THE ENZYMATIC HYDROLYSIS OF INULIN AND RELATED FRUCTOSANS

by

CURTIS J. M^c^KAY

submitted to the Faculty of Graduate Studies
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University of Manitoba
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ABSTRACT

With the increasing use of fructose as a commercial sweetener, interest has been directed at the hydrolysis of the highly polymerized form of fructose known as inulin. Hoehn et al. (1981) have developed a processing technique for the production of high fructose syrups from root crops such as Jerusalem artichoke, (Helianthus tuberosus). A key step in this procedure is the enzymatic hydrolysis of inulin and related fructosans. However, there were no commercial inulase preparations available, therefore the objective of this study was to find such a preparation.

Several commercially available clarification enzyme preparations were screened for their ability to hydrolyse inulin. The Irgazyme M-10 preparation from Aspergillus sp., as manufactured by Novo Industries Ltd. was found to possess both inulase and sucrase activity. The preparation showed a temperature optimum of 55°C and a pH optimum closer to pH 4.5 then 6.0. The nature of these results suggested the presence of more than one inulase enzyme in the preparation with a molecular weight in the range of 50-100,000. Concurrent to this investigation a commercial inulase preparation was developed by Novo Industries Ltd. This preparation shows several similarities to the Irgazyme M-10 preparation, being derived from Aspergillus sp. and having temperature and pH optima of 55°C and pH 5.0.

The ability, of mixtures of this inulase preparation and invertase to hydrolyse inulin was evaluated. It was found that there was a specific ratio of enzymes which had a highest

activity in comparison to either enzyme alone, and that the formulation of this ratio was dependent on the average degree of polymerization of the substrate. Analysis of the progression of hydrolysis by gas liquid chromatography suggested this complementary hydrolysis was due to hydrolysis of "preferred polymer lengths" by respective enzymes, and that inulase may outcompete the invertase for sucrose as a substrate when other polymer lengths are not available.

A computer model was designed to evaluate the potential economic benefits of this complementary hydrolysis. Depending on the substrate average DP and the cost ratio between the enzymes, there could be a significant economic benefit to operating with a mixture of inulase and invertase, in comparison to either enzyme alone.

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Few things are a singular effort it seems, this thesis being no exception. At the outset I would like to thank Dr. E. Hoehn, my advisor, whose tireless efforts and recommendations were directed toward standards of excellence. I also appreciate the willingness and flexibility of Dr. M. Henderson and Dr. B. Watts to examine this thesis in spite of severe time limitations. I would also like to thank my sister in the Lord, Regina Lau who typed this paper. Thanks also to the students and staff of the Food Science Department for their encouragement during the course of this study.

This thesis has been a great educational opportunity in many ways, not the least of which has been the spiritural. Two guiding principles come to mind here: "He who is slack in his work is a brother to him who destroys." (Proverbs 18:9), and "But seek first the kingdom of God and his righteousness..........," (Matthew 6:33).

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CHAPTER 1 INTRODUCTION

Recent reports indicate that within the next three or four years, the use of high fructose syrups, (HFS) will capture approximately one third of the commercial sweetener market. Fructose has become preferable to other sweeteners largely because of its higher sweetness rating in comparison to sucrose, (1.5 - 2.0x). This fact allows manufacturers to use less sweetener to achieve the same degree of sweetness. This is of considerable attraction considering the popularity of calorie-reduced foods. Other advantages include a reduced capacity to support the growth of those oral bacteria which induce dental caries, (Vannian and Doty, 1973), and functional properties such as a higher solubility and lower viscosity in comparison to sucrose (Sharman, 1976).

Fructose is utilized in two main forms, the high fructose corn syrup, and of secondary importance, the crystalline fructose. The manufacture of "high fructose corn syrup", (HFCS) is a highly technical process involving the enzymatic hydrolysis of corn starch to glucose, followed by its isomerization to fructose. The product of this process is a stream of 42% fructose and 58% glucose. Through fractionation, a 90% fructose stream is derived and used to enrich the 42% fructose stream to the commercially popular 55% fructose stream.

Another potential source of high fructose syrups is inulin and its related fructosans. First identified in the early 19th century, inulin is found as a reserve carbohydrate particularly

in the tuber and root portions of members of the compositae family, notably Jerusalem artichoke, (Helianthus tuberosus L.). Inulin and related fructosans from the tuber may be hydrolysed in a singular step to high fructose syrups.

Hoehn et al. (1981) have developed a processing technique for the production of high fructose syrups from inulin containing tubers or roots, such as those of Jerusalem artichoke. Inulin is extracted from the tubers with water. This crude extract is partially purified through removal of low molecular weight contaminants by ultrafiltration. The partially purified inulin and related fructosans are enzymatically hydrolysed to fructose and glucose. These are separated from high molecular weight contaminants again by ultrafiltration. The resulting fructose-glucose stream may then be concentrated if necessary. The process relies on enzymatic hydrolysis rather than acid hydrolysis. Enzyme hydrolysis is essential because the enzyme can be successfully segregated from the final product by ultrafiltration membrane whereas the acid could not.

At the time of development of this technique there was no inulase preparation commercially available. Therefore, as part I of this study, several presently available commercial clarification enzyme mixtures were evaluated in regard to their ability to hydrolyse inulin.

As part II of this study, a recently developed, commercially available, inulase preparation, (Zittan, 1981) was evaluated with respect to the economic benefit of using blends

of inulases and invertases to hydrolyse inulin in Jerusalem artichoke extracts.

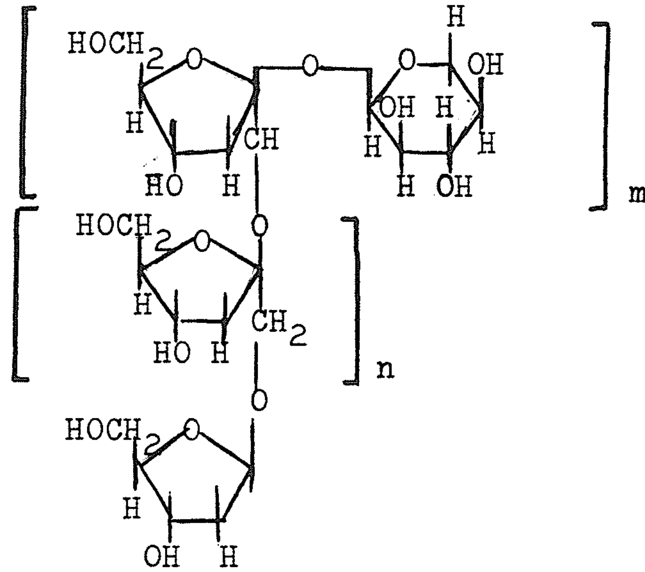
CHAPTER 2 REVIEW OF LITERATURE

2.1 STRUCTURE AND FUNCTION OF INULIN AND RELATED FRUCTOSANS

2.1.1 STRUCTURE

Inulin is a high molecular weight polysaccharide consisting of a chain of β-D-fructofuranose residues and a terminal α-D-glucopyranose unit. The fructose units are linked with a β(2→1) bond while the glucose molecule is attached by a β2→α1 bond (Figure 1). The term inulin typically applies to chain lengths (degree of polymerization or DP) of 30 or more residues (Edelman and Jefford, 1967). In plants however a homologous series of related fructosans with variable DP. From sucrose (DP 2) and Inulin (DP 35). Edelman and Jefford (1964) characterized the related fructosans of commercial inulin as being greater or equal to about DP 15. The molecular weight of commercial preparations of inulin has been determined by Guiraud et al. (1980) at about 5,000 based on an average DP of about 30. Higher values, such as 7250 (Phelps, 1965) have been reported. Phelps (1965) investigated the nature of polyfructosan components in inulin solutions and found them to be very heterogeneous. Specifically he indicates the presence of polyfructoside compenents of variable size, reducing oligosaccharides, and free fructose which could not be removed by recrystallization of the inulin from water. The highly polymeric nature of fructose in inulin suggests the usefulness of inulin as a source of high fructose syrups. Complete hydrolysis by acid or enzyme of commercial inulin for example, with an average DP of 30

FIGURE 1 INULIN MOLECULE



m: terminal sucrosyl unit

n: fructofuranose unit

units, (1 glucose and 29 fructose) would result in a syrup of 97% fructose. This is in comparison to the 55% stream of the corn process, developed through hydrolysis plus isomerization, and enrichment with the 90% stream, (obtained by hydrolysis and fractionation).

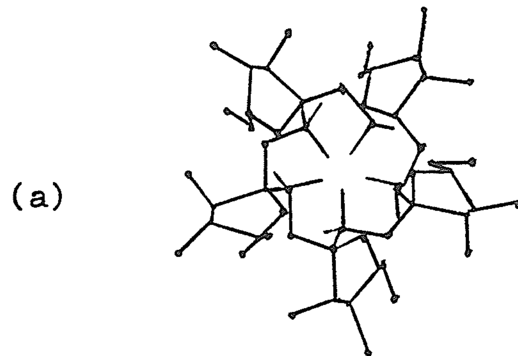
2.1.2 CONFORMATION AND PROPERTIES

In studies on the gross shape of the solvated molecule, Middleton (1977) describes the conformation of inulin as being cylindrical, specifically a helix with a rise of 3.9 residues per turn and a solvated dimension of 25Å semilength, radius 10Å and pitch 10Å, (Figure 2). A unique feature of this molecule is that as the DP increases, radius of the molecule remains constant. This is of advantage in purification systems such as ultrafiltration where rejection of inulin by the membrane will be essentially constant, irrespective of its DP.

