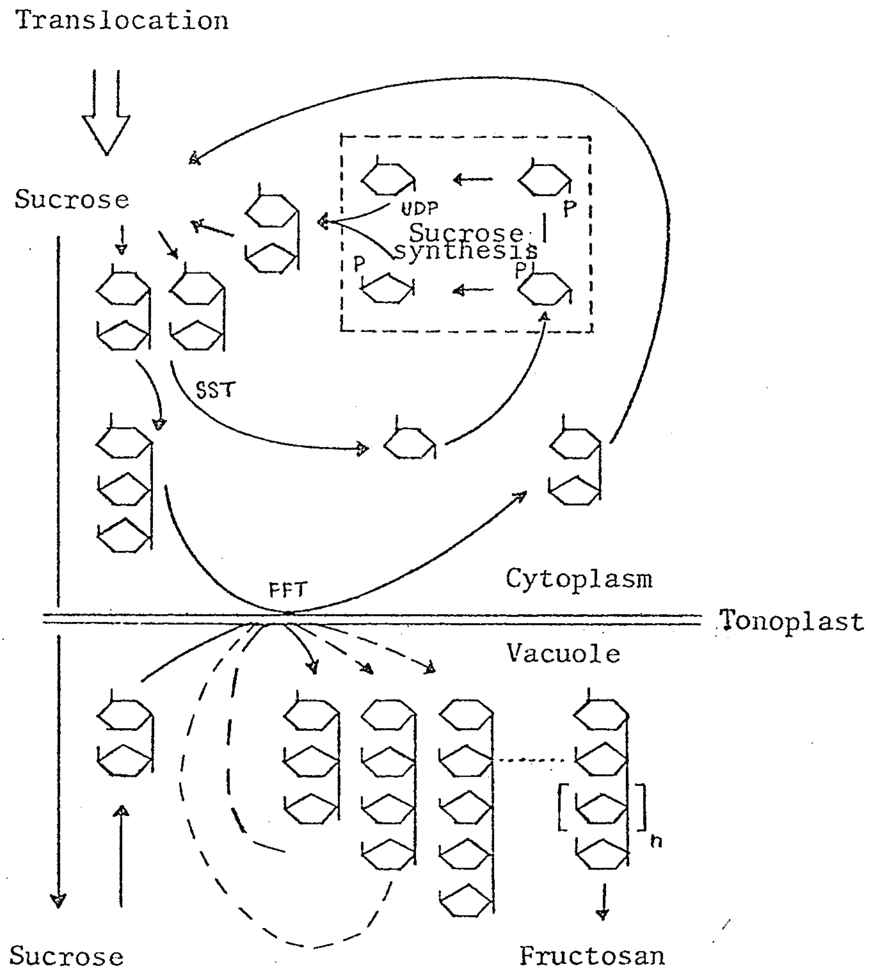
However, the fructose content in the tuber determines the quality of the high fructose syrup produced from them. Production of high fructose syrups with a maximal level of fructose relies therefore on the control of these enzymes, in particular during storage and processing of the tubers.

2.5.2 ENZYME ACTIVITY IN THE BIOSYNTHESIS OF POLYFRUCTANS AND INULIN DURING TUBER FORMATION

Synthesis of the polyfructans and inulin from sucrose takes place during tuber formation and seems to be most pronounced towards the end of the growing season. An overall scheme of the synthesis as proposed by Edelman and Jefford (1967) is presented in Figure 2.

Polyfructan and inulin syntheses are initiated by the formation of the trisaccharide 1^F-fructosylsucrose from sucrose. 1^F-fructosylsucrose is the primary donor molecule for the transfer of fructosyl moieties. This transfer activity is catalysed by two distinct transferases; sucrose-sucrose 1 - fructosyl transferase (SST) [Scott, Jefford and Edelman, 1966] and B(2-1') fructan 1 - fructosyl transferase (FFT) [Edelman and Dickerson, 1966]. SST forms the trisaccharide 1^F-fructosyl-sucrose from sucrose by the reaction: G-F+G-F→G-F-F+G. SST shows high specificity for sucrose, having little or no activity against the trisaccharide as donor or with glucose as acceptor; thus its action is essentially irreversible (Edelman and Jefford, 1967). The enzyme does not promote polymerization above the trisaccharide level as the tri-saccharide and higher polymers are practically inactive as acceptors for further transfer (Scott, 1968). Further polymerization of the trisaccharide to higher D.P. fructans is catalysed by FFT. This enzyme is highly specific for the transfer of single terminal B(2-1') linked fructofuranosyl residues to the same position on other molecules as shown by the equation: $G\text{-}F\text{-}F_n + G\text{-}F\text{-}F_m \rightleftharpoons G\text{-}F\text{-}F_{n-1} + G\text{-}F\text{-}F_{m+1}$ (Edelman and Jefford, 1967). 'SST' is active in the cytoplasm of the tuber cell where it catalyses the formation of 1^F-fructosylsucrose

FIGURE 2 SCHEME FOR POLYMERIZATION OF SUCROSE TO FRUCTOSAN IN CELLS OF DEVELOPING ARTICHOKE TUBER



FROM: EDELMAN AND JEFFORD, 1967

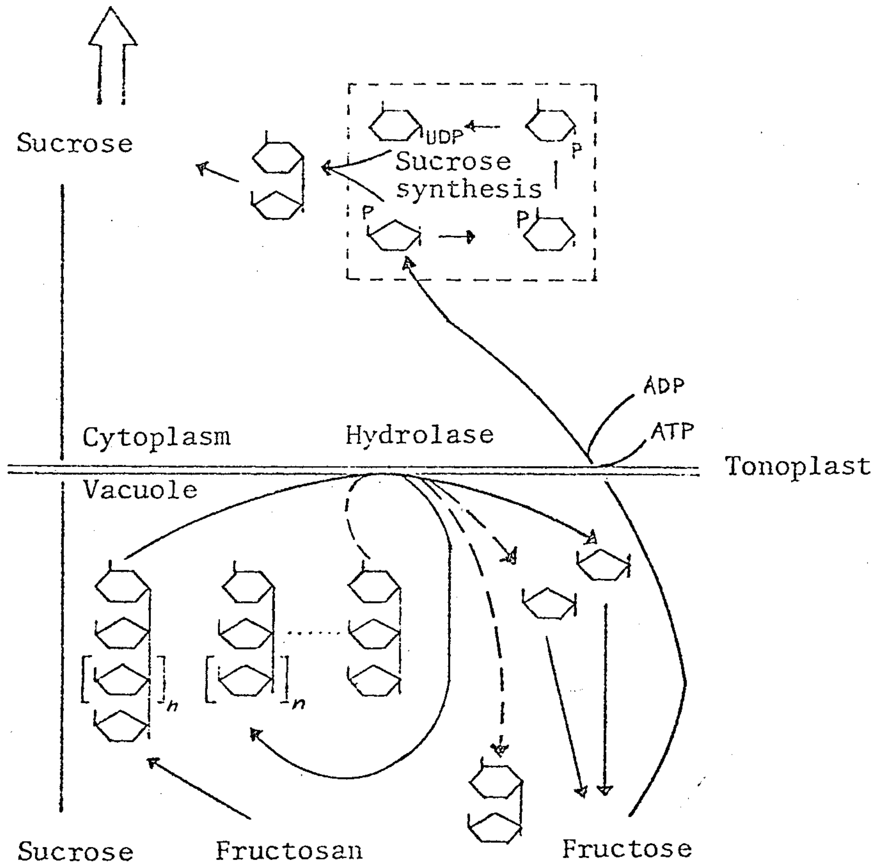
releasing glucose in the process (Figure 2). Glucose produced is converted to sucrose by the UDPG pathway in the cytoplasm. Sucrose is translocated from the cytoplasm to the vacuole; the site of fructan synthesis. The terminal fructosyl group of the trisaccharide is transferred to a sucrose molecule in the vacuole by FFT situated at the tonoplast. Further transfer of terminal fructosyl groups from trisaccharide molecules to the growing chain takes place until chain termination occurs to give an 'inulin' molecule (Edelman and Jefford, 1967).

2.5.3 ENZYME ACTIVITY DURING DORMANCY

During cold storage, the tuber remains in a dormant state. However, significant catabolic activity results in important changes in the polyfructans. These changes determine the fructose content of the stored tubers by lowering the average D.P. of the polyfructans and the fructose/glucose ratio.

The active enzymes during this period are FFT and the hydrolases; inulase A and inulase B. SST disappears during the initial stages of dormancy resulting in the loss of the trisaccharide 1^F-fructosyl sucrose which is probably related to the high rate of polyfructan degradation during this period as mentioned in paragraph 2.3.3. FFT transferes fructosyl groups among the chain ends in the vacuole. At the same time, inulases A and B hydrolyse fructose from the polyfructans, liberating free fructose which is converted to sucrose by the UDPG pathway (Edelman and Jefford, 1967). This results in a reduction in the average chain length of the polyfructans and a concurrent reduction in the fructose/glucose ratio without a net change in the carbohydrate content.

FIGURE 3 SCHEME FOR DEPOLYMERIZATION OF FRUCTOSANS TO SUCROSE IN CELLS OF SPROUTING ARTICHOKE TUBER



FROM: EDELMAN AND JEFFORD, 1967

The action of FFT during storage (dormancy) does not seem to affect the fructose/glucose ratio in the tubers in a significant way. On the other hand, the reduction in the fructose/glucose ratio is directly related to the actions of the inulases. Therefore, the control of these enzymes is important in maintaining a high fructose/glucose ratio during cold storage. Sucrose, in high enough concentration at the reaction site, inhibits the inulases. Low temperature and high humidity also seem to limit polyfructan breakdown, probably as a result of direct action on the enzymes and/or the polyfructans, or some other factor. It is also possible that reduction in the fructose/glucose ratio might also be minimized if the conversion of free fructose to glucose and sucrose is prevented by blocking the UDPG pathway.

2.5.4 ENZYME ACTIVITY DURING SPROUTING

When sprouting occurs, the polyfructans are consumed in supporting the growth of the new plant. The hydrolases (Inulases A and B) convert the polyfructans to fructose and sucrose (Figure 3). The fructose and sucrose move into the cytoplasm where fructose is converted to sucrose by the UDPG pathway and sucrose is translocated from the cell (Edelman and Jefford, 1967).

2.5.5 IMPORTANCE OF THE TUBER ENZYMES REGARDING TUBER QUALITY FOR PRODUCTION OF HIGH FRUCTOSE SYRUP

SST, FFT and the inulases are the enzymes which control the synthesis and breakdown of the polyfructans in Jerusalem Artichoke tubers. The presence of inulin and other high D.P. polyfructans in the tubers is as a result of SST and FFT activity. This activity seems to vary among

strains resulting in variations in sugar content among tubers of different varieties. It is important therefore, to select strains with high SST and FFT activities and high sugar content for the processing of high fructose syrup. Another tuber characteristic which can determine tuber quality and hence its selection for high fructose syrup processing is inulase activity. Once under cold storage, the polyfructans in the tuber are subject to breakdown by the inulases; lowering the fructose/glucose ratio. This can be controlled to some extent by selection of tuber strains with low inulase activity and by storage under conditions previously mentioned.

Apart from these major enzymes, the Jerusalem Artichoke tuber contains invertases which are present in the tuber throughout its life cycle. The effects of these enzymes are relatively small compared to those of the major enzymes. Edelman and Jefford (1967) stated that invertases are present in intact Jerusalem Artichoke tubers only in small amounts and show highest activity when degradation of the polyfructans to monomers proceedsvery rapidly during sprouting. Invertase degrades sucrose, therefore, it may interfere with the production of sucrose through the UDPG pathway by breaking down the sucrose produced, thereby reducing the amount of sucrose available for polyfructan synthesis. By degrading sucrose, invertase may also reduce direct inhibition of the inulases by sucrose, resulting in higher polyfructan breakdown. It is desirable therefore, to also select a tuber strain with the lowest possible invertase activity, for high fructose syrup production.

SST, FFT and the invertases determine the sugar content in a tuber during the growth and maturation stages. These enzymes are not significantly affected by normal external factors during these periods and their

activities are of little importance in terms of polyfructan breakdown during storage. During cold storage, the inulases degrade the polyfructans. This action, and the fact that it can be controlled by simple management practices, makes the inulases the key enzymes in the system with regard to maintaining a high fructose content in the tuber for high fructose syrup production.

2.6 ROLE OF SUCROSE IN POLYFRUCTAN METABOLISM IN JERUSALEM ARTICHOKE TUBERS

Sucrose acts as a control point for polyfructan deposition during tuber formation, for conversion of high D.P. polyfructans to lower D.P. polyfructans during cold storage and for polyfructan degradation and transport as sucrose during sprouting. Edelman and Jefford (1967) observed that during tuber growth, polyfructan synthesis is initiated by the translocation of sucrose and formation of the trisaccharide, 1^F-fructosylsucrose from sucrose. Depolymerization of the polyfructans to sucrose during sprouting of the tubers results in sucrose being translocated from the tuber cell to support growth. Edelman and Jefford (1967) also found that sucrose can control the D.P. of fructans to some extent during polymerization. When the trisaccharide, 1^F-fructosylsucrose acted as both donor and acceptor during polyfructan synthesis, the sucrose liberated accepted free fructosyl residues more readily than did the trisaccharide. The sucrose competed successfully with the trisaccharide, reforming the substrate, thereby affecting the length and number of fructosyl chains. Sucrose can also inhibit depolymerization of the polyfructans by direct action on inulases A and B, being more affective against inulase B. The terminal sucrose moiety on the fructan chain also plays a major part in the rate of attack by the inulases on the lower members of the

polyfructan series. Edelman and Jefford (1964) observed that activity increased markedly with increasing D.P. However,above D.P. 8, this effect disappeared. Probably, at this chain length, the sucrose moiety was too remote to be effective.