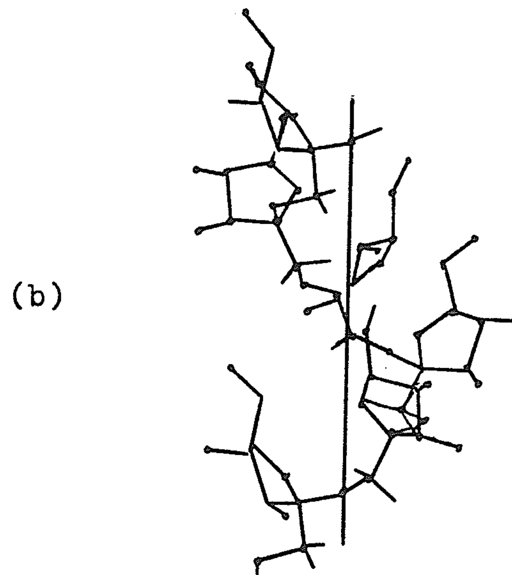
Middleton (1977) notes that water associated with the inulin helix was not appreciably modified from the free form. In other words, inulin possesses a low degree of hydration, specifically a monolayer of attracted water molecules. This implies that water is not tightly bound. This is possibly reflected in the solubility of inulin at various temperatures, (Figure 3). Phelps (1965) observed that inulin supersaturated at high temperatures, then cooled, will remain stable for several days. In contrast, unsaturated solutions may throw down a precipitate upon standing for several days. Phelps (1965) suggests this behavior is due to polymerization into larger

FIGURE 2 CONFORMATION OF INULIN - helical configuration

(a)



(b)



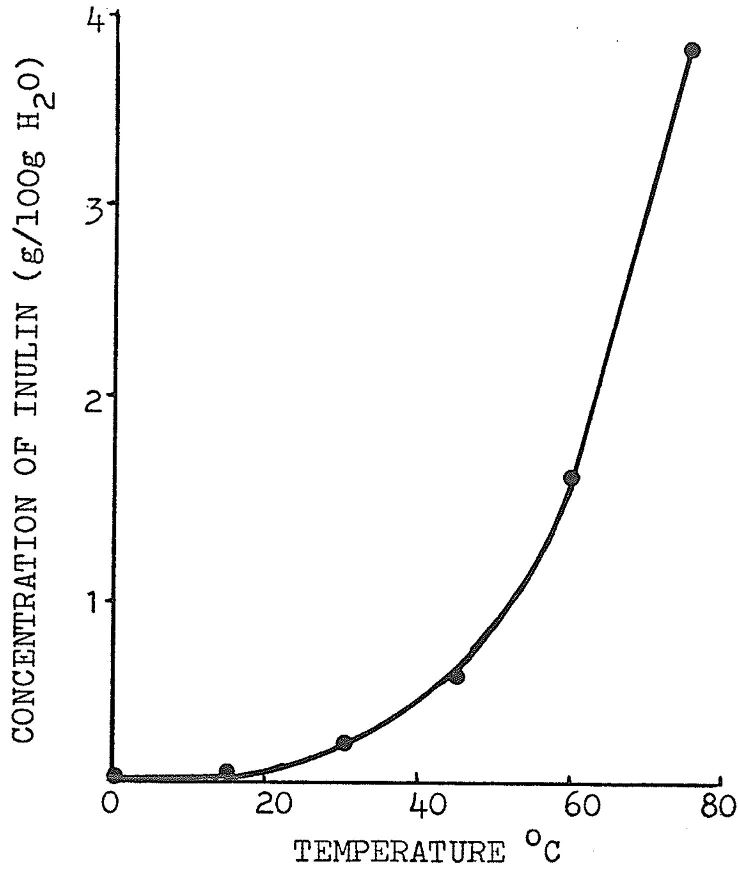
(a) Dorsal view

(b) Lateral view, (after Marchessault et.al. 1980)

FIGURE 3

INULIN SOLUBILITY VS TEMPERATURE.

(AFTER PHELPS, 1965)

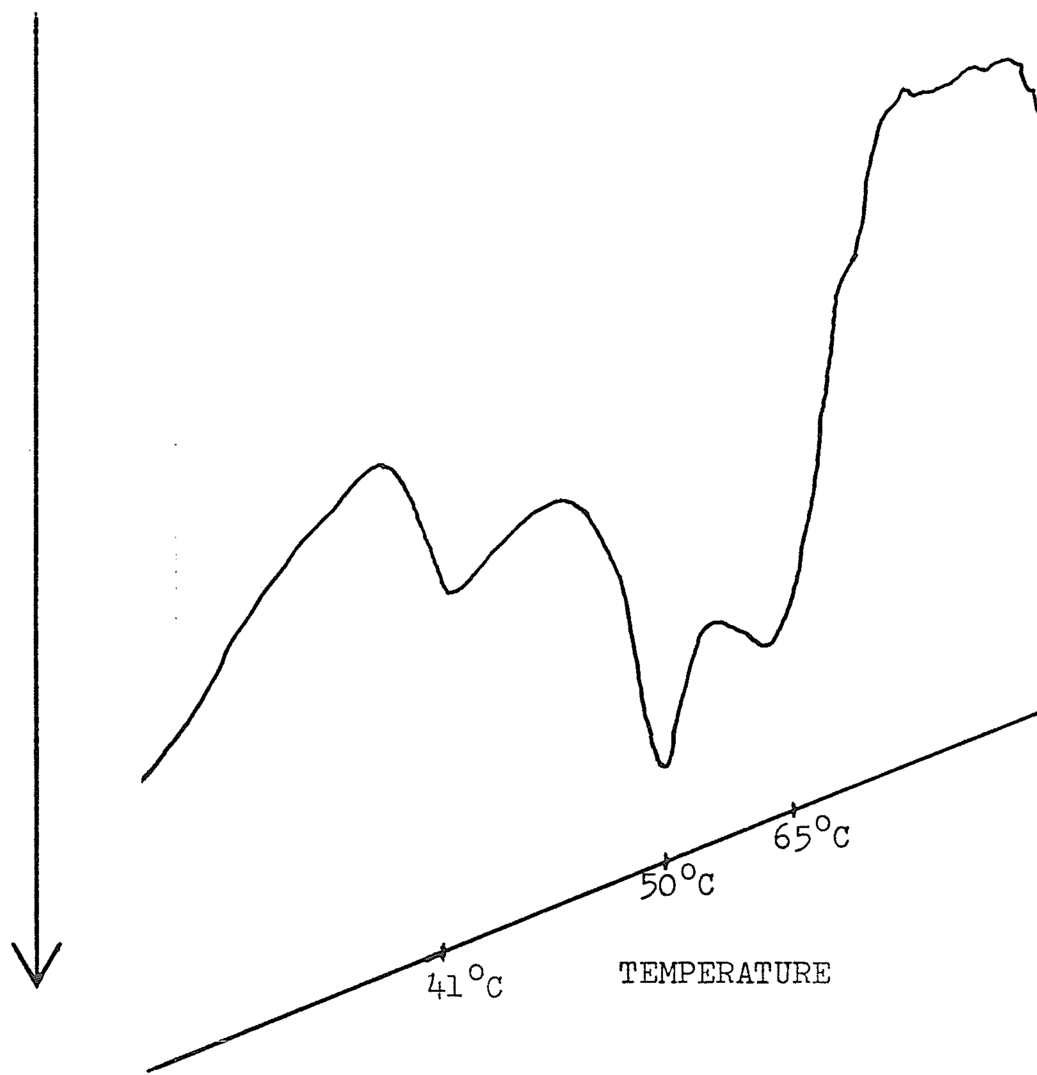


units. Phelps (1965) also investigated the solubility of inulin as a function of temperature. At temperatures below 50°C - 60°C inulin solubility is poor, (about 0.5g/100g.H_2O at 45°C), while at higher temperatures, solubility is greatly increased (36.5g/100g.H_2O at 100°C). Phelps (1965) suggests that a major change in structure must occur to account for the large increase in solubility of inulin above 60°C. The same structural change is observed by Guiraud et al. (1980) who investigated the effect of various sugars as substrates, on the thermal stability of an inulase enzyme, (from Candida salmenticensis). Specifically, the enzyme was stable in saccharose, raffinose, and inulin at 50°C. But a rise in temperature to 55°C resulted in a loss of activity, (thermal denaturation) of about 33%, after 25 minutes when in the presence of raffinose or saccharose. And a complete loss of activity, before 25 minutes, when in the presence of inulin. In studies on the change in structure of inulin over temperature, by differential scanning calorimetry, McKay (1980) observed minor changes at 41°C, 56°C and 65°C, (Figure 4).

Solubility is best explained on the basis of available polar groups, in this case, the hydroxyl groups of the fructosyl rings. On the basis of so many hydroxyl groups a high affinity for water would be expected. This effect seems to be "masked" below 60°C. Such a masking effect could be accounted for by formation of multi-helical aggregates. The minor changes observed by McKay (1980) are possible indicators of the disassociation of these aggregates at 56°C and 60°C. One implication of the increased solubility of inulin would be

FIGURE 4

DIFFERENTIAL SCANNING CALORIMETRY CHROMATOGRAM OF INULIN, (McKAY, 1980)



the facilitated extraction from tubers or roots at these higher temperatures.

2.2 POTENTIAL SOURCES OF INULIN

Inulin in the tubers and roots of plants, (Jerusalem artichoke, Dahlia, Chicory) exists as a homologous series of related fructosans. Although inulin content varies with maturity and source, inulin content of Jerusalem artichoke ranges from 10% - 20% on a fresh weight basis, and fructose content ranges from 75% - 80%, (Kim et al., 1979). In Canada, carbohydrate yields from Jerusalem artichoke can be as high as 74,100 Kg/ha. compared with 20,200 Kg/ha. for corn, or 32,100 Kg/ha. for sugar beets (Fleming and GrootWassink, 1979). The average DP is dependent on the source and maturity of the tuber. Ota and Mino (1980) have reviewed the seasonal changes in inulin content that occur in the underground portions of the carpacculaceae and composidae families due to tuberal enzyme action.