2.7 JERUSALEM ARTICHOKE INULASES: PROPERTIES AND CHARACTERISTICS

Since the inulases are the key enzymes in the Jerusalem Artichoke tuber in terms of producing high fructose syrup, generating detailed information about the enzymes is important in understanding them. Edelman and Jefford (1963,1964) isolated the inulases by precipitation with $(NH_4)_2SO_4$ followed by purification and separation on DEAE cellulose. Detection of enzyme activity was through the liberation of fructose measured by the increase of total reducing sugar, using the method of Somogyi (1951), as described by Ashwell (1957). The mode of action of inulases A and B is thought to be by exo-attack from the non-reducing end of the B-D-fructofuranoside. Edelman and Jefford (1964) demonstrated that a multichain mechanism was operating, where the enzyme attacks the end of various polymer chains at the same time in a completely random fashion, removing single fructosyl residues. These workers also found that Inulase A attacked substrates of lower D.P. faster than did Inulase B. The optimum chain length for Inulase A was D.P. 5 while that for Inulase B was D.P. 6-8. Michaelis constants (Km) measured for inulin and 1^F-fructosylsucrose were 3.3×10^{-2} M while that for inulopentose was 2.2×10^{-2} M. The optimum temperature for Inulase A was 30°C and that for Inulase B was 35°C. Edelman and Jefford (1964) found that Inulase B was more labile at high temperatures than was Inulase A. Optimum pH for

Inulases A and B were pH 5.0 and pH 5.5, respectively. Edelman and Jefford (1962) found that both enzymes were stable at pH 5.0 for long periods at room temperature and -25°C. Sucrose, 1^F-fructosylsucrose, raffinose and methylfructoside inhibit the inulases (Edelman and Jefford, 1964). Sucrose inhibited Inulase B about three times as much as it inhibited Inulase A. The inhibition is non-competitive, suggesting that the site on the enzyme, sensitive to sucrose inhibition, is different from the active site.

CHAPTER 3 MATERIALS AND METHODS

MATERIALS

3.1 JERUSALEM ARTICHOKE TUBERS

Jerusalem Artichoke tubers, *Helianthus tuberosus* L., were provided by Dr. B. B. Chubey, Canada Department of Agriculture Research Station, Morden, Manitoba. The tubers (crop years 1978, 1979 and 1980) were harvested in October and stored in plastic bags under refrigeration at 2 - 4°C. from October to April. The tubers remained in the dormant state during this period.

Characterization of the inulases was performed on tubers of the Manitoba strain #M6. Variability of inulase activity was investigated on six new strains; NC 10 16, NC 10 22, NC 10 28, NC 10 31, NC 10 39 and NC 10 40, generated by the breeding program at the Morden Research Station.

3.2 CHEMICALS

Inulin (derived from chicory) was obtained from Fisher Scientific Co., New Jersey. Reagent grade cysteine and diethyldithiocarbamate were also purchased from Fisher Scientific Co. Crystalline bovine serum albumin, analytical grade, was obtained from Sigma Chemical Co., Missouri. DEAE cellulose (DE23) was obtained from Whatman Ltd. "Stox" oxime internal standard reagent (containing 6 mg/ml phenyl-B-D-glucopyranoside) and trimethylsilylimidazole were purchased from Pierce Chemical Co., Rockford, Illinois. All other chemicals and reagents were of analytical grade.

METHODS

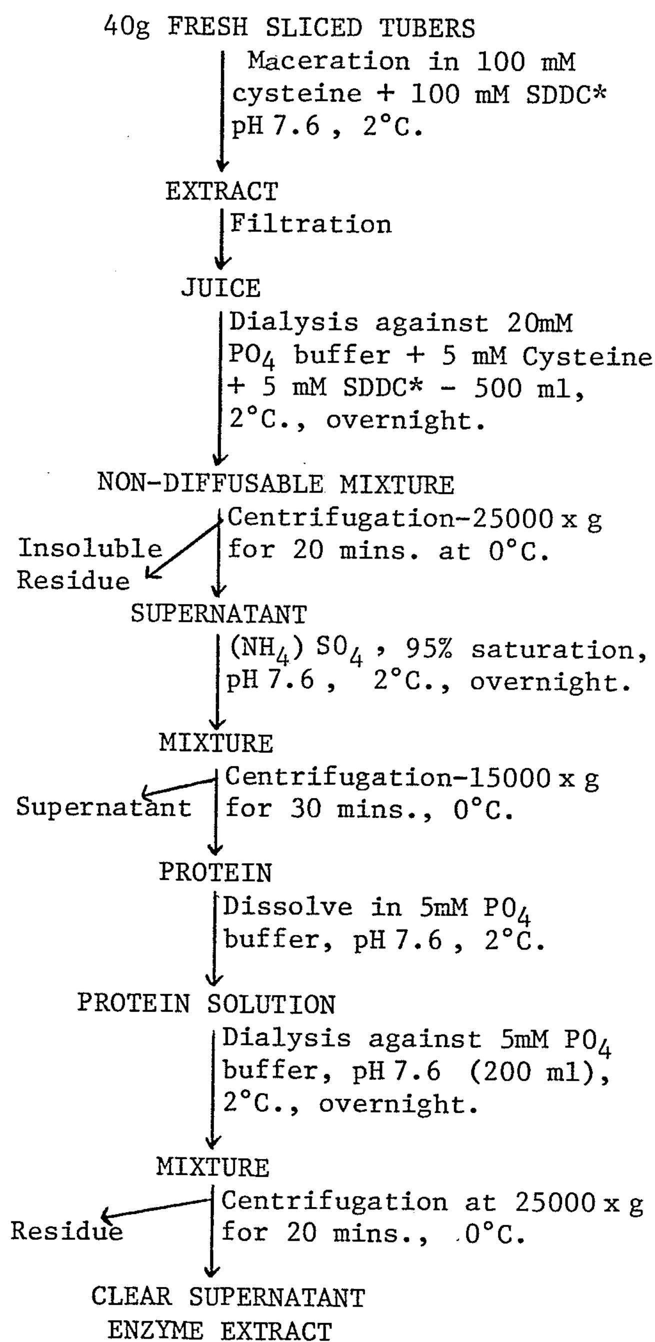
3.3 EXTRACTION OF INULASES

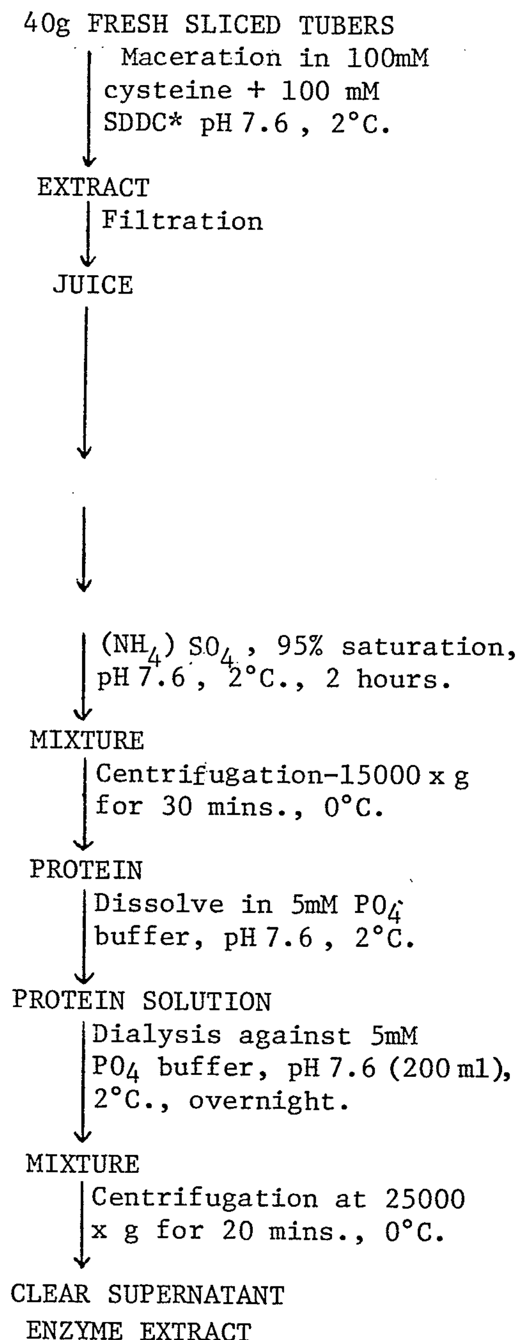
Inulase extract was prepared according to the method described by Rutherford et al (1969) and is outlined in Fig. 4 (a). Tubers were washed, peeled and sliced (thickness 3 mm ± 1 mm). Forty g of the sliced tubers were macerated in an Oster blender in the presence of 25 ml of a blending medium containing 100 mM phosphate buffer, pH 7.6, 100 mM cysteine and 100 mM sodium diethyldithiocarbamate. The extract was then filtered through four thicknesses of muslin and the filtrate dialysed overnight against 20 mM phosphate buffer, pH 7.6, containing 5 mM cysteine and 5 mM sodium diethyldithiocarbamate. The non-diffusable material was centrifuged at 25,000 x g for 20 minutes with a Sorvall Superspeed RC2-B centrifuge to remove insoluble residue. Protein was precipitated from the supernatant by the careful addition of $(NH_4)_2SO_4$ to 95% saturation, the pH being maintained at 7.6 . After standing at 2°C., 16 hr. (overnight), the precipitated protein was separated by centrifugation at 15,000 x g for 30 minutes and then dissolved in 5 mM phosphate buffer, pH 7.6 . This solution was dialysed overnight against 5 mM phosphate buffer, pH 7.6 . The non-diffusable material was centrifuged at 25,000 x g for 20 minutes and the clear supernatant (25 ml) used for analysis. Centrifugation was at 0°C. All other operations were at 4 ± 1°C.

3.4 ABRIDGED METHOD FOR ENZYME EXTRACT PREPARATION

The method described by Rutherford et al (1969) was shortened to allow faster screening of Jerusalem Artichoke tubers for inulase activity. Fig. 4 (a) shows the published method and Fig. 4 (b) shows the abridged procedure. This procedure was used in the screening of new strains for inulase activity.

FIG. 4 ENZYME EXTRACT PREPARATION





*SDDC = sodium diethyldithiocarbamate

3.5 CHROMATOGRAPHY OF CRUDE EXTRACT ON DEAE CELLULOSE

The crude extract was passed through an ion exchange column to effect both purification of the extract and separation of inulases A and B.

The procedure used was described by Edelman and Jefford (1964). A 1 g. column (95 mm x 11 mm) of DEAE cellulose (DE23) was prepared and an aliquot of protein solution containing 5 mg protein was placed on the column and fractionated by stepwise elution with the eluant systems shown in the following table.

TABLE 3 ELUANT SYSTEM FOR THE FRACTIONATION OF CRUDE EXTRACT

Eluant No.	mM PO_4	M NaCl	pH	Volume (Ml)
1	5	0	7.6	20
2	20	0	7.6	20
3	50	0	7.6	20
4	50	.1	7.6	20
5	50	.2	7.6	20
6	50	.3	8.0	20

10 ml fractions of the column effluent were collected automatically using a LKB 7000 Ultarac Fraction Collector. Each fraction was assayed for inulase and invertase activities.

3.6 PREPARATION OF SUBSTRATE M

A standard mixture of oligosaccharide (Substrate M) was prepared from Jerusalem Artichoke tubers according to the method described by Edelman and Jefford (1964). 250 g of peeled tubers were disintegrated in an Oster blender with 100 ml of 5 mM sodium diethyldithiocarbamate.

The homogenate was filtered through cheese cloth and boiled for four minutes to deactivate enzymes. The resulting juice was then filtered through a Whatman No. 4 filter while hot. Fractional precipitation was then carried out with ethanol. Three volumes of ethanol were added to the extract and the mixture was left at 2°C. overnight. The precipitate was separated by centrifugation at 9000 g for 10 minutes. A further 5 volumes of ethanol was added to the supernatant and the mixture was allowed to stand overnight at 2°C., after which the precipitate was separated by centrifugation at 9000 g for 10 minutes. The precipitate was dissolved in water and 8 volumes of ethanol was added. The material which precipitated overnight at 2°C. was separated as before, dissolved in water and made up to 50 ml. The solution ("Substrate M") contained 52.1 mg/ml total reducing sugar.

3.7 DETERMINATION OF FRUCTOSE/GLUCOSE RATIO IN POLYFRUCTANS BY GAS CHROMATOGRAPHY

3.7.1 HYDROLYSIS OF "SUBSTRATE M" AND INULIN

A 25 ml sample of "Substrate M" or inulin (5%) was placed in an Erlenmeyer flask and the pH was adjusted to 1.5 with 2.5 N HCl. The flask was placed in a water bath at 80°C. for one hour, after which the contents were cooled and adjusted to pH 5.6 with 2.0 N NaOH.

3.7.2 PREPARATION OF SYLYL DERIVATIVES

The hydrolysed samples were freeze-dried and derivatives prepared as follows:

10 - 15 mg of the freeze-dried sugar mixture were weighed into a 3.0 ml Reacti-Vial. 1.0 ml "stox" oxime internal standard reagent was added

to the sugar. The vial was then heated for 30 minutes at 75°C., forming the sugar oximes. It was then cooled to room temperature, after which 1.0 ml trimethylsilylimidazole was added. The mixture was capped, shaken for 30 seconds and allowed to stand at room temperature for 30 minutes allowing silylation of the oximes to the trimethylsilyl ethers (Pierce Chemical Co.).

3.7.3 GAS CHROMATOGRAPHY

Separation of the fructose and glucose derivatives was carried out on a Varian Model 3700 Gas Chromatograph equipped with a Varian Aerograph Model 477 Integrator. The column characteristics and operational parameters for the chromatograph are given in the tables following.

TABLE 4

COLUMN CHARACTERISTICS		
Length	-	46 cm
Int. Diameter	-	2.0 mm
Support Material	-	Chromosorb W-HP(80-100)
Liquid Phase	-	3% SP2100

TABLE 5

OPERATIONAL CHARACTERISTICS		
Injector Temperature	-	300°C
Detector Temperature	-	350°C
Oven Temp. Program	-	6°C/min. from 110°C to 350°C
Holding Time at Final Temp. (350°C)	-	2 mins.
Carrier Gas (N_2) Flow Rate	-	20 ml/min.

Peaks representing glucose and fructose were quantified by comparison with the internal standard phenyl-B-D-glucopyranoside.

3.8 DETERMINATION OF REDUCING SUGARS

3.8.1 TOTAL HYDROLYSED REDUCING SUGARS

The total reducing sugar content of sugar samples, after total acid hydrolysis of the samples, was determined as follows.

The sample was diluted as necessary with water and then placed in an Erlenmeyer flask, after which the pH was lowered to pH 1.5 by the dropwise addition of 2.5 N HCl. The flask was then placed in a water bath at 80°C for one hour. The contents were cooled and adjusted to pH 5.6 with 2.0 N Na OH. Aliquots of the resulting mixture were then analysed for reducing sugar by the method described by Ashwell (1957) using the standard curve described below.

3.8.2 FREE REDUCING SUGARS

Free fructose and free glucose are responsible for the reducing powers in unhydrolysed sugar samples. Determination of this reducing sugar content was as follows.

The sample was suitably diluted with water and aliquots were then analysed by the method described by Ashwell (1957) using the standard curve described below.

3.8.3 STANDARD CURVE (FRUCTOSE)

A standard curve for the determination of reducing sugar content was prepared as described by Ashwell (1957). Fructose was used as the standard reducing sugar.

A series of fructose sugar solutions in the concentration range 0 - 350 ug/ml were prepared. 1.0 ml of each solution was placed in a test tube and 1.0 ml of a mixture of Copper Reagent A and Copper Reagent B

(25:1) was added. The contents were well mixed and the tubes placed in a boiling bath for 20 minutes. The tubes were then rapidly cooled and 1.0 ml of arsenomolybdate reagent was added to each tube and mixed. The contents of each tube was then diluted with water to 25.0 ml in a volumetric flask and the absorbance of the resulting blue solution was determined at 535 nm on a spectrometer.

3.9 DETERMINATION OF THE AVERAGE MOLECULAR WEIGHTS OF "SUBSTRATE M" AND INULIN

The average molecular weight or degree of polymerization of polyfructans could be determined from the fructose/glucose ratio. Each inulin or polyfructan molecule contains n fructose molecules and one terminal glucose molecule. Complete hydrolysis of the polyfructans gives free fructose and glucose. The average D.P. is obtained from the equation:

$$\text{Average D.P.} = \frac{\text{No. of fructose}}{\text{No. of glucose}} + 1 \quad .$$

The fructose /glucose ratio for "Substrate M" was 16/1. From the equation, Average D.P. $= \frac{16}{1} + 1 = 17$. The Molecular Weight of "Substrate M" was determined to be 2756 (M.W. "Substrate M = M.W. Fructose x 0.9 x 17). Total reducing sugars for "Substrate M" was determined to be 52.1 mg/ml (g/l) by the Somogyi method. The molarity of "Substrate M" could now be determined from the equation:

$$\text{Molarity "Substrate M"} = \frac{\text{Total reducing sugar "Substrate M"}}{\text{Molecular Weight "Substrate M"}} \quad .$$

However, the total reducing sugar value for "Substrate M", measured in aqueous solution as free fructose and glucose must be adjusted if it must accurately represent the polyfructans in "Substrate M". When

free fructose polymerize to form polyfructans, one water molecule is lost in order to accommodate bonding between the fructose units, resulting in a 10% loss from the fructose molecule. This 10% reduction is reflected in the factor of 0.9. Therefore, the adjusted equation would be:

$$\text{Molarity of "Substrate M"} = 0.9 \times \frac{\text{Total reducing sugar "Substrate M"}}{\text{Molecular Weight of "Substrate M"}}$$

The molarity of "Substrate M" was 0.017 M.

3.10 PROTEIN DETERMINATION

Protein concentration was determined by the method of Lowry *et al*, 1951. Crystalline bovine serum albumin served as the standard for the construction of the calibration curve. The absorbance of the blue solution was determined on a Bausch and Lomb Spectronic 710 spectrometer.

3.11 ASSAY PROCEDURES

3.11.1 INULASE ASSAY

Digests contained 3.0 ml enzyme extract, 2.0 ml substrate (10 mM Inulin or 17 mM "Substrate M") and 1.0 ml of 0.5 M acetate buffer, pH 5.0 . Incubation was at 35°C. for four hours. Activity was determined by the increase in reducing sugars in the digest, measured by the Somogyi procedure as described by Ashwell (1957)(3.8). Units of inulase activity were expressed as nkatals.

3.11.2 INVERTASE ASSAY

The procedure was identical to that for inulase activity (3.11.1) except that 0.1 M sucrose was used as substrate. Units of invertase activity was expressed as nkatals.

3.12 CHARACTERIZATION OF THE INULASES

3.12.1 EFFECT OF TEMPERATURE ON ACTIVITY

The optimum temperature of the enzymes were determined by incubating the crude enzyme extract, inulase A and inulase B at 5, 15, 20, 25, 30, 35, 40, 45, 55, 60 and 65°C. for two hours at pH 5.0 after which they were assayed for activity.

3.12.2 EFFECT OF pH ON ACTIVITY

The optimum pH of the crude enzyme extract, inulase A and inulase B were determined by incubating each at pH 3, 4, 4.5, 5, 5.5, 6, 7 and 8. Temperature was 35°C. and incubation time was two hours. Each digest was adjusted to pH 5.0 before the Somogyi sugar determinations were made. This was to prevent erroneous results due to acid hydrolysis of the substrate in the digests during the heating phase of the test.

3.12.3 DETERMINATION OF MICHAELIS CONSTANT

The Michaelis Constant (Km) of inulase A and inulase B were established by determination of their initial velocities at 1.7, 3.4, 8.5 and 17 mM "Substrate M". A reciprocal plot of velocity versus substrate concentration was used to determine the Km (Lineweaver and Burk, 1934).

3.12.4 SUCROSE INHIBITION

The inhibition constant (Ki) for inulase A and inulase B were determined by incubating digests containing 3.4 mM and 17 mM "Substrate M" with sucrose concentrations of 2, 4, 8 and 20 mM. Incubation temperature was 35°C. Aliquots of the digests were withdrawn at 5, 15, 30, 60 and 90 minute intervals and assayed for activity. The initial velocities were

determined and a plot of 1/V versus sucrose concentration was made for each substrate concentration according to Dixon (1953) and the K_i determined.

3.12.5 EFFECTS OF METAL IONS

Investigations of inhibition or activation of inulase A and inulase B by metal ions were done by incubating the enzymes for four hours at 35°C. with varying concentrations (10, 20, 30, 40, 100 mM) of $CaCl_2$, KCL, NaCl, ZnCl, $MgSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 2H_2O$. The digests were then assayed for a decrease or increase in inulase activity. The digests were compared against a control which contained no added metal ion.

3.13 HEAT STABILITY OF THE INULASES

Heat stability of the enzyme preparations were determined by exposing aliquots of each enzyme, at pH 5.0 and in the absence of substrate, to 40, 50 and 60°C. 3.0 ml aliquots were removed at ten-minute intervals and directly assayed for residual activity at 35°C. and pH 5.0 .

3.14 EFFECTS OF DRYING AND FREEZING OF TUBER MATERIAL ON INULASE ACTIVITY

3.14.1 EFFECTS OF DRYING

Jerusalem Artichoke tubers were washed, peeled and cubed. Four (4) 40g samples were weighed out in aluminum dishes and then dried in a vacuum oven at 35°C. and 700 mm Hg for 72 hours, to 3.8% moisture. Enzyme extracts were prepared from the samples using 25 ml blending medium and 20 ml added water to compensate for water lost during drying, and then assayed for inulase activity.

Similarly, 200g of cubed tubers were dried in a fluidized bed drier (Aeromatic STS6) at 65°C. for eight hours, to 3.2% moisture. Four (4) 8g samples were extracted as described above and the enzyme extracts assayed for inulase activity.

3.14.2 EFFECTS OF FREEZING

Five lots of tubers, two of M#6 and three of NC 10 16, about 300g each, were washed, dried and sealed in plastic bags. These were then placed in a freezer at -40°C. for 48 hours, after which they were removed and placed in a water bath for 2 1/2 hours at 35°C. The tubers were then peeled and enzyme extracts prepared. Each extract was assayed for residual inulase activity. Fresh tubers of both strains were used as controls.

3.15 SCREENING OF SIX (6) STRAINS FOR INULASE ACTIVITY

Six strains of Jerusalem Artichoke tubers were used to prepare inulase extracts using the abridged method mentioned in (3.4). Each extract was then assayed for inulase activity as outlined in (3.11.1).

CHAPTER 4 RESULTS AND DISCUSSION

4.1 EXTRACTION AND SEPARATION OF THE INULASES FROM JERUSALEM ARTICHOKE TUBERS

Crude enzyme extract was prepared from Jerusalem Artichoke tubers; Manitoba strain M#6, and its inulase and invertase activity determined. The extract was then separated on a DEAE cellulose column as illustrated in Figure 5. Inulase activity was found in fractions 4 to 7 (inulase A) and fractions 10-12 (inulase B) (Fig. 5). Both showed activity when inulin and Substrate M were used as substrates. There was also slight invertase activity in fractions 4 to 12. However, it was evident only after prolonged incubation for 24 hours. The resolution of the enzyme extract into inulase A and B (Fig. 5) was concordant with findings reported by Edelman and Jefford (1964) who found two inulases and an invertase whose activity slightly overlapped that of the inulases.

The specific enzyme activity in the crude extract was 0.218 nkatals/mg protein while that for the separated and purified enzymes were 0.854 nkatals/mg and 0.918 nkatals/mg for inulases A and B respectively (Table 6). This represents a 3.9 fold increase in activity upon purification for inulase A and a 4.2 fold increase for inulase B. The ratio of inulase A activity to inulase B activity was 0.73. Edelman and Jefford (1964) found also that inulase B was dominant. However, their data showed that the activity ratio was dependent on the D.P. of the substrate. It was evident from their results that the ratio of inulase A to inulase B was 0.45 for $(1^F\text{-fructosyl})_3$ sucrose (D.P.5) and 0.37 for inulin (D.P.35). However, considering substrate effect, there appears to be a difference of activity ratio found in the Manitoba strain M#6 and the strain used by Edelman and Jefford (1964).

FIG. 5

SEPARATION OF INULASES A AND B BY ION EXCHANGE CHROMATOGRAPHY

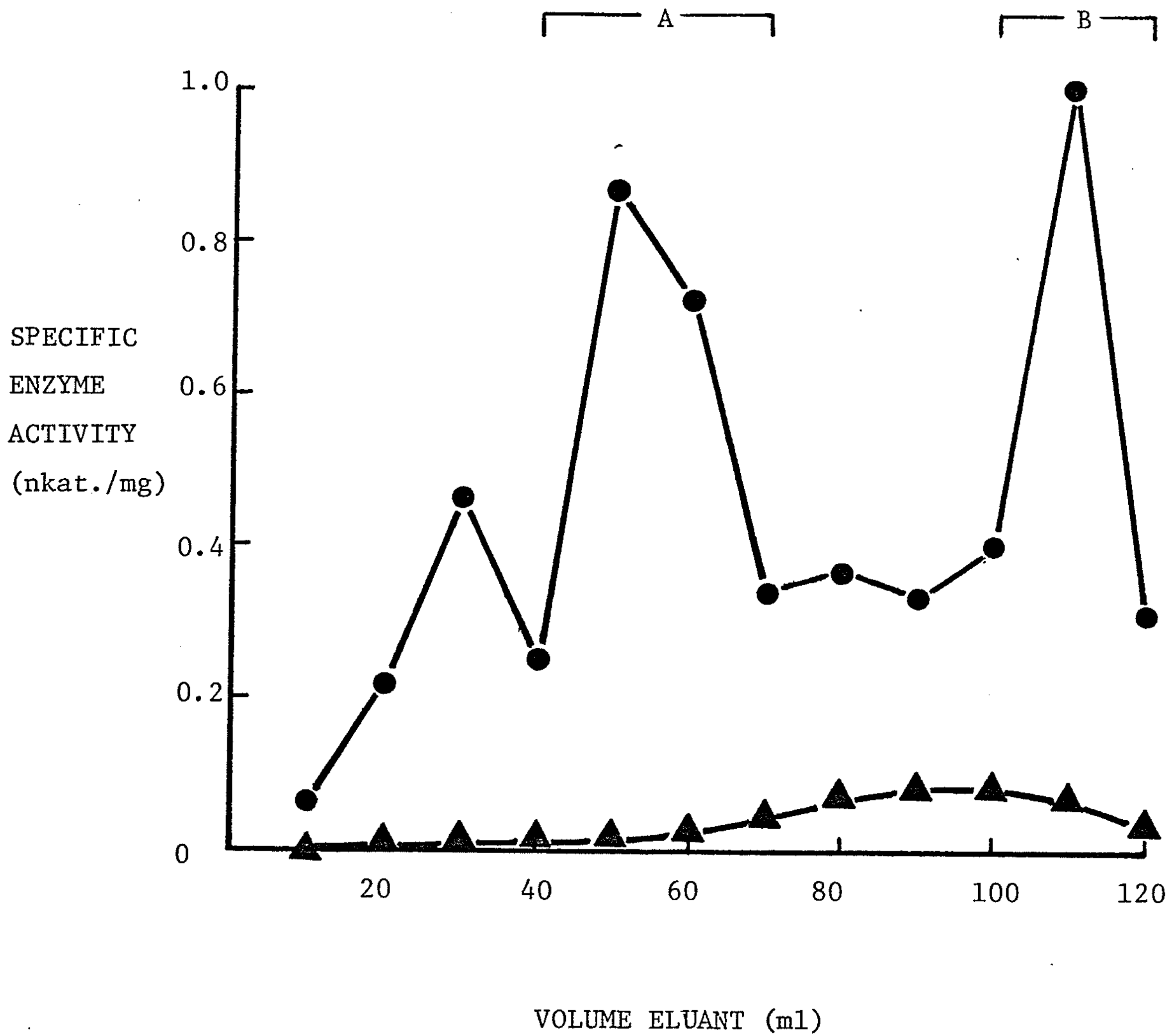


TABLE 6

INULASE AND INVERTASE ACTIVITY IN CRUDE EXTRACT AND IN INULASE A AND INULASE B PREPARATIONS

	PROTEIN (mg/g dry tuber)	ACTIVITY (nkat./g dry tuber)	SPECIFIC ACTIVITY (nkat./mg protein)	RECOVERY (%)
CRUDE EXTRACT	1.5			
Inulase Activity		.327	.218	100
Invertase Activity		.017	.0113	
INULASE A	.135	.115	.854	35.2
Invertase Activity		.00336	.0249	
INULASE B	.171	.157	.918	48.0
Invertase Activity		.0106	.0617	

4.2 SUBSTRATES AND THE INFLUENCE OF DEGREE OF POLYMERIZATION (D.P.) ON INULASE ACTIVITY

Commercial inulin, with an average D.P. of 33 was initially used as substrate for the inulases. However, the inulases from Manitoba strain, M#6, had very low activities when inulin was used as a substrate. Fractional precipitation of commercial inulin solutions with 95% ethanol indicated that this preparation was almost void of lower D.P. fructans. Substrate M was then prepared from Jerusalem Artichoke tubers and used as a substrate. The average D.P. of Substrate M was 17. The activities of the inulases with Substrate M as substrate were much higher than with inulin. Substrate M was therefore used as inulase substrate for all further study.