Tuberal enzymes hydrolyse the higher polymers of inulin during storage of the tubers, thus increasing the concentration of lower related fructosans. Fructose, released sequentially from the fructoside end of the molecule, is converted to sucrose by the uridine diphosgluceric acid cycle, (Edelman and Jefford, 1964, 1967). Such changes reduce the average DP which furthermore, results in a decreased fructose/glucose ratio, (F/G). Both changes are significant in view of high fructose syrup production. Altering the F/G ratio from 29:1 to 20:10 for example, would lower the final fructose concentration in a

syrup from 97% to 67%. In addition, such changes in the substrate affect the ability of the enzymes to hydrolyse the substrate.

2.3 POTENTIAL SOURCES OF INULASE

2.3.1 INULASES AND HIGH FRUCTOSE SYRUP PRODUCTION

The use of inulin degrading enzymes in high fructose syrup production has been considered superior to acid, avoiding the off-color and off-flavor problems typical of acid hydrolysis (Fleming and GrootWassink, 1979). Zittan (1981), reporting on by-product formation notes a 5% degradation of fructose to difructosedianhydride, thereby reducing fructose yield. In consideration of the ultrafiltration process, once added to the substrate to hydrolyse the inulin polymers, the acid cannot be easily removed from syrup.

Enzymes that hydrolyse inulin are a diverse group known as inulases, (E C 3.2.1.7). These are similar to the invertases, (E C 3.2.1.26), hydrolysing the same bonds. True inulases show a marked preference for the $\beta(2\rightarrow1)$ bond, (between fructosyl residues) in comparison with the $\beta2\rightarrow\alpha1$ bond, (between the terminal glucosyl and adjacent fructosyl residue).

2.3.2 INULASES FROM PLANTS

The enzyme systems used by plants that store carbohydrates as inulin have been investigated by several authors. Their intent was to find an explanation for the changes observed in the nature of inulin stored in the plant. Edelman and Jefford (1964, 1967) isolated three β- fructofuranosidases

from the tubers of Jerusalem artichoke. One was termed an invertase because it hydrolysed sucrose, and two hydrolases, designated A and B, which were considered inulases. These two inulases showed slight difference in physical properties, both being inhibited by sucrose, (non-competitive), hydrolase B being the more susceptible. The apparent Km values for the inulases are given at 33 mM inulin. In comparison, is an inulase from germinating garlic bulbs (Allium sativum L.) which has an apparent Km of 10 mM inulin (Bhat and Pahabiraman, 1980). These high apparent Km values suggest a weak affinity of enzyme for substrate, requiring a large concentration of substrate active sites on the enzyme. Normally a concentration of about 10x Km is necessary for an enzyme to achieve maximum velocity, (V_{max}). Based on a molecular weight of 5000, 10x the Km of the artichoke inulases would correspond to a concentration of about 165% inulin. From the solubility curve discussed earlier, even half this concentration would be practically impossible to obtain. The rate of reaction is therefore substrate dependent.

Reference is made to apparent Km rather than Km, primarily due to the effect of polymer size on the Km value. The apparent Km of Jerusalem artichoke inulases A and B is reported by Edelman and Jefford, (1964) as 33 mM commercial inulin, average DP 30-35. Bacchus, (1981) however, working with a substrate with a standized DP of 17, reports an apparent Km of 7.65 mM for the same enzymes. As hydrolysis of a substrate proceeds the average DP of the substrate is reduced thus lowering the average molecular weight of a polymer. As a result, the concentration required to satisfy the Km value is also decreased.

Bacchus (1981) has also investigated the properties of the inulases from Jerusalem artichoke with regard to their activity during storage and processing of the tubers. The energy of inactivation of inulases A and B has been determined to be 158 KJ/mole and 148 KJ/mole respectively. These are considerably lower than the normal range of 210 KJ/mole to 630 KJ/mole typical of most enzymes (Athanosupoulos and Heldman, 1980). Similar to the inulases from Jerusalem artichoke, the garlic inulase is very heat labile, being completely inactivated within five minutes at 55°C and PH 7.0 (Bhat and Pahabiraman, 1980). An inulase from chicory (Chickorium intybus) with similar properties has been identified (Flood et al., 1967). The heat sensitivity of these enzymes is reflected in their temperature optima. Inulases A and B from Jerusalem artichoke have an optimium of 30°C and 35°C respectively, (Bacchus, 1981). Inulases A and B from the root of the common dandelion, (Taraxacum officinale Weber) have an optimum of 25°C, (Rutherford and Deacon, 1972). These enzymes would be inadequate in a process using higher temperatures to limit microbial growth, if indeed they were able to survive the high temperatures used to extract the inulin from the tubers.

Rutherford and Deacon (1967) investigated inhibition of Jerusalem artichoke inulases by sucrose and determined the Ki to be 2 mM sucrose. This corresponds to a concentration of about 0.7% sucrose. Such levels are frequently encountered in extracts, either due to sucrose initially present or due to the development of sucrose as hydrolysis of higher polymers proceeds. In either event, sucrose is present in sufficient concentrations

to inactivate the inulases from the Jerusalem artichoke tubers.

2.3.3 MICROBIAL INULASES

It has been known, since about the year 1900, that a variety of yeast organisms will ferment inulin, (Lindner, 1900). However, the presence of a distinct microbial inulase was not confirmed until 1941 (WeidenHagen, 1941). Since that time there has been much investigation into the characterization of the microbial inulases, especially those from yeast and fungal sources. Since investigators were interested in these enzymes from a biochemical point of view, their work has focused on defining the general properties of the individual inulases, (pH, temperature optimum, Km etc.), and their pattern of action.

Generally, microbial inulases may exist in an external and an internal form. The enzyme may be "endo" or "exo" acting. "Endo" type hydrolysis results in essentially random hydrolysis of the inulin molecule, whereas "exo" type hydrolysis results in a stepwise degradation from the furanose end of the chain. When both enzymes are native to a particular organism, their physical properties vary only slightly. Properties of these enzymes vary with source. The range in pH optima is from about 3.0 to 6.5, commonly being 4.0 - 5.0, (table I). This range is compatible with the range in pH encountered in typical Jerusalem artichoke extracts, which may vary between pH 4.5 and 6.0.

The range in temperature optima for microbial inulases is commonly between 45°C and 55°C, (table I) but inulases are

found with optima as low as 30°C, and as high as 60°C. These higher temperature optima, relative to plant inulases, are important. The higher temperatures are necessary to dissolve the inulin and maintain commercially sterile processing conditions.

TABLE I RANGE IN pH AND TEMPERATURE OPTIMA FOR INULASES FROM A VARIETY OF SOURCES (SUBSTRATE INULIN)

ORGANISM	pH OPTIMUM	TEMPERATURE OPTIMUM (extracellular/ intracellular)	REFERENCE
Kluyveromyces fragilis	5.1	55/60	Fleming and GrootWassink (1980)
Saccharomyces lactis	5.0	37	Yurkevich *et al.* (1972-73)
Candida kefyr	4.5	50/27-30	Negoro and Kito (1973)
Candida salmenticensis	3.5-4.0	46	Guiraud *et al.* (1980)
Aspergillus niger	5.0-5.3	55/45	Nakamura *et al.* (1978)
Debaromyces cantarelli	4.0	30	Beluche *et al.* (1980)
Dabaromyces phaffi capriotti	4.0	50/50	Guiraud *et al.* (1981)
Streptomyces chibaensis	6.5	-	Kim (1975)

An important advantage of the microbial inulases, over plant inulases is their ability to hydrolyse sucrose. Both inulase and sucrase functions have been demonstrated from the same active site, (Yurkevich and Kovaleva, 1972). In comparison to the invertases, the inulases are more active on the higher polymers of inulin, but show a preference for the lower range of polymers, about DP2 - DP18, (Byun and Nahm, 1978). The S/I ratio is a ratio of activity on sucrose in comparison to inulin. An invertase from Baker's yeast for example, will hydrolyse sucrose 14000 times faster than inulin, (Snyder and Phaff, 1960). A typical S/I ratio for an inulase, for example that from *Saccharomyces lactis*, is reported to be 3.5, (Yurkevich *et al.*, 1972-73).

The apparent Km of inulases from several organisms is given in table II. Although there is a large range in values the Km values for microbial inulases are generally 10-100 times smaller than the ones found for plant inulases. Lower Km values mean a lower substrate concentration for half maximal activity, thus reducing the dependence of reaction rate on the substrate concentration. From the example of the Km for *Kluyveromyces fragilis* inulase on sucrose, it appears the invertase Km's are about 5x higher. This implies that inulase may have a greater affinity for sucrose than the invertase.

TABLE II RANGE IN Km AND S/I VALUES FOR INULASES FROM A VARIETY OF SOURCES IN COMPARISON TO SOME INVERTASES

ORGANISM	Km(SUBSTRATE)	S/I	REFERENCE
Inulases:			
Kluyveromyces fragilis	8.0 mM (Inulin)	25	Fleming and GrootWassink(1980)
	6.7 mM (Sucrose)	-	Nahm and Byun (1977)
Saccharomyces lactis	0.85 mM (Inulin)	3.5	Yurkevich *et al.* (1972-73)
Candida kefyr	-	50-43	Negoro and Kito (1973)
Candida salmenticensis	0.17 mM (Inulin)	18	Guiraud *et al.* (1980)
Aspergillus niger	1.25 mM (Inulin)	1.26	Nakamura *et al.* (1978)
Debaromyces phaffi capriotti	12.0 mM (Inulin)	-	Guiraud *et al.* (1981)
Strepotmyces chibensis	0.45 mM (Inulin)	-	Kim (1975)
Invertases:			*Snyder and Phaff (1960)
Saccharomyces cerevisiae	26 mM (Sucrose)	14000*	Nahm and Byun (1977)
Neurospora crassa	6.1 mM (Sucrose)	-	"
Kluyveromyces fragilis	11 mM (Sucrose)	-	"
Aspergillus awamori	48 mM (Sucrose)	-	"
Lactobacillus plantarum	320 mM (Sucrose)	-	"

2.4 APPLICATION OF ENZYMES TO HIGH FRUCTOSE SYRUP PRODUCTION

A few investigations have been conducted concerning the enzymic hydrolysis of inulin from Jerusalem artichoke for the production of high fructose syrups. All procedures have employed the use of microbial inulases.

Recognizing the need to develop a temperature stable inulase for HFS production, Zittan (1981) has conducted a screening program of microbial inulases. A commercial inulase preparation has been developed for use, with a temperature optimum of 60°C. Zittan (1981) also investigated the effect of blending two inulases. Both derived from a strain of *Aspergillus* sp, the optimum ratio of an "exo" and an "endo" acting inulase was found to be about 1:1. This is very close to the ratio normally produced by the organism.

Using an extracellular inulase from *Kluyveromyces fragilis*, Byun and Nahm, (1978) have investigated the hydrolysis of inulin from Jerusalem artichoke. A batch hydrolysis procedure was used, where the fructose was separated from the reaction mixture by Dowex 1-x8 ion exchange chromatography. Hydrolysis was reported to be about 90% efficient. This illustrates a shortcoming in the use of microbial inulases. Specifically, these inulases exhibit difficulty in completely hydrolysing the tuberal inulin in a reasonable amount of time. Commenting on the use of invertases to hydrolyse inulin, Fleming and GrootWassink (1979) conclude the rate of hydrolysis with an invertase simply would not be fast enough for a commercial process.

Kim *et al.* (1979) recognized the necessity of removal of the enzyme used in hydrolysis from the final product. It was

pointed out that since few soluble enzymes are on the G.R.A.S. list, their use in food processing has been hampered. Immobilization was suggested as a solution. A partially purified inulase from *Kluyveromyces fragilis* was immobilized on Tygon tube and on amino ethyl-cellulose with a retention of 22.5% and 53.4% original activity on inulin extracts from Jerusalem artichoke. In the process described by Hoehn *et al.* (1981), the ultrafiltration membrane acts as a physical barrier to separate the enzyme from the final product. Obviously then, the enzyme must be large enough to be rejected by the membrane used. Fortunately, the molecular weight of inulases, (76,000, Bhat *et al.*, 1980), (60,000, Nakamura *et al.*, 1978) are far in excess of the molecular weight cut-off, (1000) of the membrane employed. This feature could possibly be developed to a form of immobilization through entrapment.

CHAPTER 3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 JERUSALEM ARTICHOKE TUBERS

Jerusalem artichoke tubers, (*Helianthus turberosus* L.), (fall harvest, 1979) were obtained from the Canada Department of Agriculture Research Station, (Morden, Manitoba) courtesy Dr. B.B. Chubey.

3.1.2 ENZYME PREPARATIONS AND CHEMICALS

Commercial clarification enzymes and the commercial inulase preparations were obtained from Novo Industries, Enzyme Division, (Bagsvaerd, Denmark), Courtesy J.L. Bayard. An inulase preparation, (derived from *Kluyveromyces fragilis*) was supplied by Dr. J. GrootWassink, National Research Council, Prairie Regional Laboratory, (Saskatchewan). The two invertases used were purchased from the Sigma Chemical Co., (St. Louis, Missouri). Gas chromatographic reagents used were: "Stox" oxime internal standard reagent - a pyridine solution containing 25 mg/ml hydroxylamine hydrochloride and 6 mg/ml phenyl-β-D-glucopyranoside as an internal standard and "HMDS" - hexamethyl-disilazane. These were obtained from the Pierce Chemical Co., (Rockford, Illinois). The column packing and support phase, 80/100 mesh chromosorb W, and Sp 2100 were obtained from Supelco Inc. (Bellefonte, Pennsylvania). All other chemicals were of reagent grade or better.

3.2 METHODS

3.2.1 PREPARATION OF JERUSALEM ARTICHOKE EXTRACTS

Jerusalem artichoke tubers were washed and sliced, (3mm thickness). The slices were extracted with water at 90°C in a ratio of 1:20, (W/V). Batches of 4 l of crude extract were filtered through 80g celite on a glass fiber filter, (Whatman grade #934 AH) over a Buchner funnel, and then freeze-dried.

3.2.2 DETERMINATION OF REDUCING SUGARS

3.2.2.1 FREE REDUCING SUGARS

The free reducing sugar content of a sample was determined by the Folin-Wu method, (A.O.A.C., 1975). Fructose was used as the standard sugar. A standard calibration curve was prepared over the range in concentration 0 mg/ml to 200 mg/ml, with a sensitivity of 2 mg/ml. For preliminary screening studies the method described by Ashwell (1957) was used. The standard curve was prepared with fructose in concentrations 0 mg/ml to 110 mg/ml, with a sensitivity of 1 mg/ml.

3.2.2.2 TOTAL HYDROLYSED REDUCING SUGARS

The total content of reducing sugars after hydrolysis, was defined as the total hydrolysed reducing sugars. The pH of a sample was lowered to 1.5 by adding 4N HCl. The sample was then placed in a water bath at 80°C for one hour. After cooling, the pH of the solution was adjusted to 5.5 and the reducing sugar content was determined as outlined in Section 3.2.2.1.

3.2.3 ANALYSIS OF SUGARS BY GAS CHROMATOGRAPHY

3.2.3.1 PREPARATION OF SUGAR DERIVATIVES

Gas chromatography was employed to determine the fructose/ glucose ratio in hydrolysed samples, (3.2.3.2) and for qualitative analysis of unhydrolysed samples, to determine oligomers. The method employed is a modification of that described by Brobst and Lott (1966). In principle, the oxime derivatives of the sugars were prepared to prevent anomer peak formation during subsequent silylation. These derivatives were silylated to the respective trimethylsilyl ethers by catalysis with trifluoroacetic acid. The method is outlined below, (Anonymous, 1981/82). Ten to twenty mg of freezedried sample was weighed into a 3.5 ml screw-cap septum vial. To this, was added 1.0 ml of "Stox" oxime internal standard reagent. This mixture was heated for 30 minutes at 70 - 75°C. After cooling to room temperature, 1.0ml of "HMDS", (hexamethyldisilazane) was added and mixed. To this 0.1 ml of trifluoroacetic acid was added. The mixture was capped and shaken on a vortex for 30 seconds. The mixture was then allowed to stand at room temperature for 30 minutes to allow completion of the reaction and complete settling of the white precipitate, formed in the previous step. The clear solution was injected directly into gas chromatograph.

3.2.3.2 GAS CHROMATOGRAPHY OF SUGAR SILYL DERIVATIVES

Separation of various sugar derivatives was carried out on a Varian, (model #3700) gas chromatograph, equipped with a flame ionization detector. A Hewlett Packard Reperting Integrator, (model #3390A) was used to quantify peak areas. Column parameters are outlined in table III. The operational program is outlined in table IV.

TABLE III GAS CHROMATOGRAPH COLUMN PARAMETERS

LENGTH	46 cm
INTERNAL DIAMETER	2 mm
COMPOSITION	glass
PACKING	80/100 mesh Chromosorb W
SUPPORT PHASE	3% SP 2100

TABLE IV GAS CHROMATOGRAPHY OPERATIONAL PROGRAM

INJECTOR TEMPERATURE	$200^{o}C$
DETECTOR TEMPERATURE	$300^{o}C$
INITIAL TEMPERATURE	$120^{o}C$ for 2 min.
OVEN TEMPERATURE PROGRAM	$10^{o}C$/min.
FINAL TEMPERATURE	$280^{o}C$ for 2 min.
CARRIER GAS/FLOW RATE	N_2/40 ml/min.

3.2.3.3 EVALUATION OF GAS CHROMATOGRAMS

Quantitative evaluation of the chromatograms was based on the peak area response of each sugar, (fructose, glucose, sucrose) and phenyl-B-D-glucopyranoside, (internal standard). Calculation of the amount of each sugar in any given sample was determined by the formula:

$$Ws = (As/RFs)(WIs/AIs)$$

where: Ws=weight of sugar (mg, anhydrous basis), As=peak area of sugar unknown, RFs=response factor of sugar, WIs=weight of internal standard (mg) in unknown, AIs=peak area response of internal standard in unknown. Response factors were determined using the following formula; and 5 mg of each sugar:

$$RFs = (As/Ws)(WIs/AIs)$$

where: RFs=response factor of sugar, As=peak area response of sugar, Ws=weight of sugar (mg), WIs=weight of internal standard (mg), AIs=area response of internal standard. Sample calculations are provided in appendix 1(a) and (b).

A semi-quantitative analysis of higher polymers was obtained by the same method, using the sucrose response factor. This will lead to an underestimation of the amount of higher polymer present.

Fructose/glucose ratios in hydrolysed samples was based on comparison of the peak area response, since the response factors for each was the same, (appendix 3). A standard curve of peak area response over a range of 0 -15.0 mg was also prepared for fructose, glucose and sucrose (appendix 4). The peak area response was found to be linear and equal in magnitude.

Inverson and Bueno (1981) have evaluated the oxime-TMS procedure and report the accuracy to be about 81-116% recovery.

3.2.4 PROTEIN DETERMINATION

An estimate of protein content was obtained by the method described by Lowry et al. (1951). Crystalline bovine serum albumen was the standard protein used for establishing a calibration curve. Absorbance was measured at 650 nm with a Bausch and Lomb 710 spectrophotometer.

3.2.5 ASSAY PROCEDURE

Substrates were prepared by dissolving crystalline sucrose or inulin or freezedried Jerusalem artichoke extract in buffer. The standard buffer used was a 0.1M citrate - 0.2M phosphate buffer at pH 4.5. The use of a similar buffer for use with plant inulases is described by Rutherford and Deacon (1972), and for use with microbial inulases by Negoro (1973).