The difference in activities between the substrates was not surprising since Edelman and Jefford (1964) observed that the inulases in Jerusalem Artichoke tubers reacted faster with fructans of lower D.P. They found an optimum D.P. of 5 for inulase A and a D.P. of 6-8 for inulase B.

4.3 EFFECTS OF TEMPERATURE ON INULASES A AND B

Temperature profiles of the crude extract, inulase A and inulase B indicate an optimum temperature range of 30°C to 35°C (Figures 6 and 7). Optimum temperature for inulase A was 30°C , inulase B was 35°C and that of the crude extract was 35°C. Inulase A had activity higher than 85% within the temperature range of 20°C to 40°C. Inulase B had a narrower profile, with activities of 85% and higher occurring between 28°C and 38°C. Therefore, it seems that inulase A was less affected by temperature change than inulase B, within the temperature range investigated.

FIG. 6

EFFECT OF TEMPERATURE (OPTIMUM TEMPERATURE) ON CRUDE INULASE EXTRACT

100% = .219 nkat./mg

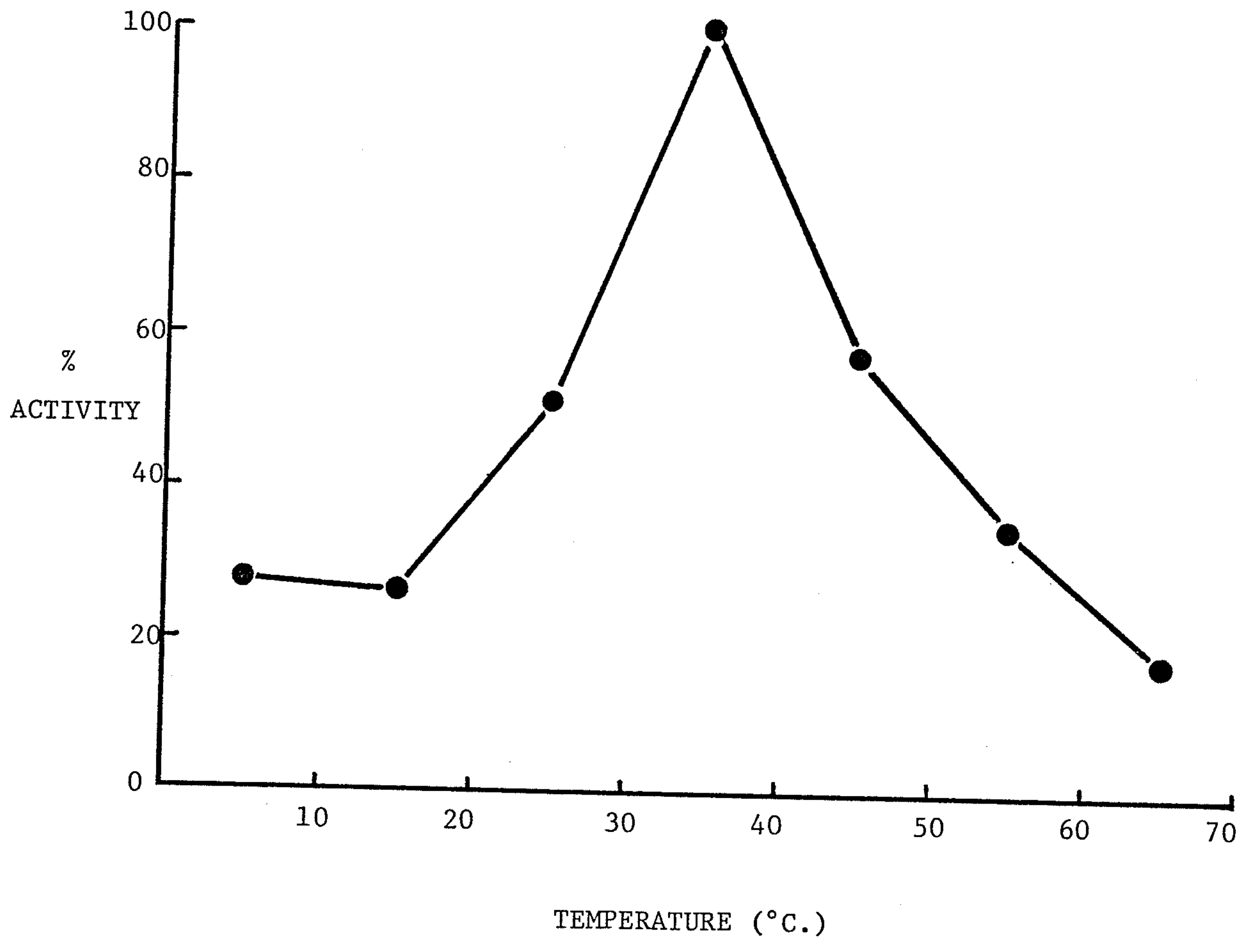
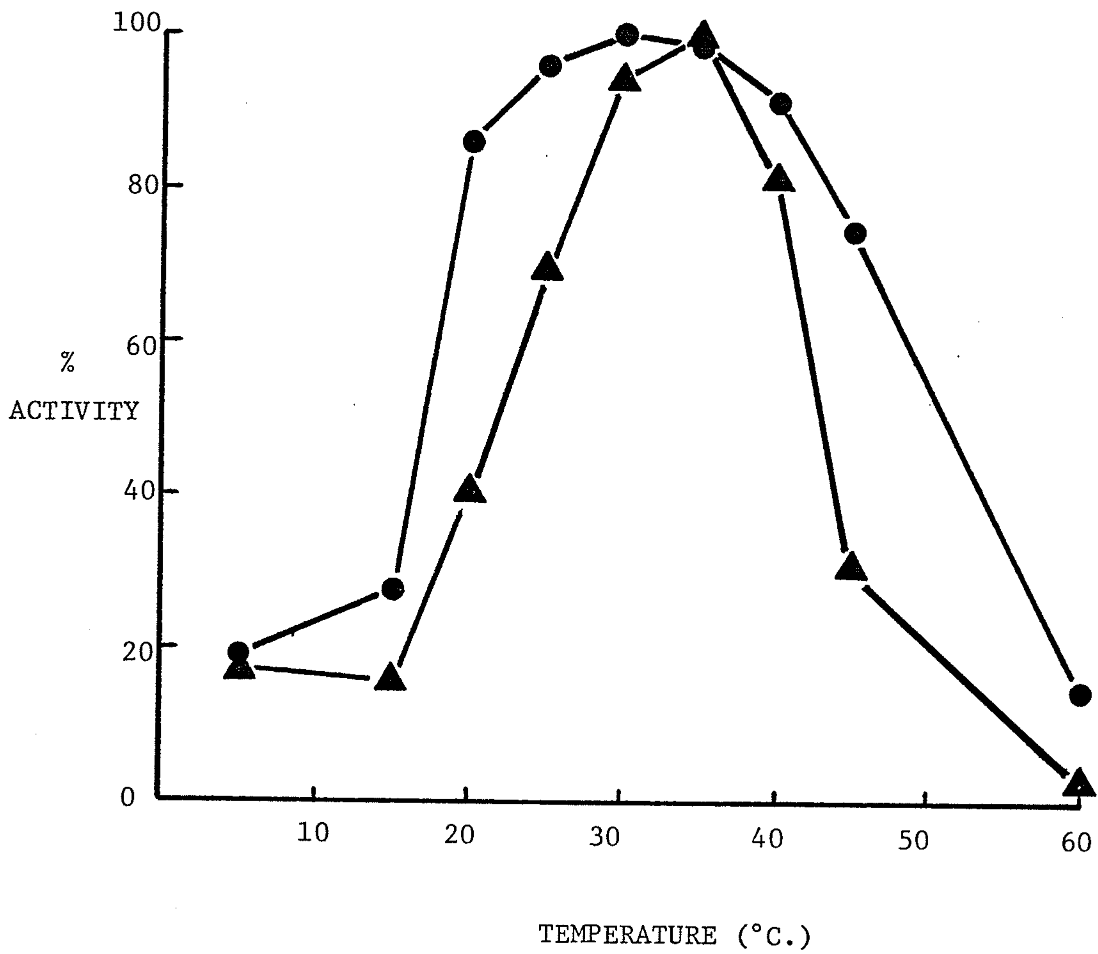


FIG. 7

EFFECT OF TEMPERATURE (OPTIMUM TEMPERATURE) ON INULASES A AND B

INULASE A - ● INULASE B - ▲

100% = 854 nkat./mg (Inulase A); .918 nkat./mg (Inulase B)



Temperature effects on the enzymes may be more complex than indicated. Edelman and Jefford (1963) observed that storage of tubers at 20°C resulted in less depolymerization of the polyfructans than at 2°C. This suggested that the inulases were more active at 2°C than they were at 20°C. In this study, no investigations of the enzymes' activities were done at temperatures below 5°C. However, Figures 6 and 7 do show a marked upturn in the temperature profiles at 5°C. This may be interpreted as an upward trend in the enzymes' activities at temperatures below 5°C and indicate that these enzymes may, in effect, have two temperature optima.

4.4 EFFECTS OF pH ON INULASES A AND B

The effects of pH on enzyme activity are illustrated in Figures 8 and 9. An optimum at pH 5.0 was found for the crude extract. Inulases A and B had optima at pH 5.0 and pH 5.5 respectively. This was in agreement with the optima for these enzymes reported by Edelman and Jefford (1964). Inulase A also had a broader profile and might be less affected by pH changes between pH 4.5 and pH 7.0.

4.5 EFFECTS OF SUBSTRATE CONCENTRATION ON THE INULASES AND THE DETERMINATION OF MICHAELIS CONSTANT (Km)

In all cases, the effects of substrate concentration on enzyme activity were as expected. Plots of velocity versus substrate concentration resulted in rectangular hyperbole as shown in Figures 10 and 11. Lineweaver-Burk plots gave straight lines (Figures 12, 13 and 14) and the apparent Michaelis Constants (Km) were determined to be:

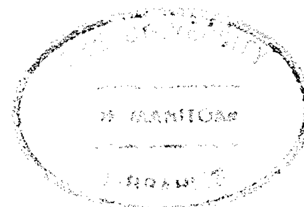


FIG. 8

EFFECT OF pH (pH OPTIMUM) ON CRUDE INULASE EXTRACT

100% = .223 nkat./mg

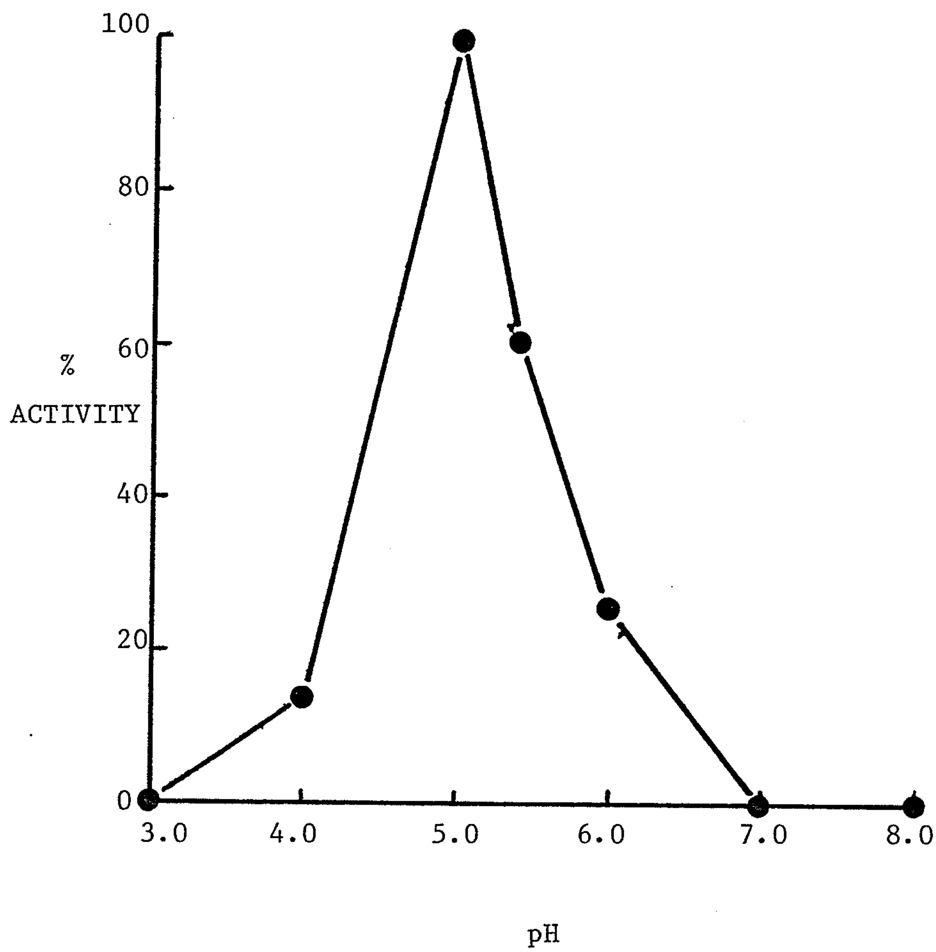


FIG. 9

EFFECT OF pH (pH OPTIMUM) ON INULASES A AND B

INULASE A – ●   INULASE B – ▲

100% = .866 nkat./mg (Inulase A); .912 nkat./mg (Inulase B)