Enzyme activity was determined in a solution containing 5.0 ml enzyme solution in buffer and 5.0 ml substrate. This mixture was incubated in a water bath for 60 minutes at temperatures, which included 25°C, 35°C, 50°C, 55°C, 60°C and 70°C. A substrate blank was prepared using 5.0 ml substrate and 5.0 ml of buffer. An enzyme blank was prepared using 5.0 ml of enzyme and 5.0 ml buffer. Release of reducing sugars was used as an index of enzyme activity. Enzyme activity was calculated as given in the following equation:

$$\begin{pmatrix}\text{mg/ml corrected}\\ \text{Reducing sugar}\end{pmatrix} = \begin{pmatrix}\text{mg/ml reducing}\\ \text{sugar in test}\\ \text{sample}\end{pmatrix} - \left[\begin{pmatrix}\text{mg/ml reducing}\\ \text{sugar in}\\ \text{enzyme blank}\end{pmatrix} + \begin{pmatrix}\text{mg/ml reducing}\\ \text{sugar in sub-}\\ \text{strate blank}\end{pmatrix}\right]$$

A sample calculation is provided in Appendix 1(c). The unit of activity adopted is the Katal, (kat) (Moss, 1978) and is defined as that catalytic activity which produces a catalyzed reaction rate of 1 mole reducing sugar per second.

3.2.6 EVALUATION OF ENZYME PREPARATIONS

3.2.6.1 PURIFICATION TECHNIQUES

Investigations were conducted into purification of the clarification enzyme preparation, and the invertase through some simple one step procedure. The following methods were employed.

3.2.6.1.1 ULTRAFILTRATION

Purification of the inulase from other components of the clarification enzyme preparation was examined by ultrafiltration using a standard stirred cell, (Amicon model #202) under a pressure of 53 psi. A variety of membranes, (supplied by Amicon Corp, Lexington, Massachusetts) were employed with varying nominal molecular weight cut-off. Membranes used, (and their molecular weight cut-offs) were: PM 10 (10,000), XM 50 (50,000), and XM 100A (100,000).

3.2.6.1.2 AMMONIUM SULPHATE PRECIPITATION

The method described by Takewaki *et al.* (1980) was employed to isolate the inulase from the clarification enzyme mixture. A solution of the enzyme mixture was saturated with ammonium sulphate then centrifuged. The resultant pellet was redissolved in buffer and saturated to 95% with Ammonium

Sulphate. The solution was then mixed with celite and packed into a column. The precipitated protein was eluted with a gradient of ammonium sulphate in buffer from 100% - 0% saturation. Absorbance of the effluent was monitored at 280 nm with a Bausch and Lomb 710 spectrophotometer. Subsequent peaks were examined for enzyme activity.

3.2.6.2 PURIFICATION OF INVERTASE

Partial purification of the invertase activity was accomplished by suspending 20 gm of the enzyme pellets in buffer and diluting to a total volume of 100 ml. This mixture was stirred overnight at $5^{o}C$. The resultant slurry was then centrifuged at 10,000xg for 10 minutes at $5^{o}C$. Analysis revealed essentially all the activity was in the supernatant, thus the pellet fraction was discarded.

3.2.7 EVALUATION OF ENZYME BLENDS

The effect of using blends of inulase and invertase to hydrolyse inulin and the related fructosans was examined. An inulase, (from a clarification enzyme mixture) was mixed with an invertase, (from *Saccharomyces cerevisiae*) to determine the effects of blends in differing proportions. The two enzymes were mixed in varying proportions from 0%x/100%y to 100%x/0%y, and examined on a variety of related substrates reflecting a variety of degrees of polymerization.

CHAPTER 4 RESULTS AND DICUSSION

4.1 SELECTION OF ENZYMES FOR EVALUATION

At the onset of this study there were no commercially available inulase enzymes. However it was considered that there could be some preparations of industrial enzymes where inulase activity would be present as contaminating or side activity. Such preparations would necessarily be derived from micro-organisms known to possess inulase activity. Inulase activity is commonly found in members of the *Kluyveromyces*, *Candida* and *Aspergillus* genera. Two examples of the industrial use of enzymes from one of these sources are the pectolytic enzymes used in the clarification of wines and fruit juices, and the hemicellulases used in the starch industry and biscuit manufacture. Prepared from the selected species of the *Aspergillus* genera, these are often crude preparations containing a mixture of enzymes. On this basis, three preparations were selected and screened for inulase activity. They are known commercially as Irgazyme M-10, (a clarification enzyme mixture), Ultrazym 100, (a more highly purified preparation of Irgazyme M-10) and pentosanase (a hemicellulase preparation). These enzyme preparations were evaluated in comparison to a crude inulase from *Kluyveromyces fragilis*, and an invertase from *Saccharomyces cerevisiae*.

4.2 SCREENING FOR INULASE ACTIVITY

On Jerusalem artichoke extract and on a 1% sucrose solution, only the Irgazyme M-10 and Ultrazym 100 preparations demonstrated activity. Since the Pentosanase preparation showed no activity it was deleted from further screening procedures. The two clarification enzyme preparations were evaluated on 2% sucrose and 2.0% commercial inulin. These two substrates represent the extremes of the range in degree of polymerization, (DP) of inulin and related fructosans found in Jerusalem artichoke extracts. Specific activities of these preparations on sucrose (2%) at different temperatures are compiled in table V. Assays were performed at pH 4.5 which represents the lower limit of pH found in artichoke extracts. For the Irgazyme M-10 preparation the highest activity was observed at 50°C, whereas for the Ultrazym 100 preparation it was highest at 60°C. Specific activity was higher in the Ultrazym 100 preparation at all temperatures, than in the Irgazyme M-10 preparation. Since the Ultrazym 100 is a more purified form of the Irgazyme M-10 it appears that the invertase activity was purified along with the pectolytic activity. The Irgazyme M-10 demonstrated a broader optimum than the Ultrazym 100. At 35°C the Irgazyme M-10 has 34% of the maximum activity compared with 12% of the maximum for the Ultrazym 100. At 70°C the comparison was 57% of maximum to 30% of maximum, respectively. This broader temperature optimum likely reflects the presence of more than one enzyme, probably two invertases. It appeared that one of these has been removed in the purification to form the Ultrazym 100 preparation.

TABLE V SPECIFIC ACTIVITY OF CLARIFICATION ENZYME PREPARATIONS ON 2% SUCROSE (pH 4.5).

ENZYME (0.6% W/V)	ACTIVITY (n KATALS/mg protein)* 35°C	50°C	60°C	70°C
Irgazyme M-10	(12%)	61%)	(100%)	(30%)
(% of maximum)	24±1	121±4	200±10	59.2±.7
Ultrazym 100	(33%)	(100%)	(81%)	(57%)
(% of maximum)	21.74±.03	65±3	52.57±.07	37.0±.1

* corrected for loss of activity during storage (avg. 5%/month).

TABLE VI SPECIFIC ACTIVITY OF CLARIFICATION ENZYME PREPARATIONS ON 0.2% INULIN

ENZYME (0.6% W/V)*		ACTIVITY (n KATALS/mg protein) 35°C	50°C	60°C	70°C
*Irgazyme M-10	pH 4.5	14.9±.3	10.9±.9	7.5±.4	0.57±.02
	pH 6.0	4.4±.1	2.8±.3	1.8±.0	1.10±.2
*Ultrazym 100	pH 4.5	37.0±1	33.1±.7	34±2	16.4±.5
	pH 6.0	3.3±.2	0.0±.0	0.0±.0	0.0±.0
INULASE	pH 4.5	21.7±.2	22.1±.2	1.8±.0	1.0±.1
	pH 6.0	28.1±.1	35.3±.3	6.0±.1	2.7±.1
INVERTASE	pH 4.5	8.6±.3	3.4±.2	1.4±.0	-
	pH 6.0	17.0±.2	11.4±.2	8.0±.5	0.5±.2

Table VI tabulates the specific activities of these enzymes on 0.2% commercial inulin (pH 4.5 and 6.0), in comparison to an invertase from Saccharomyces cerevisiae and an inulase from Kluyveromyces fragilis. The Irgazyme M-10 demonstraed greater stability at the lower pH (4.5) than that at pH 6.0. At all the temperatures investigated there is about a two thirds loss in activity at pH 6.0 as compared with pH 4.5. At both pH the highest activity was found at 35°C. Specific activity dropped by 50% from 35°C to 60°C at pH 4.5, then dropped off almost completely at 70°C. A similar trend was observed at pH 6.0, but with more activity remaining at 70°C. The Ultrazym 100 showed greater stability at pH 4.5 being completely destabilized at pH 6.0, which indicated a narrow pH optimum. Highest activity was found at 35°C. The activity being 10x higher at pH 4.5 than pH 6.0. There was little change in activity at 35°C, 50°C, or 60°C at pH 4.5, but a 50% drop in activity at 70°C.

The trends observed for the inulase enzyme from Kluyveromyces fragilis confirmed the literature reports of a pH optimum near 6.0 and a temperature optimum near 50°C, (Fleming and GrootWassink, 1980). Similarly, trends observed for the invertase from Saccharomyces fragilis confirm the literature reports of a temperature optimum near 35°C and a pH optimum near 6.0, (Anonymous, 1980).

In terms of a comparison between the Irgazyme M-10 and Ultrazym 100 preparations, trends appear similar to those observed on sucrose, (table V). The broad temperature optimum of the Irgazyme M-10 at both pH values on inulin, suggest the presence of more than one enzyme. Also the results tend to confirm the literature reports for inulases from Aspergillus of a pH optimum

of about 5.0, and the temperature stability of these enzymes, (see table II). Although the Ultrazym 100 preparation had a higher inulase activity, the Irgazyme M-10 preparation appeared to be the best potential source of inulases and thus the preferred candidate for further examination. Because of the presence of more than one enzyme with inulase activity it would be expedient to utilize them both.