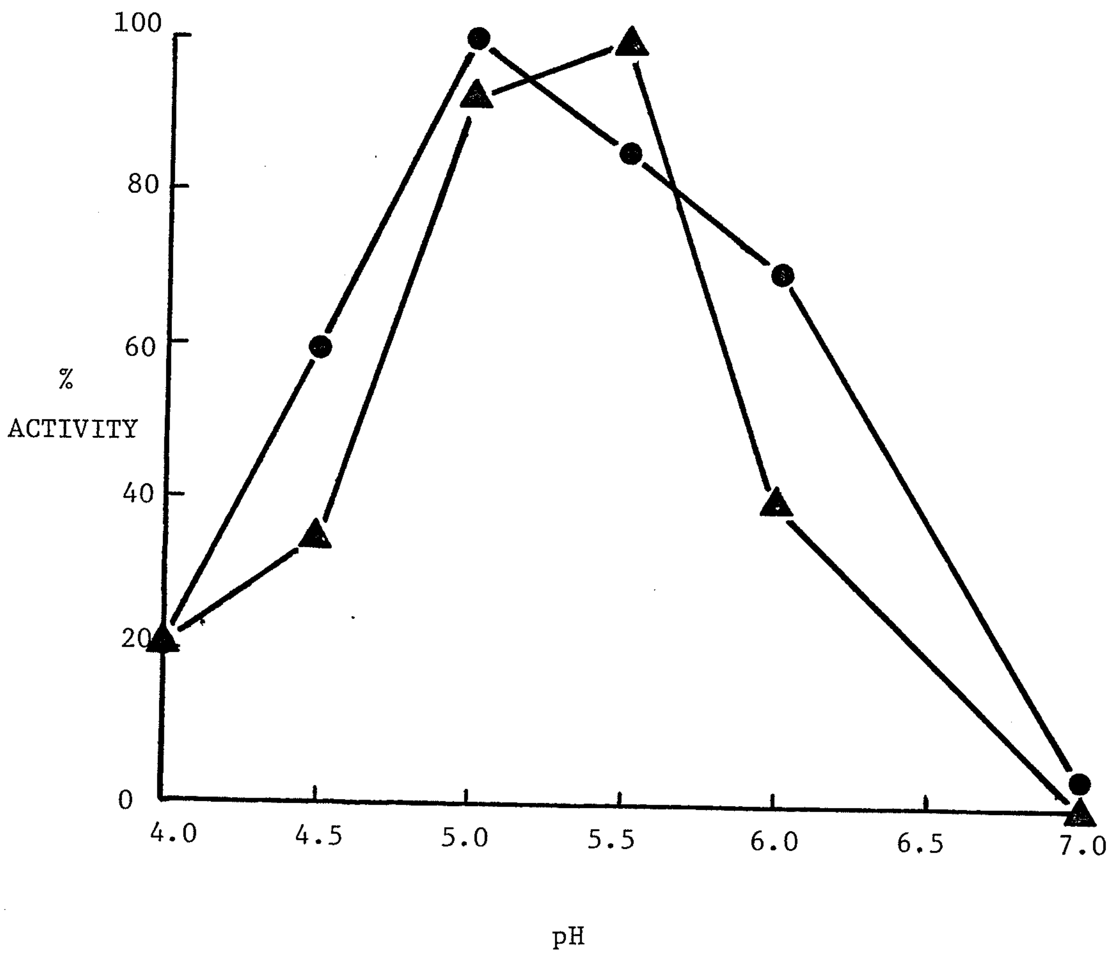


FIG. 10

EFFECT OF SUBSTRATE CONCENTRATION ON JERUSALEM ARTICHOKE ENZYME EXTRACT

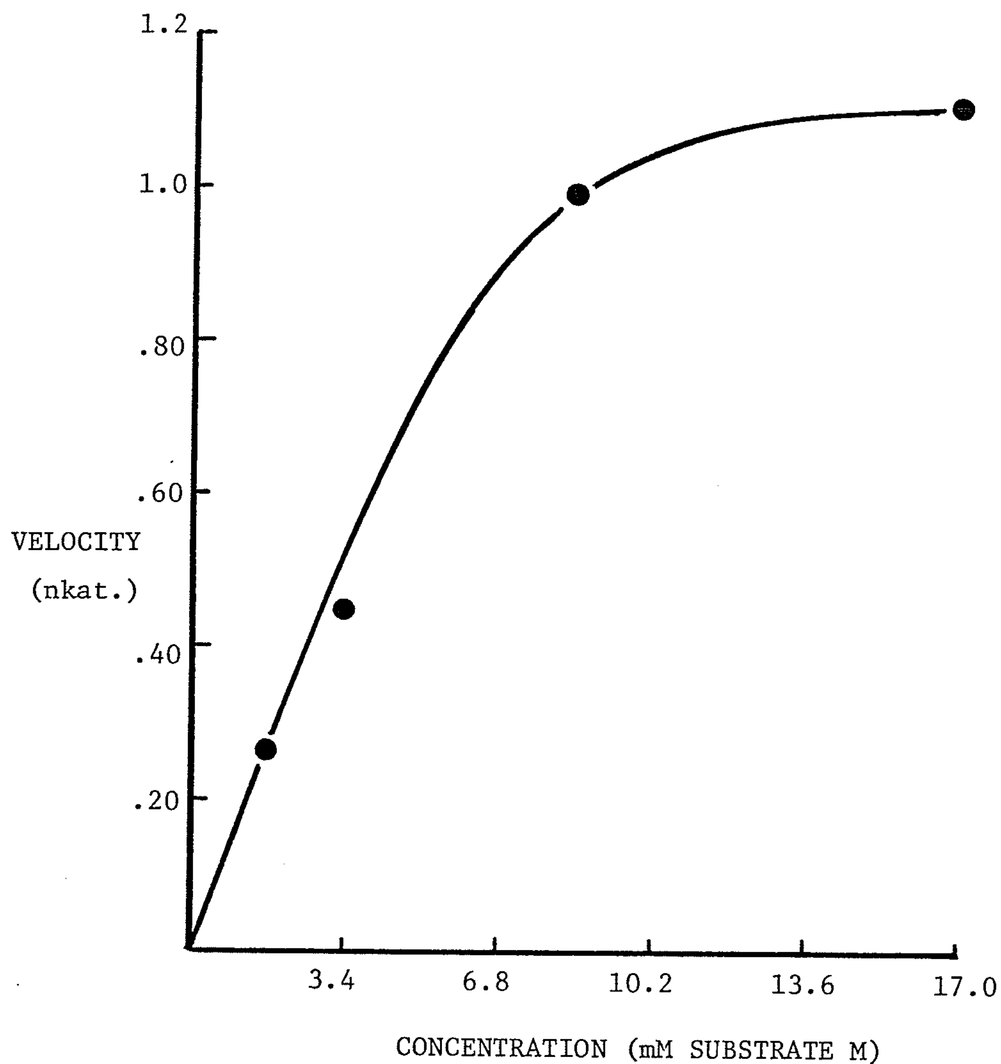


FIG. 11

EFFECTS OF SUBSTRATE CONCENTRATION ON INULASES A AND B OF JERUSALEM ARTICHOKE

● INULASE A △ INULASE B

.015 mg/ml Protein .019 mg/ml Protein

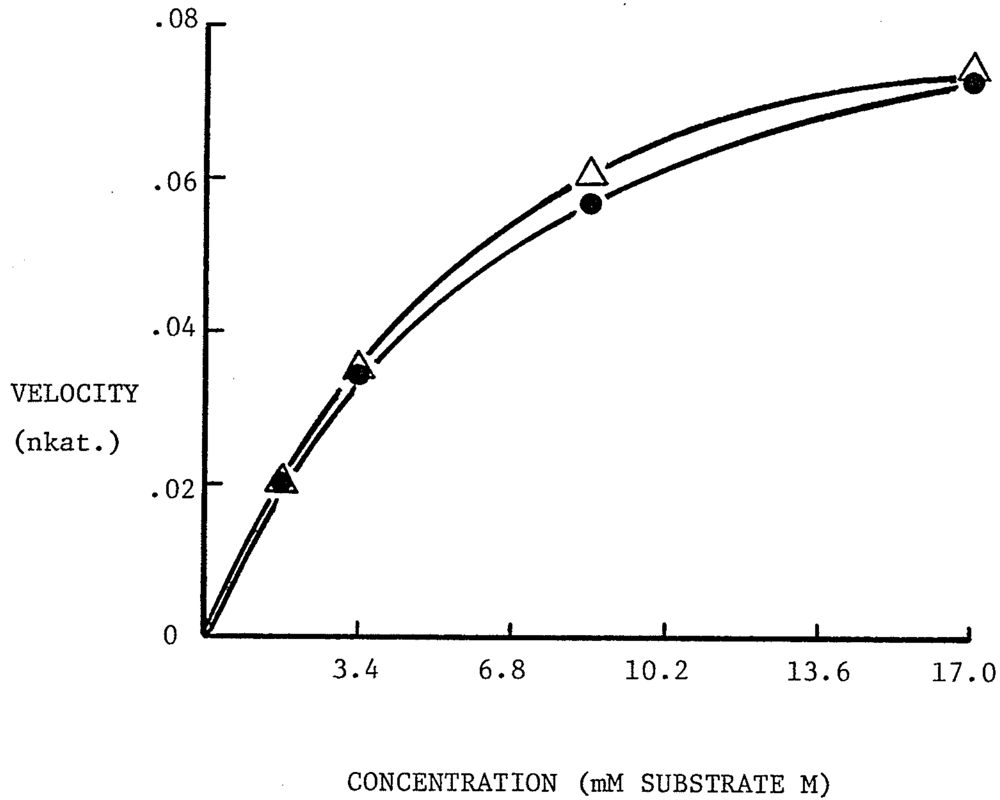


FIG. 12

LINEWEAVER-BURK PLOT OF THE EFFECT OF SUBSTRATE CONCENTRATION (SUBSTRATE M) ON JERUSALEM ARTICHOKE ENZYME EXTRACT

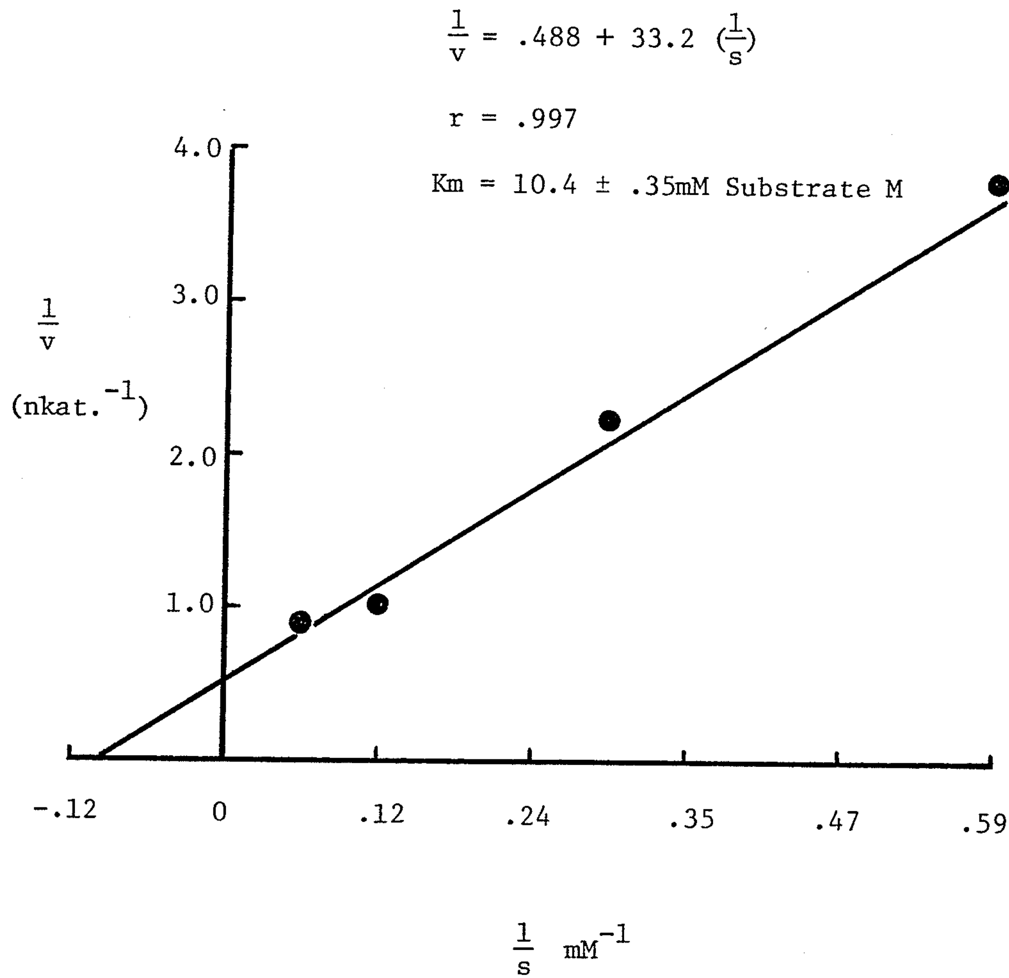


FIG. 13

LINEWEAVER-BURK PLOT OF THE EFFECT OF SUBSTRATE CONCENTRATION (SUBSTRATE M) ON JERUSALEM ARTICHOKE INULASE A

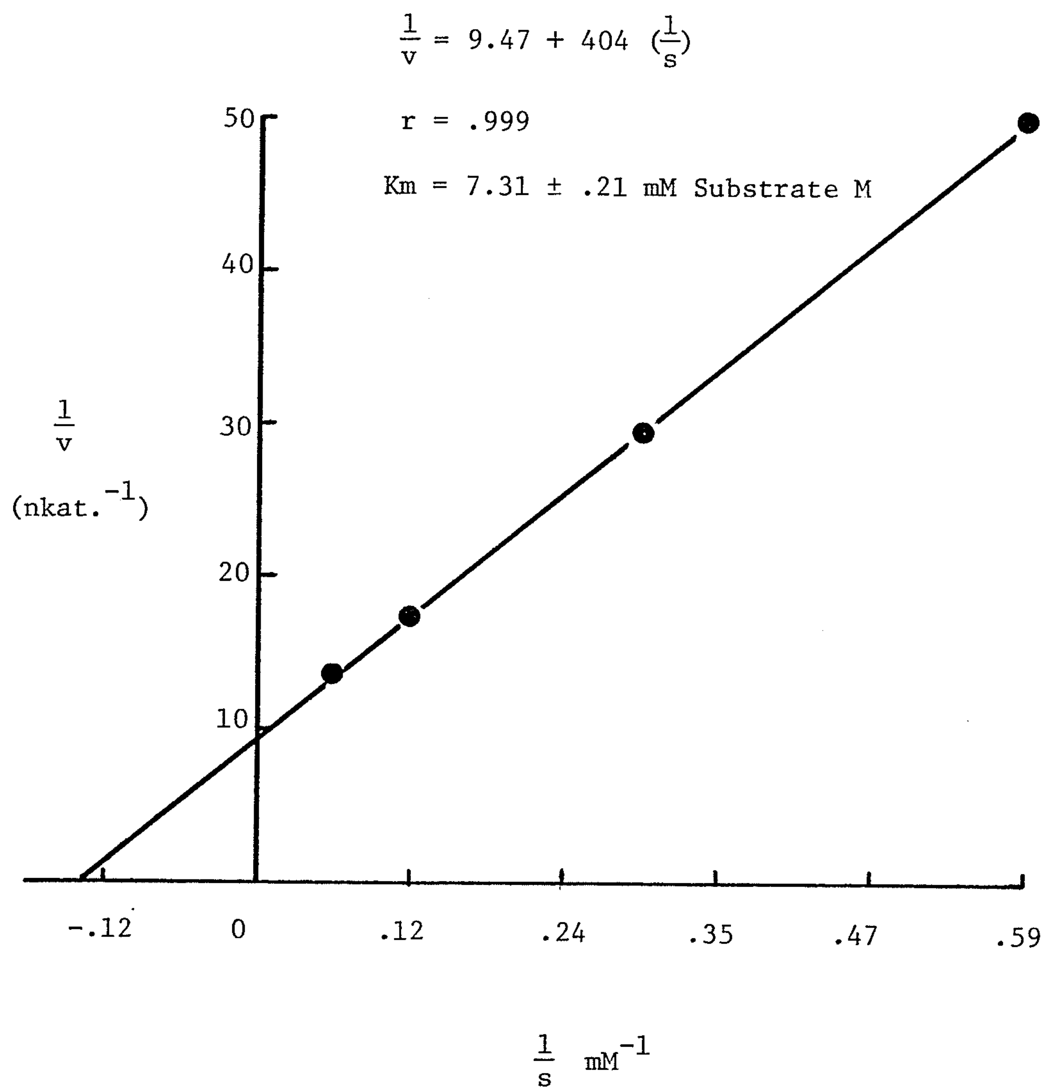
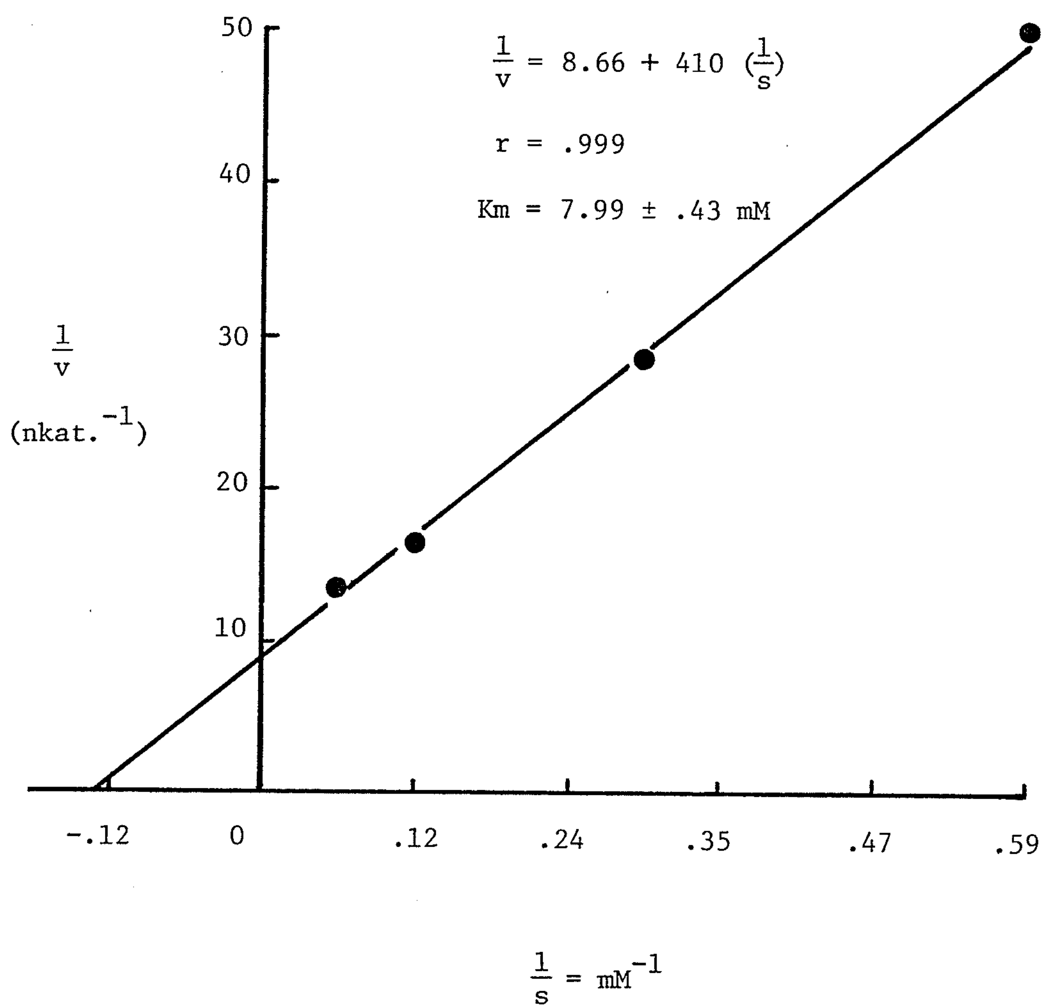


FIG. 14

LINEWEAVER-BURK PLOT OF THE EFFECT OF SUBSTRATE CONCENTRATION (SUBSTRATE M) ON JERUSALEM ARTICHOKE INULASE B



$\frac{1}{s} = mM^{-1}$

10.4 ± .35 mM Substrate M for the crude extract, 7.31 ± .21 mM Substrate M for inulase A and 7.99 ± .43 mM Substrate M for inulase B. Inulases A and B were similar with respect to Km. However, a significantly higher ($p < .05$) value of 10.4 ± .35 mM Substrate M was found for the crude extract. This may be due to the presence in the crude extract of a competitive inhibitor which is removed during the chromatographic process. Edelman and Jefford (1964) reported a Km value of 33 mM inulin and 22 mM inulopentaose for inulase B but no data were reported for inulase A.

4.6 EFFECTS OF METAL IONS ON INULASES A AND B