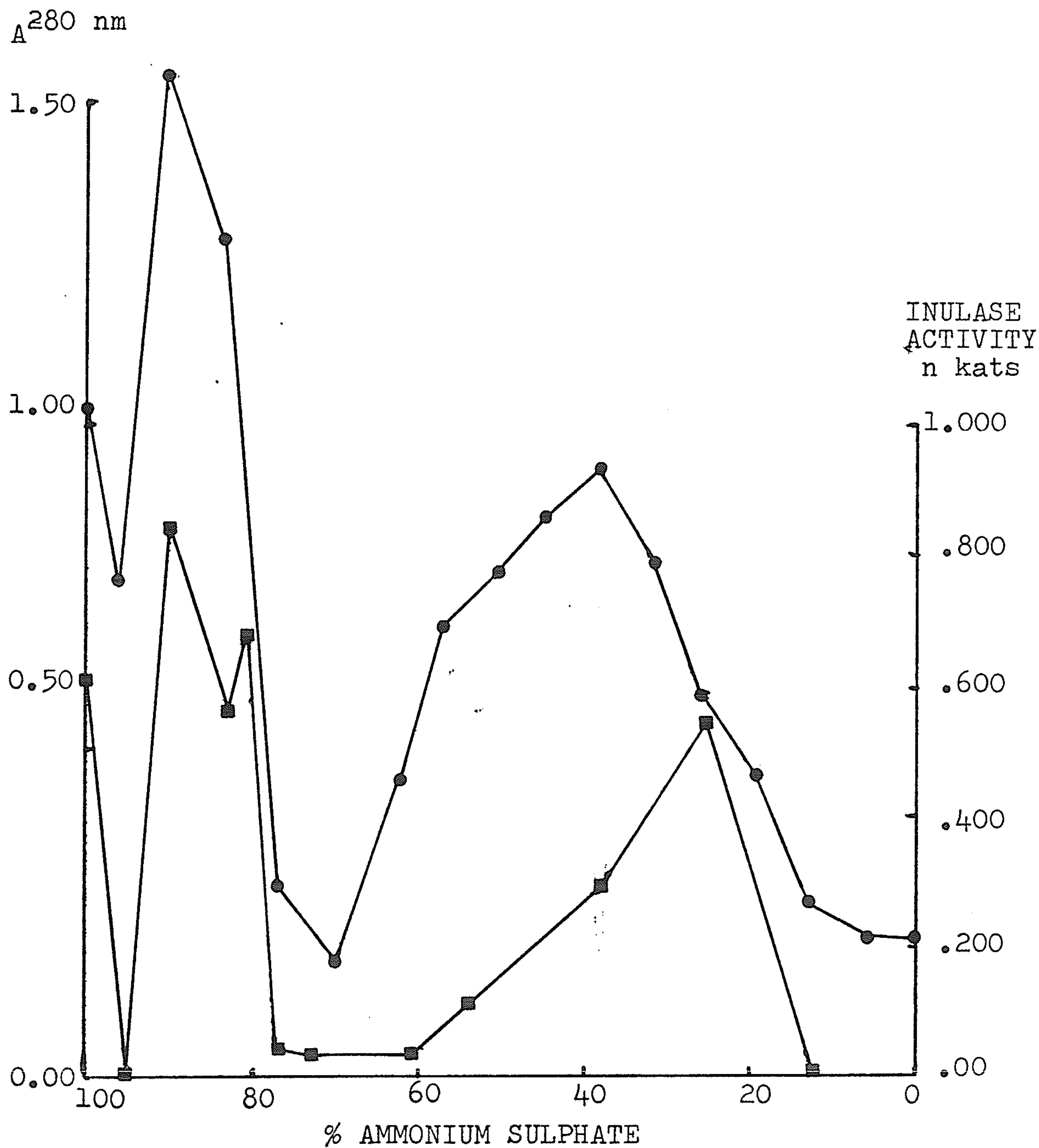
4.3 PARTIAL PURIFICATION OF ENZYMES

Due to the low activity of the enzymes preparations, particularily of the inulase activity, (0.1 n kats/mg preparation) it was decided to investigate the possibility of partial purification of the enzymes through some simple one-step procedure. At this point also, an invertase, (Baker's yeast) with a temperature optimum compatible with the inulase (55°C) was used. Partial purification of invertase enzyme was achieved through extraction with a citrate/phosphate buffer. The solubilized enzyme was separated in the supernatant by centrifugation at 10,000 xg for 10 minutes. This resulted in an increase in specific activity of 18 times the original.

Attempts were made to isolate the inulase enzyme from the Irgazyme M-10 preparation through the use of an ammonium sulphate concentration gradient. The results illustrated in figure 5 indicate the presence of two inulase enzymes eluted at different levels of saturation, This was consistent with observations of the presence of two enzymes obtained in the screening studies, (section 4.2). It is not apparent what the distinction between these two inulases is. Being derived from the same

FIGURE 5

PURIFICATION OF INULASE FROM IRGAZYME M-10 BY ELUTION WITH AN AMMONIUM SULPHATE GRADIENT. ACTIVITY (■) (nkats), (●) ABSORBANCE OF EFFULUENT AT 280 nm.



source, (Aspergillus sp.) they may represent an "exo" and an "endo", (Zittan, 1981) or, an intracellular and an extracellular enzyme. Attempts to isolate the enzymes, either together or separately, by batch precipitation with ammonium sulphate were not successful.

Purification of the inulase activity was also investigated by the use of ultrafiltration. The results are illustrated in table VII. A PM 10 membrane with a molecular weight, (MW) cut-off of 10,000 was used. The rejection coefficient, (appendix 1(d) of the protein component was determined to be 1.00, indicating complete rejection of the protein component by the membrane. On this basis, no increase in specific activity could be expected. As would be expected, very little activity was found in the filtrate, (1.3% of the original). There was however a loss of activity in the retentate of nearly two thirds of that initially present, (62.3%). The possibility that the enzyme was denatured by the pressure or stirring employed in the procedure does not seem likely. Subsequent experiments under the same conditions, but with a different membrane resulted in no net loss of activity. This significant loss of activity can possibly be explained by the tendency of the PM series membranes to absorb hydrophobic macromolecules.

On the basis of the above results, it was decided to repeat the experiment with a different membrane series and a higher molecular weight cut-off. The XM 50, a moderately hydrophilic membrane, with a molecular weight cut-off of 50,000 was chosen. As would be expected with a membrane with a higher molecular

TABLE VII PURIFICATION OF THE INULASE FROM IRGAZYME M-10 BY ULTRAFILTRATION

Membrane/ MW cut-off	FRACTION										REJECTION COEFFICIENT	
	ORIGINAL			FILTRATE			RETENTATE			TOTAL		
	mg[4]	nkat/mg	%[2]	mg[4]	nkat/mg	%[2]	mg[4]	nkat/mg	%[2]	%[2]	CARBOHYDRATE[3]	PROTEIN
PM 10/ 10,000	818	0.210	100	41	0.055	1.3	801	0.076	33.7	35.0	0.47	1.00
XM 50/ 50,000	488	0.094	100	158	0.067	26.9	345	0.041	25.7	52.6	0.49	0.81
XM 100A/ 100,000	833	0.210	100	308	0.385	57.9	501	0.144	49.2	107.1	0.10	0.73

1 results based on averages of duplicate experiments
2 percent recovery of activity (nkat/mg protein)
3 as determined by Ashwell Test for reducing sugars
4 mg protein

weight cut-off, more of the protein component may permeate the membrane and therefore a drop in the rejection coefficient to 0.81 was observed. It is interesting to note that the rejection value for the carbohydrate component remains essentially unchanged, in spite of an increase in molecular weight cut-off of 5 times. It is also noteworthy that almost half of this component has a molecular weight larger than 50,000. The inulase activity was equally distributed in the filtrate and retentate fractions, (26.9% and 25.7% respectively). Again however, there was no net increase in specific activity, rather a net loss of 47.3% of the original activity, presumably due to binding to the membrane.

To achieve a better fractionation of the enzymes in the preparation, a membrane with a molecular weight cut-off of 100,000, the XM 100A was used. There was a further reduction in the rejection coefficient of the protein component to 0.73, indicating more permeation of the membrane by proteins. There was no net loss in activity with this membrane and a nearly equal distribution of activity between the two fractions. This indicates two possibilities. Either the enzymes are freely permeable through the membrane, becoming equally distributed on either side of the membrane, or there may be a separation of the two inulases found in this preparation. Sufficient of the non-inulase protein was rejected by the membrane so as to effect an increase in specific activity to 184% in the filtrate. At this point nearly all the carbohydrate component was freely permeable. From these observations it appears the inulase

enzymes have a molecular weight in the 50,000-100,000 range. This is consistent with the value of 60,000 for an extracellular inulase from *Aspergillus niger*, (Nakamura *et al*., 1978).

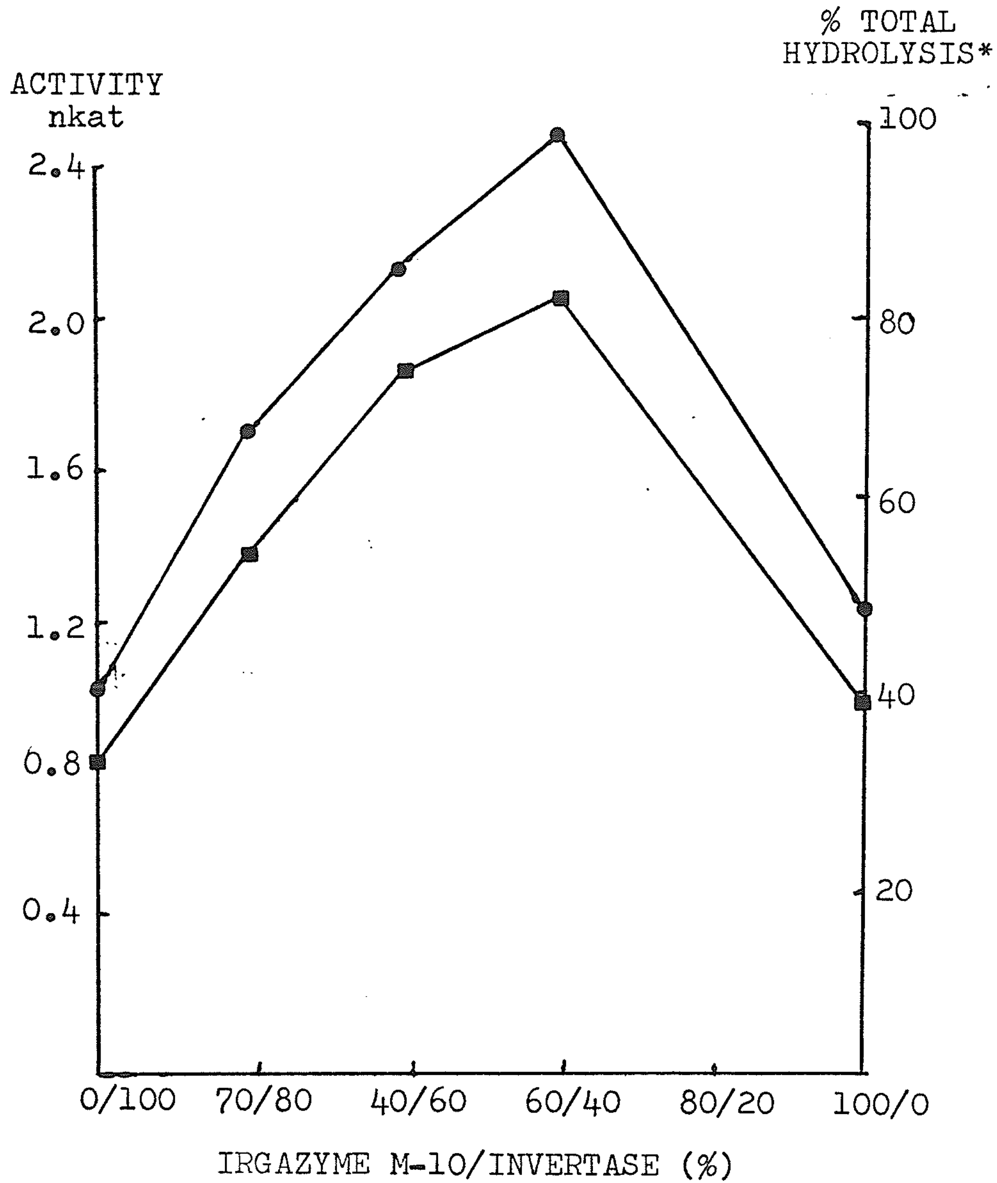
4.4 COMPLEMENTARY HYDROLYSIS WITH IRGAZYME M-10 AND INVERTASE

It has been reported that the inulase and invertase enzyme have a preferred substrate range in terms of polymer length, (Byun and Nahm, 1978). The invertase preferring the dimer sucrose, the inulase preferring the intermediate polymer lengths (DP 2-18). Because their ranges do not generally overlap, the effect of simultaneous hydrolysis of inulin by both enzymes was investigated.

In a preliminary study, both the unpurified invertase, (Baker's yeast, 55°C optimum) and the Irgazyme M-10 preparation, (*Aspergillus* sp.) were examined by using concentrations of each to give approximately balanced levels of activity. Different volumes of each enzyme were blended to create various ratios between two enzymes. These were evaluated on 2.0% inulin at 55°C and pH 5.0. Figure 6 illustrates the change in activity of the mixture as the ratio of enzymes changes. Both enzymes individually, have approximately equal levels of activity. (At point 0/100, 100% invertase, activity is 0.8n Katals, while at point 100/0, 100% Irgazyme M-10, activity is 1.0n Katals). As the proportion of Irgazyme M-10 preparation increases, there is a rise in activity, above that which could be attributed to either enzyme alone. This increase becomes maximal near a ratio of 60% Irgazyme M-10/40% invertase. All mixtures of these enzymes

FIGURE 6

CHANGE IN ACTIVITY[1] (■) AND CHANGE IN % TOTAL HYDROLYSIS (●) AS RATIO OF ENZYMES CHANGES



[1] hydrolysis of 2.0% Inulin after 1 hour, compared to complete hydrolysis by acid.

result in an increased activity above the level of either enzyme alone, but there appears to be an optimum ratio. At the point 60/40 the increase in activity is approximately 2.3 times the activity of either enzyme alone. The degree of total hydrolysis reached after one hour is also plotted on the same graph, (figure 6). At the optimum point, (60/40) there was nearly complete hydrolysis (97.4%, compared to complete hydrolysis with acid).

The enhanced activity of the mixtures of enzymes is not likely due to a true synergistic effect. Rather, there is an increased efficiency in utilization of preferred substrates. The inulase enzyme has a greater effectiveness on the higher polymers than does the invertase. The inulase enzyme also hydrolyses the polymers, (DP 3-18) faster than it hydrolyses sucrose. Conversely the invertase hydrolyses sucrose much faster than the inulin polymers, (typical S/I of 14000). The reader will recall the inulase preparation may be actually a mixture of an exo and an endo-inulase (Zittan, 1981). The increased activity can be explained on the basis that the invertase and the exo-inulase are capable of "recognizing" a preferred polymer length. Thus, their active sites are "preoccupied" by the most preferred substrate. The endo-inulase supplies polymers of variable length by its random hydrolysis, thus building up the concentration of polymers of shorter and intermediate length. This also supplies the necessary "preferred" substrates for the invertase and exo-inulase.

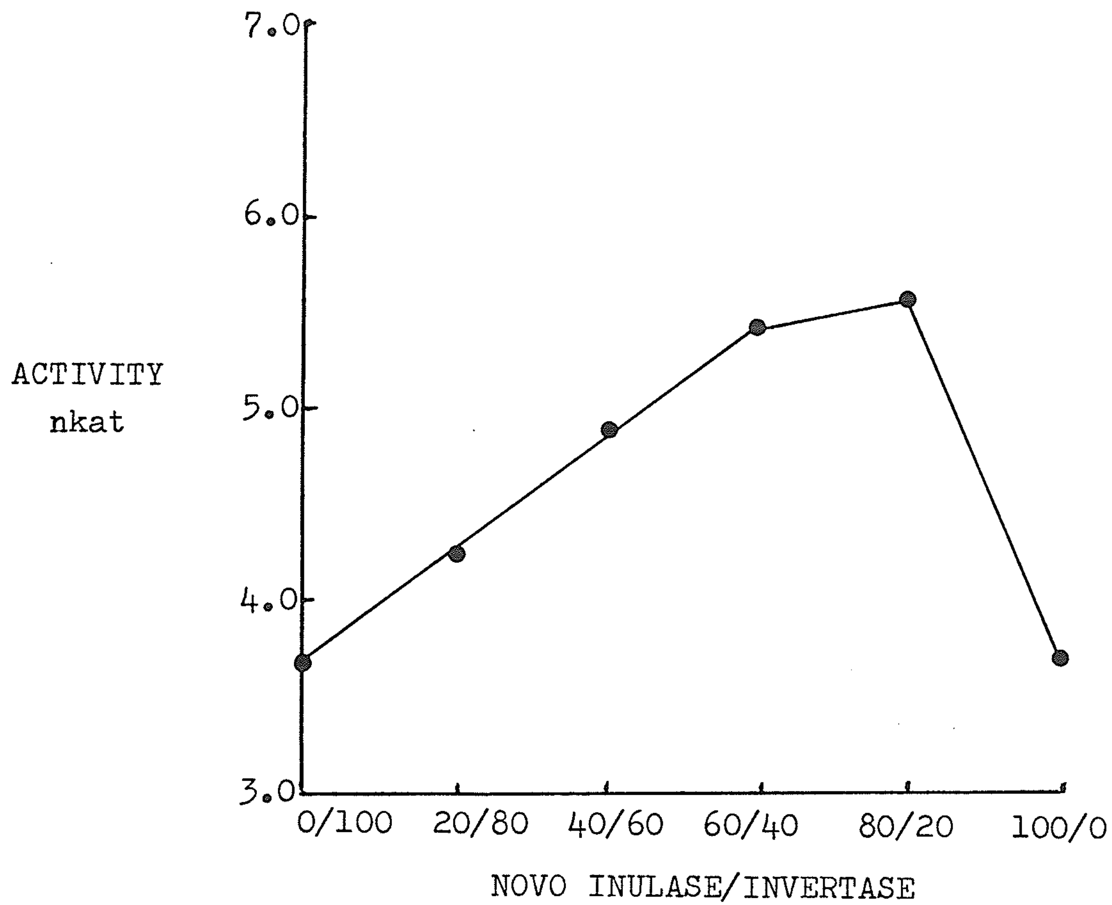
4.5 COMPLEMENTARY HYDROLYSIS WITH INULASE AND INVERTASE

It was observed that hydrolysis of inulin proceeded at a faster rate when inulase and invertase were used together. This phenomenon of complementary hydrolysis was further investigated with partially purified invertase, (section 4.3) and the Novo inulase preparation, (from Aspergillus sp. courtesy Novo Industries Ltd, Zittan, 1981). Mixtures of the above two enzymes were evaluated on substrates which included: commercial inulin, Jerusalem artichoke extract, and mixtures of the two. This variety of substrates was used to investigate the effect of the average DP on the hydrolytic pattern.