The enzymes (inulases A and B) were exposed to several compounds at varying concentrations (3.12.5) to determine the effects of the metal ions on the activity of the enzymes. There were no significant ($p < .05$) activation or inhibition effects on the enzymes by any of the metals investigated (Table 7). This confirmed observations by Edelman and Jefford (1964), who claimed that metals were not required and did not inhibit the inulases. They came to these conclusions after dialysis, which presumably removed metal ions from the enzyme preparations, but did not result in changes in activities of the inulases.

4.7 SUCROSE INHIBITION OF INULASES A AND B

Dixon plots of sucrose inhibition of inulase A, inulase B and the crude extract are presented in Figures 15, 16 and 17. The apparent inhibitor constant (K_i) were determined directly from these plots; being the point of intersection of the lines representing substrate concentrations of 3.4 mM and 17 mM Substrate M. The K_i for the crude extract was 4.96 ± .040 mM sucrose; that for inulases A and B were 10.4 ± .55 mM sucrose and 3.56 ± .095 mM sucrose, respectively. The lines intersect on

TABLE 7

EFFECTS OF METAL IONS ON THE INULASES

(a)

INULASE A

SPECIFIC ENZYME ACTIVITY (nkat./mg)

Ion	10 mM	20 mM	30 mM	40mM	100 mM
Ca^{++}	.970	.954	.962	.982	.954
K^{+}	1.00	.990	.990	.954	.982
Mg^{++}	1.02	.996	.996	.996	.990
Mn^{++}	.982	.996	.982	.982	.996
Na^{+}	.968	.990	.982	1.02	1.00
Zn^{++}	.996	.976	1.01	.982	.968
None	.939 nkat./mg				

(b)

INULASE B

SPECIFIC ENZYME ACTIVITY (nkat./mg)

Ion	10 mM	20 mM	30 mM	40 mM	100 mM
Ca^{++}	.932	.962	.982	.954	.962
K^{+}	.990	.982	.948	.954	.962
Mg^{++}	.982	.948	.982	.962	.948
Mn^{++}	.934	.962	.976	.968	.962
Na^{++}	.990	.948	.968	.968	.976
Zn^{++}	.982	.940	.976	.948	.990
None	.960 nkat./mg				

Average of 3 Trials

FIG. 15

DIXON PLOT OF THE EFFECT OF SUCROSE ON JERUSALEM ARTICHOKE ENZYME EXTRACT

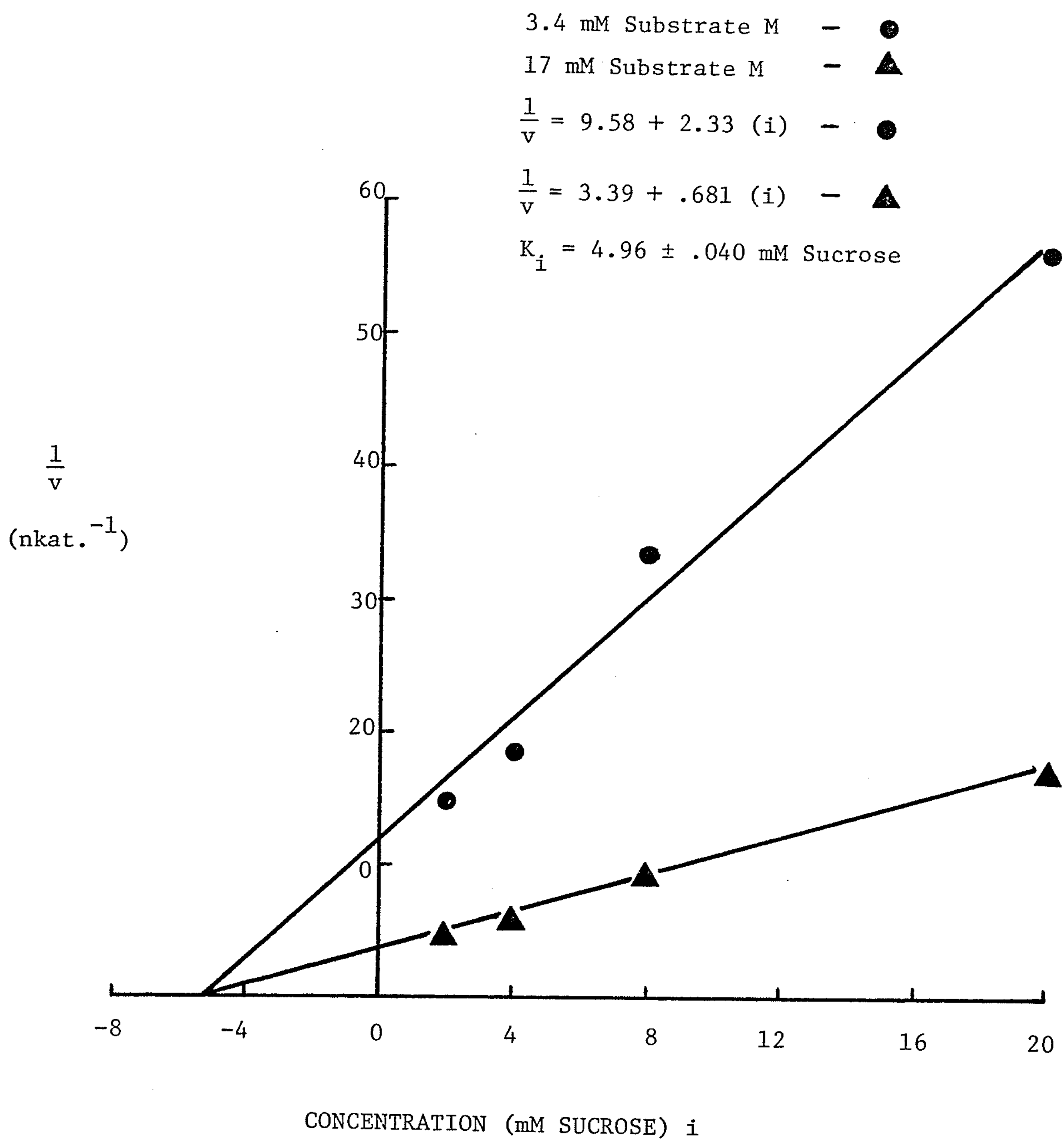


FIG. 16

DIXON PLOT OF THE EFFECT OF SUCROSE ON INULASE A

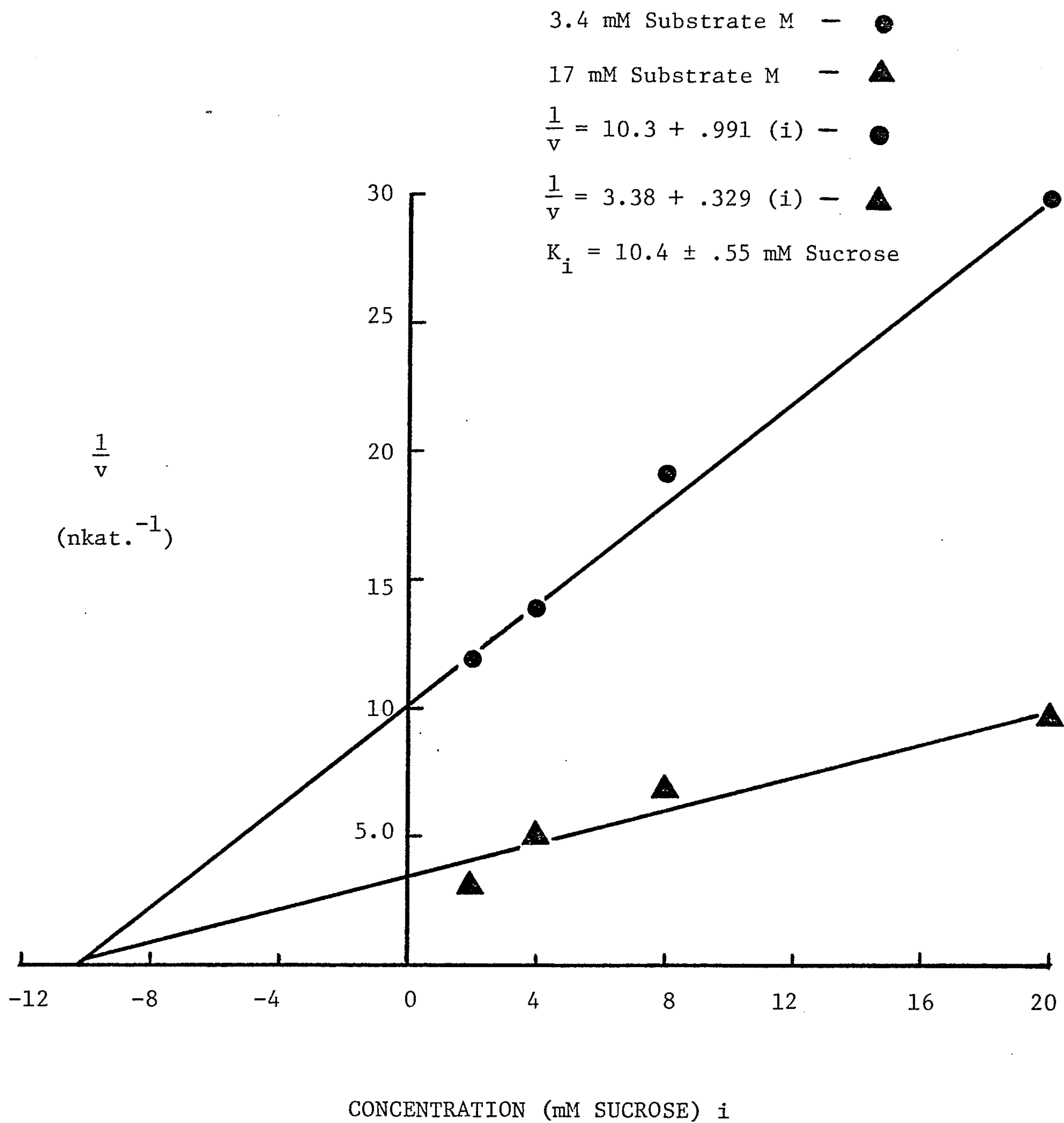
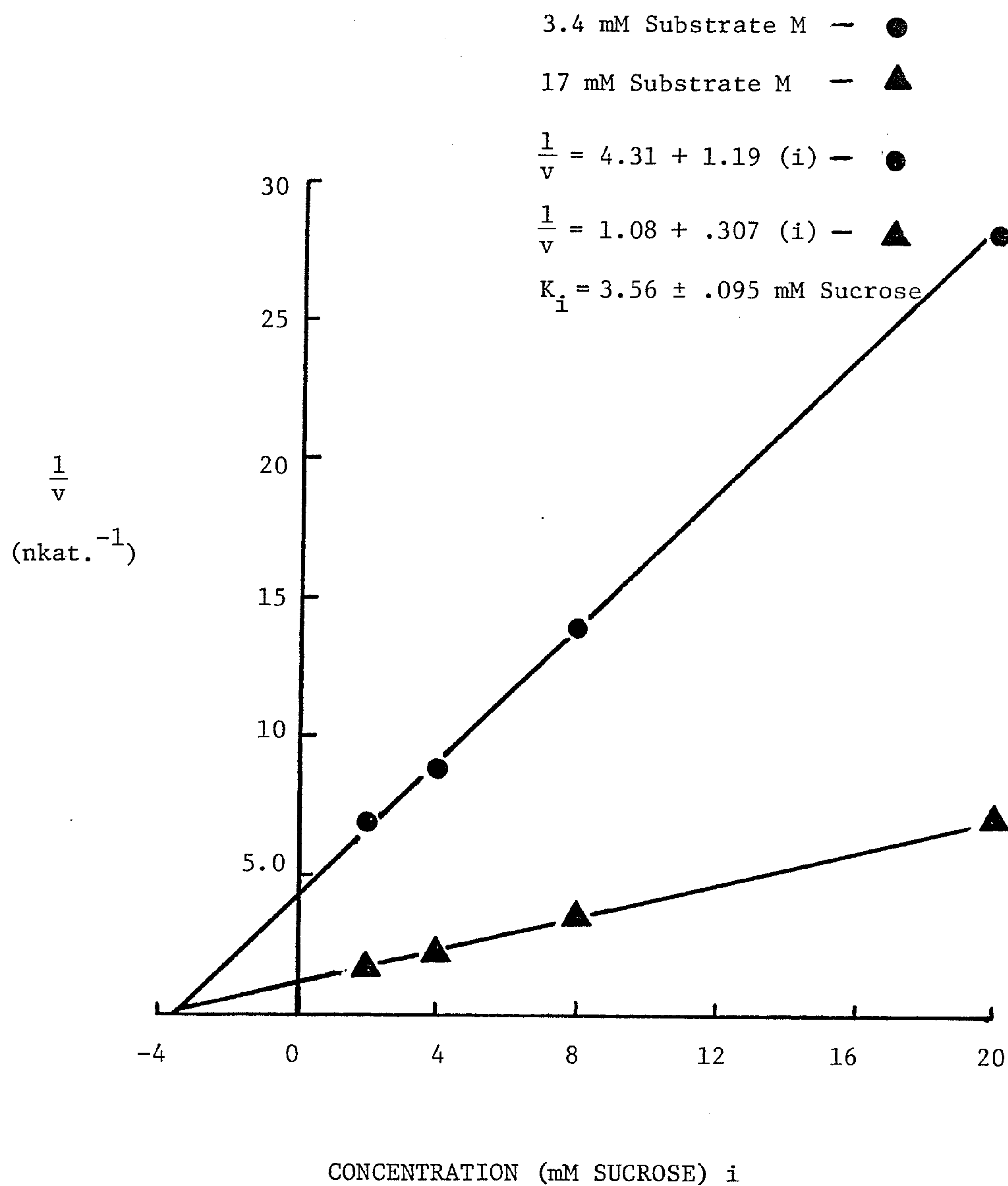


FIG. 17

DIXON PLOT OF EFFECT OF SUCROSE ON INULASE B



the abscissa, indicating non-competitive inhibition by sucrose of the inulases. The inhibitor constants are significantly different ($p < .05$) for the three preparations. The K_i is three times lower for inulase B than for inulase A. This suggested that inulase A was more resistant to sucrose inhibition than inulase B. Edelman and Jefford (1964) obtained K_i values of 2.0 - 5.0 mM sucrose with inulase B, using inulin as substrate. However, Rutherford and Weston (1967) found that the sucrose content in the tuber varied between 1.0% (29 mM) and 3.0% (88 mM) [fresh weight basis] from Fall to Spring. This increase in sucrose content during the storage period was concurrent with the observed rapid rate of polyfructan breakdown during the first five weeks of storage and a subsequent leveling off of hydrolysis. Thus, this seems to indicate that sucrose not only plays a major role in polyfructan synthesis during the growth stage of the tuber but also inhibits the inulases directly, controlling polyfructan breakdown during cold storage. Considering the K_i values obtained, both in this study and by Edelman and Jefford (1964), it would at first appear that a 29 - 88 mM sucrose content in the tuber during storage would effectively inhibit the enzymes. However, previous observations showed that this did not occur and the inulases were active during cold storage. Apparently, most of the sucrose may not be inhibiting the enzymes in the vacuole, having been translocated from the vacuole to the cytoplasm. This would have to occur by an active transport system which would move the sucrose out of the vacuole against a concentration gradient. On the other hand, activity of the inulases during storage, despite the apparently high sucrose concentration present in the tuber, may be a result of differences in the enzymes' behaviour *in vivo* as opposed to their behaviour *in vitro* with respect to sucrose inhibition. *In vivo*, the enzymes

are immobilized at the tonoplast in the vacuole as opposed to being free in solution *in vitro*. This may result in a lowering of the enzyme's affinity for its substrate *in vitro* and an exaggeration of the effect of sucrose as an inhibitor.

4.8 TEMPERATURE STABILITY OF INULASES A AND B

The temperature stabilities of inulases A and B were evaluated at 40, 50 and 60°C. as illustrated in Figures 18 and 19. From these graphs, inulase A seems to be more stable then inulase B at 50 and 60°C. Rate constants at these temperatures (Table 8) were derived from the slopes of the curves and then utilized in Arrhenius plots (Figure 20). Energy of inactivation (E_{in}) of the inulases were then derived from the slopes of the Arrhenius curves. Energy of inactivation for inulase A was found to be 158 kJ/mole while that for inulase B was 148kJ/mole. The normal range for inactivation of enzymes by heat is from 210 kJ/mole to 630 kJ/mole (Athanosopoulos and Heldman, 1980). Therefore, these enzymes are very heat labile and definitely cannot be utilized in processing.

4.9 EVALUATION OF SIX (6) JERUSALEM ARTICHOKE STRAINS FOR INULASE ACTIVITY

Six (6) strains of Jerusalem Artichoke tubers were screened for inulase activity. The enzyme extracts were prepared by the abridged method illustrated in Figure 4. The time required for extract preparation was reduced from approximately 50 hours to approximately 26 hours, enabling faster screening of the tubers for inulase activity. A comparison of the enzyme activities of the preparations shows reduction of the yield by 36% of total activity and also a decrease in specific activity (Table 9).

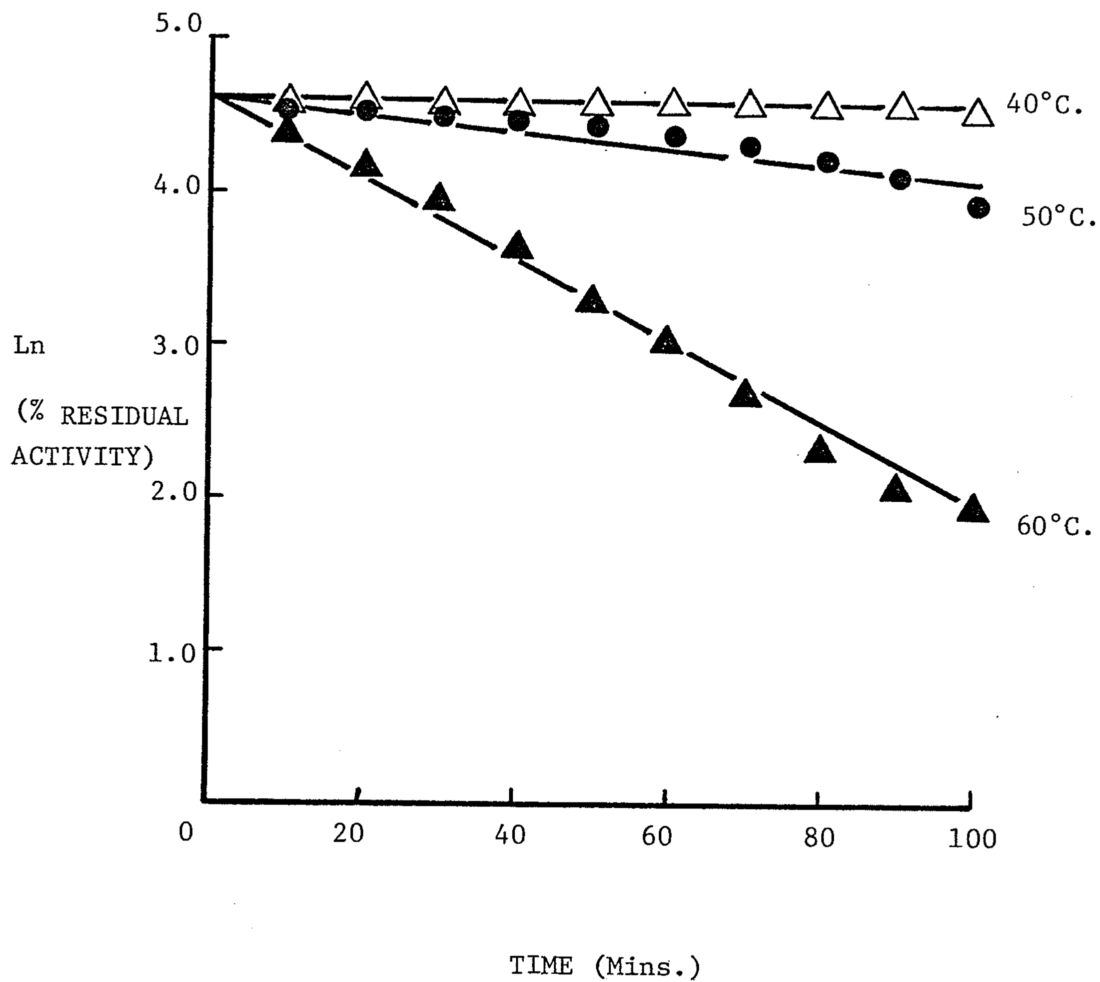
FIG. 18

TEMPERATURE STABILITY OF INULASE A

Ln (% Res. Act.) = 4.62 + (-.00077)(t) r = .927 40°C.

Ln (% Res. Act.) = 4.69 + (-.0065)(t) r = .952 50°C.

Ln (% Res. Act.) = 4.95 + (-.0291)(t) r = .997 60°C.

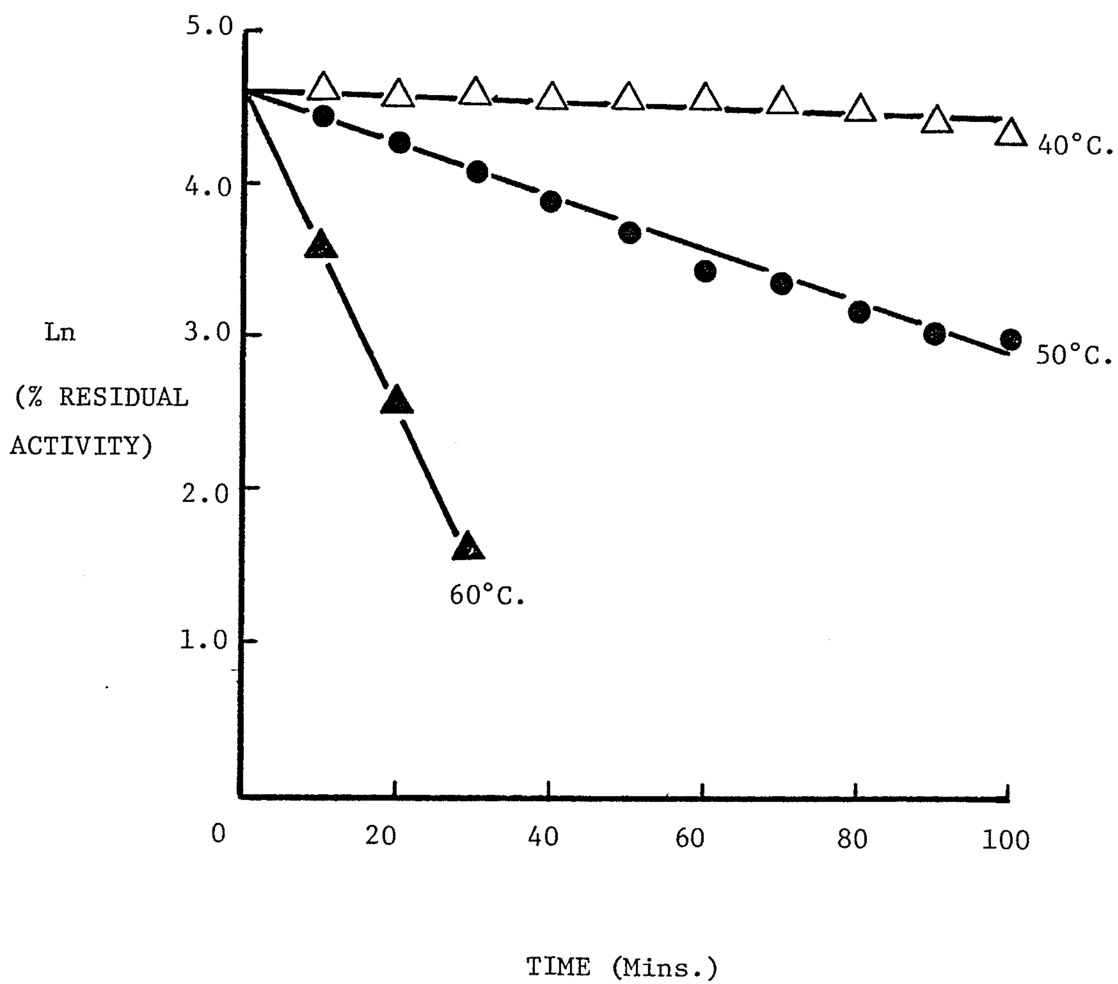


TIME (Mins.)

FIG. 19

TEMPERATURE STABILITY OF INULASE B

Ln (Res. Act.) = 4.64 + (-.0034)(t)	r = .912	40°C.
Ln (Res. Act.) = 4.56 + (-.017)(t)	r = .993	50°C.
Ln (Res. Act.) = 4.58 + (-.0995)(t)	r = .999	60°C.



TIME (Mins.)

FIG. 20

ARRHENIUS PLOT FOR DETERMINATION OF ENERGY OF INACTIVATION FOR INULASES A AND B

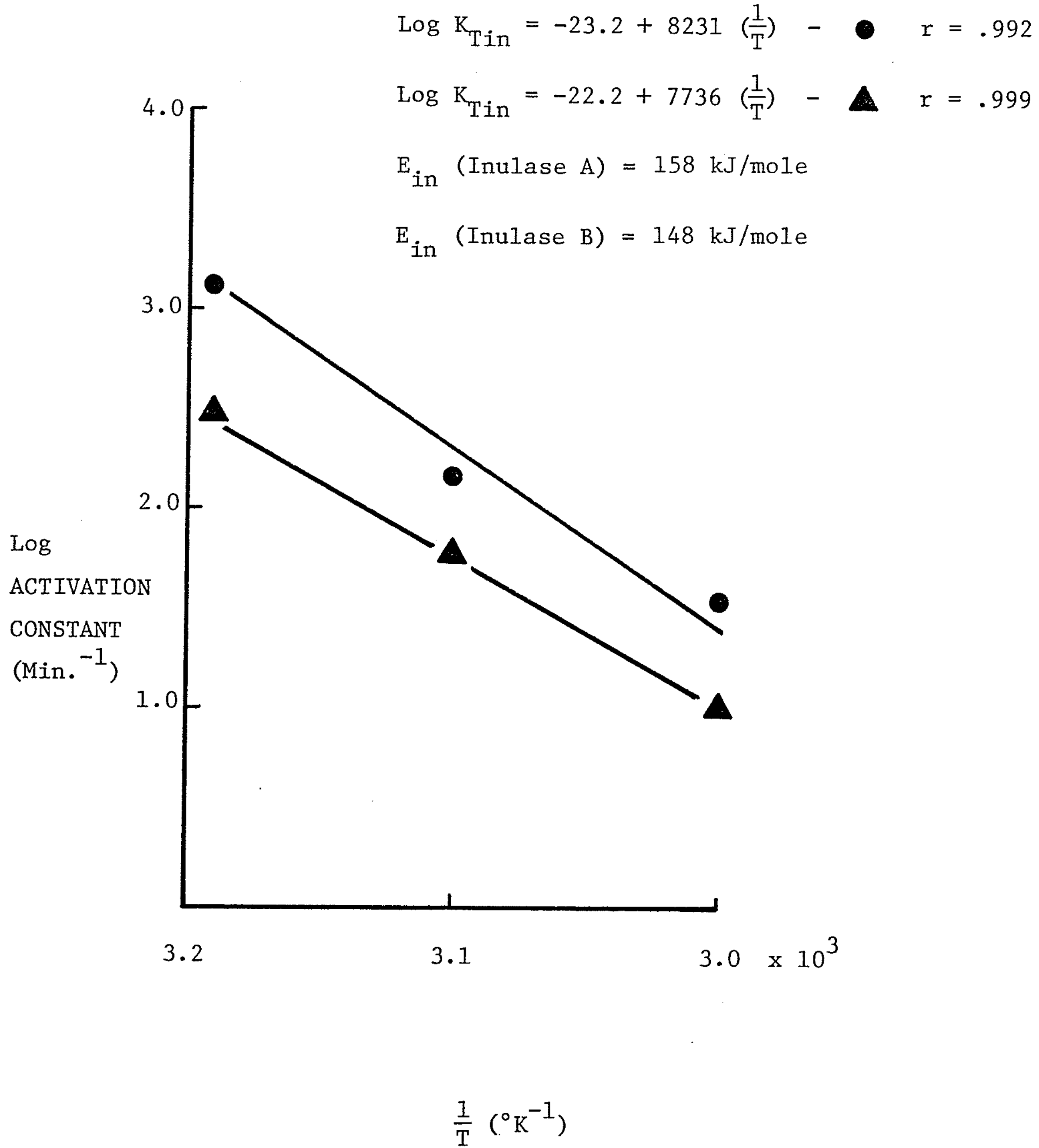


TABLE 8

RATE CONSTANTS FOR INACTIVATION OF INULASES A AND B AS INFLUENCED BY TEMPERATURE

TEMPERATURE (°C.)	CORRELATION COEFFICIENT		$K \times 10^2$	
	Inulase A	Inulase B	Inulase A	Inulase B
40	.927	.912	.0770	.335
50	.952	.993	.650	1.69
60	.997	.999	2.91	9.95

TABLE 9

COMPARISON OF ENZYME ACTIVITY IN THE PUBLISHED METHOD WITH ENZYME ACTIVITY IN THE ABRIDGED METHOD

	PROTEIN (mg/g dry tuber)	ACTIVITY (nkat./g dry tuber)	SPECIFIC ACTIVITY (nkat./mg protein)	RECOVERY (%)
Published Extraction	.910 ± .037	.200	.220	100
Abridged Extraction	.879 ± .023	.127	.144	64

AVERAGE OF 4 TRIALS

However, repeatability was good (S^d = 4.3) and data generated can be used for the comparison of inulase activity in different strains of Jerusalem Artichoke tubers.

The activities of six strains which were screened for inulase activity are shown in Table 10. The results showed similar activities for NC 10 16, NC 10 31 and NC 10 39 which as a group show a significant difference to NC 10 22, NC 10 28 and NC 10 40, which are similar in activity. This observation indicates that there is some potential for selecting strains with respect to inulase activity.

4.10 INULASE ACTIVITY OF DRIED AND FROZEN JERUSALEM ARTICHOKE TUBERS

In considering the Jerusalem Artichoke as a source of high fructose syrup, the storage of the tubers over winter becomes very important. High D.P. polyfructans and a high fructose/glucose ratio must be maintained if high efficiency is to be realized in the production of high fructose syrup. It is therefore necessary to find the best storage method which is characterized by low inulase activity and therefore low polyfructan breakdown. The tubers may be stored in several ways. These include: cold storage, dehydrated and frozen.

4.10.1 INULASE ACTIVITY OF DRIED TUBERS

Jerusalem Artichoke tubers may be stored as dehydrated cubes. Table 11 shows the results of drying cubes at 35°C. for 72 hours and 65°C. for 8 hours, to 3.22% and 3.78% moisture, respectively. The residual activities obtained from enzyme assays of the heat treated tubers were 10.2% and 7.5% for 35°C. and 65°C. treatments, respectively (Table 11). However, extrapolation of the 40°C. and 60°C. temperature

TABLE 10

INULASE ACTIVITIES OF SIX STRAINS OF JERUSALEM ARTICHOKE TUBERS

STRAIN	ENZYME ACTIVITY (nkat./g dry tuber)
NC 10 16	.121
NC 10 22	.0430
NC 10 28	.0492
NC 10 31	.110
NC 10 39	.110
NC 10 40	.0529

TABLE 11

EFFECTS OF DRYING ON INULASE ACTIVITY

(a)

TIME (hrs)	TEMPERATURE (°C)	SAMPLE	SPECIFIC ENZYME ACTIVITY (nkat./mg)	RESIDUAL ACTIVITY (%)
8	65	Dry	.024	7.5
72	35	Dry	.033	10.2
-	-	Control (Fresh)	.322	100

EFFECTS OF FREEZING ON INULASE EXTRACT ACTIVITY

(b)

VARIETY	SAMPLE	SPECIFIC ENZYME ACTIVITY (nkat./mg)	RESIDUAL ACTIVITY (%)
M # 6	Frozen	.159	76.1
	Control (Fresh)	.209	100
NC 10 16	Frozen	.116	80.6
	Control (Fresh)	.144	100

stability curves (Figures 18 and 19) to 72 hours and 8 hours respectively, showed that residual enzyme activities in the dried tubers (Table 12) were lower than those obtained from the drying study (Table 11a). Therefore, tubers stored in this manner could be expected to maintain high D.P. polyfructans over extended periods.

4.10.2 INULASE ACTIVITY OF FROZEN TUBERS

Jerusalem Artichoke tubers may also be stored frozen. However, the results shown in Table 11 indicate that this would be an unacceptable method of storage if low inulase activity and high D.P. preservation were desired during storage. The two strains of tubers investigated contained approximately 80% of their original inulase activity after freezing and thawing.

TABLE 12

RESIDUAL ENZYME ACTIVITY FOR 40°C. AND 60°C. HEAT TREATMENTS

(FIGURES 18 AND 19)

ENZYME	TEMPERATURE (°C.)	TIME (hr)	% RESIDUAL ACTIVITY
Inulase A	40	72	3.6
	60	8	9.9×10^{-5}
Inulase B	40	72	5.4×10^{-5}
	60	8	1.7×10^{-19}

CONCLUSION AND RECOMMENDATIONS

This study showed that the Jerusalem Artichoke tuber of the Manitoba strain M#6 contained two inulases. In addition, there was slight invertase activity present in the dormant tubers. The inulases differed in their mobility on a DEAE cellulose column, in their susceptibility to sucrose inhibition and in their temperature stability. Temperature and pH optima however, as well as K_ms, were similar for both enzymes. Observations by Edelman and Jefford (1964) that Jerusalem Artichoke tuber inulases hydrolyse lower D.P. fructans at a faster rate than inulin (D.P. = 35) were confirmed. Low activities were obtained when inulin was used as a substrate compared with those found for assays employing Substrate M (D.P. = 17). Activity of these two inulases seemed unaffected by metal ions since none of the investigated metals resulted in either activation or inhibition of the inulases. All of these findings concurred with reported data on the characteristics of Jerusalem Artichoke tuber inulases.

Some important relationships between the inulases and high fructose syrup production can be suggested. The inulases had low energies of inactivation which indicated that they were easily inactivated and therefore cannot be recommended for use in the hydrolysis of inulin and other polyfructans during artichoke tuber processing. If enzymic hydrolysis of inulin and the other polyfructans of Jerusalem Artichoke tubers is to be carried out, then an inulase from another source, possibly a microbial source, would have to be used. The ease of inulase inactivation *in vitro* was also observed *in vivo* as demonstrated in the drying studies. The residual activities determined in the dehydrated tubers were higher than

calculated values (Table 12) from heat treatment studies (Figures 18 and 19). Based on these results, storage of tubers in the dehydrated form can be recommended and should result in minimal polyfructan breakdown. However, further studies are required to determine if the drying process itself causes hydrolysis of inulin and the other polyfructans with related changes in the fructose/glucose ratio.

Freezing studies established that inulase activity remained in the tuber after freezing and thawing. This was not surprising, considering the winter-hardiness of the tuber. If the tubers were stored frozen therefore, it would be expected that inulin hydrolysis would occur during thawing. However, investigations into the thawing process should be conducted to determine if thawing, by rupturing of the cell organelles, would result in acceleration or inhibition of inulase activity. Inhibition constant (K_i) values for the inulases were 10.4 mM and 3.56 mM sucrose for inulase A and inulase B respectively; also, sucrose is present in the tuber at levels of 29 mM - 88 mM (fresh basis), but most may not be available to the inulases in the vacuole. However, if the organelles were ruptured during thawing, it is conceivable that the sucrose could interact with the enzymes resulting in higher inhibition.

This study has also shown that there are variations in inulase levels among different strains of Jerusalem Artichoke tubers. It is possible, therefore, to conduct a breeding program geared to selecting strains low in inulase activity. Strains may also be selected for a low inulase A to inulase B ratio, thereby exploiting the potential effectiveness of sucrose inhibition of inulase B in these strains. However, inulase activity is only one aspect involved in obtaining a tuber high in inulin

for high fructose syrup production. Further study is required on the transferases, SST and FFT, to determine if different strains have significantly different SST and FFT activity, assuming that high FFT and SST activities result in high D.P. polyfructans and high fructose/ glucose ratio. Selection could then be based on low inulase activity and high activity of SST and FFT. It may also be valuable to investigate the possibility of activating these transferases during growth and storage to increase inulin synthesis. The observation by Edelman and Jefford (1964) that degradation of inulin was faster at low temperatures than at higher storage temperatures may be a result of differences in rate of the inulases and FFT at these temperatures. It should be noted that the inulases still had about 20% of their activity at 5°C. (Figure 7). The effects of temperature in the storage of Jerusalem Artichoke tubers may be similar to those observed in potato storage, where the level of reducing sugars is a function of temperature, favouring high levels of reducing sugars at low temperatures and high levels of starch at higher temperatures. This phenomenon would be important in choosing a method for thawing tubers stored frozen. It would be necessary to minimize the time the tuber is at low temperatures thereby minimizing hydrolyis of inulin and other polyfructans. However, studies are required to determine the relationships between the inulases and FFT as they relate to polyfructan breakdown and synthesis at low and high temperatures during storage of the tubers.

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