As in the previous section, the Novo inulase and invertase were used in varying ratios, (balanced activities) on a substrate of 2.0% commercial inulin, (average DP 19). The results, illustrated in figure 7 indicate the activity of any given mixture of these two enzymes is higher than either enzyme alone. The maximum level of activity recorded was with a mixture of 80/20, (Novo inulase/invertase) and represents approximately a 35% increase in activity. Gas chromatography was used to examine the production of different sugars over the course of hydrolysis. Figure 8 depicts chromatograms of (A) unhydrolysed inulin, (B) acid hydrolysed inulin, and (C) enzyme hydrolysed inulin, (100/0 Novo inulase). The chromatogram of the unhydrolysed sample (A) reveals no free reducing sugars and no polymers below DP 4. A trace amount of sucrose (0.01% W/W) was detected on one out of three replicates. The acid hydrolysed sample (B), (completely hydrolysed) reveals fructose and glucose in a ratio 18:1, (95%

FIGURE 7

ACTIVITY[1] OF VARYING RATIOS OF INULASE/INVERTASE ON 2.0% INULIN



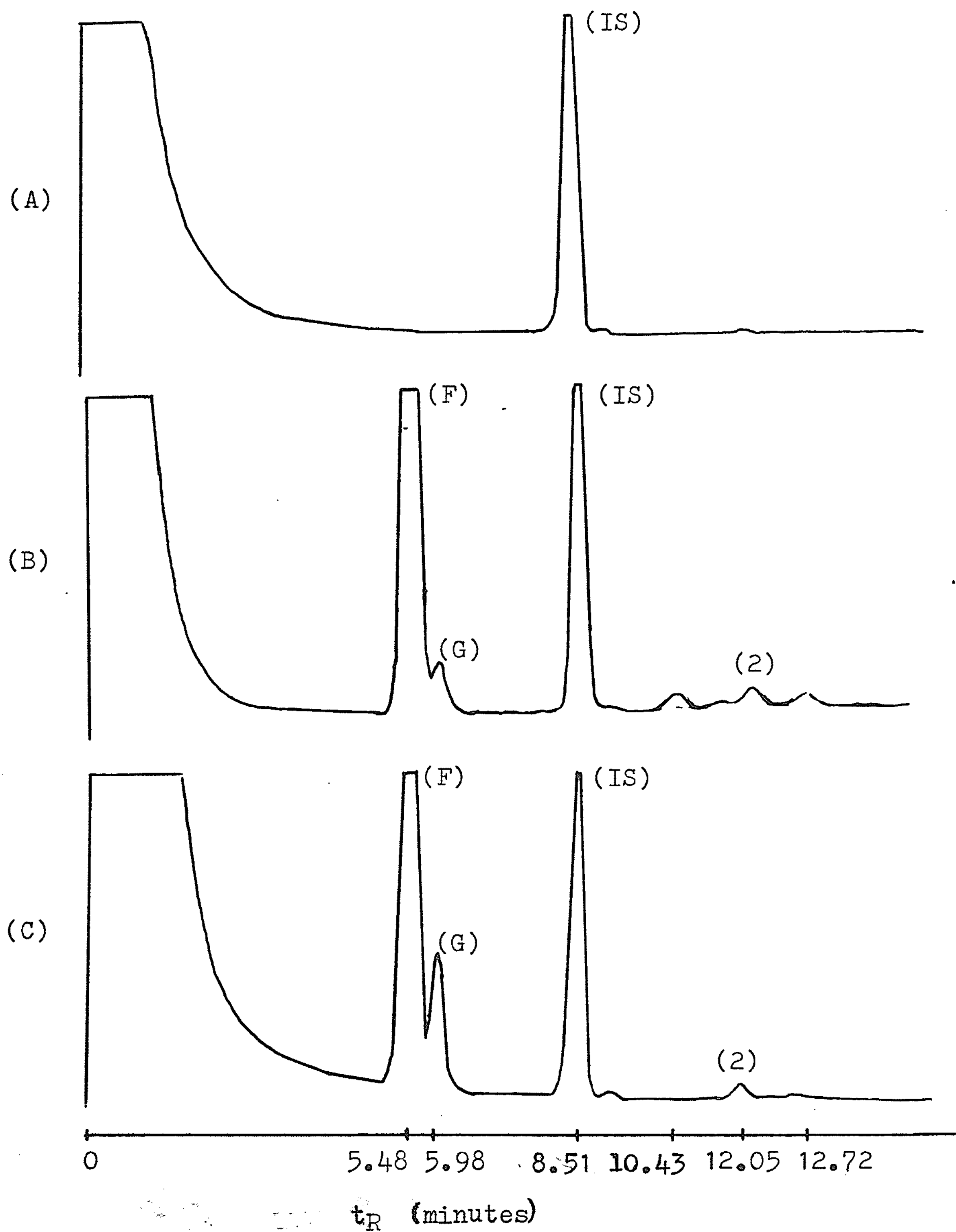
[1] activity after one hour, average of duplicate experiments

FIGURE 8

GAS LIQUID CHROMATOGRAMS OF 0.2% INULIN

(A) Unhydrolysed, (B) acid hydrolysed, (C) enzyme hydrolysed

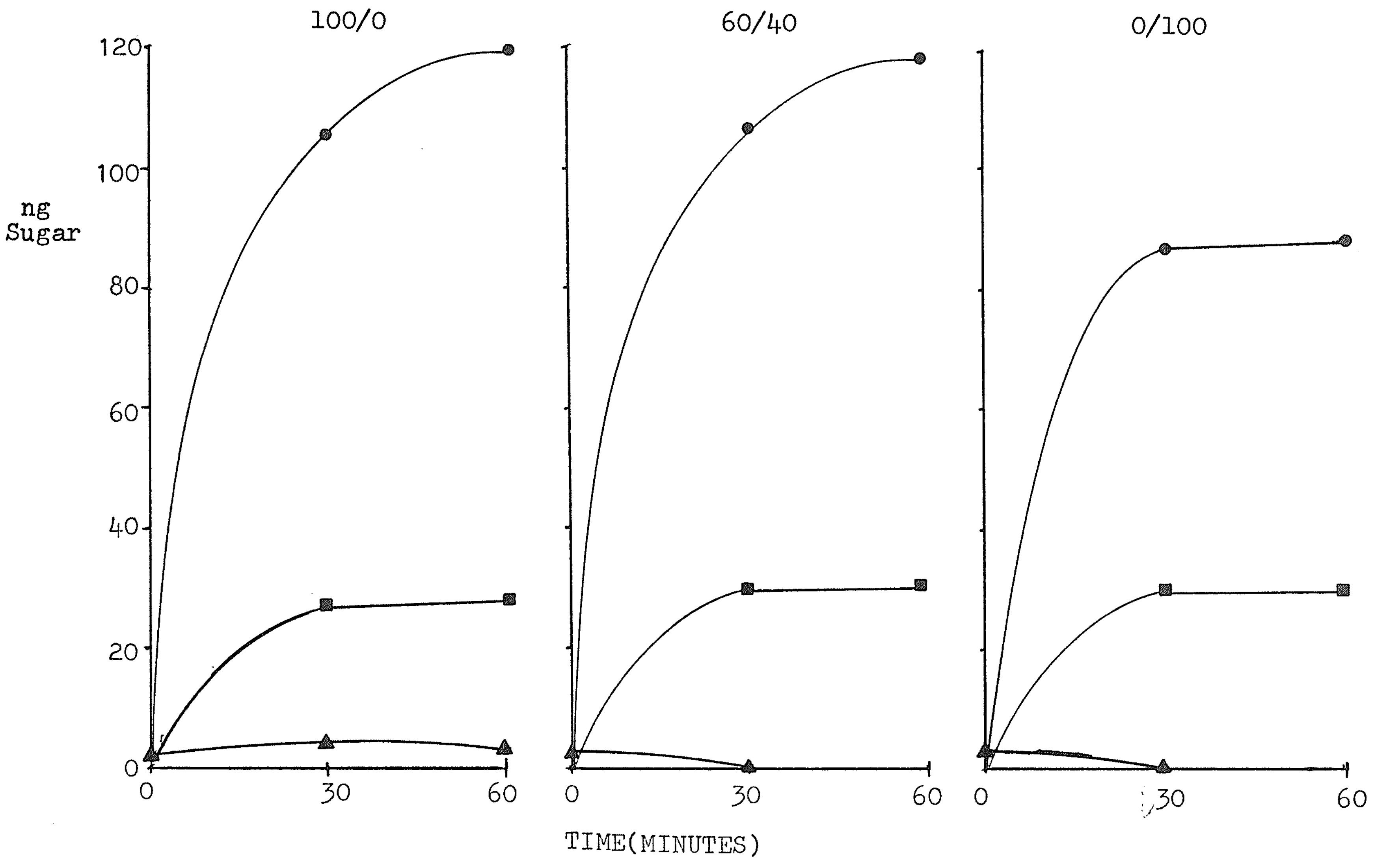
(IS)=Internal Standard, (F)=Fructose, (G)=Glucose, (2)=Sucrose



fructose) and a small amount of sucrose. In addition, there were three unidentified peaks with retention times, (t_R) of 10.43, 11.72, and 12.72 minutes. These peaks are likely not sugars, rather by products of the acid hydrolysis. The t_R of monosaccharides are much lower, (fructose t_R=5.48, glucose t_R=5.98) whereas the t_R of disaccharides are generally higher, (sucrose t_R=12.05, maltose t_R=11.98, 12.37, 13.24) and trisaccharides higher (raffinose t_R=17.03). The chromatogram of the enzyme hydrolysed sample (C) reveals the presence of glucose, fructose and sucrose. The presence of sucrose illustrates the preference the Novo inulase exhibits for the polymers DP 3-18 in comparison to the disaccharide sucrose. The unidentified peaks, as on the chromatogram of the acid hydrolysed inulin, (B) are absent. The production of various sugars over time is illustrated in figure 9. The release of glucose, fructose and sucrose is indicated as a result of hydrolysis of 0.2% inulin with 100/0, 60/40, 0/100, (Novo inulase/invertase). The significant feature of hydrolysis with the Novo inulase alone, (100/0) is the presence of sucrose in the reaction mixture, (there were also detectable levels of DP 3 present at 30 minutes). The level of sucrose remained essentially unchanged after one hour. It is apparent that as long as there are polymer lengths available preferable to sucrose, there will be unhydrolysed sucrose present. As these polymers are hydrolysed and their concentration decreases, hydrolysis will shift to sucrose. Hydrolysis with 60/40, (Novo inulase/invertase) revealed a similar pattern except that any sucrose initially present was hydrolysed and there was no net build up of sucrose as time proceeds. The hydrolysis of

FIGURE 9

RELEASE OF FRUCTOSE(●), GLUCOSE(■), SUCROSE(▲) BY HYDROLYSIS OF 0.2% INULIN WITH VARIOUS BLENDS OF NOVO INULASE/INVERTASE

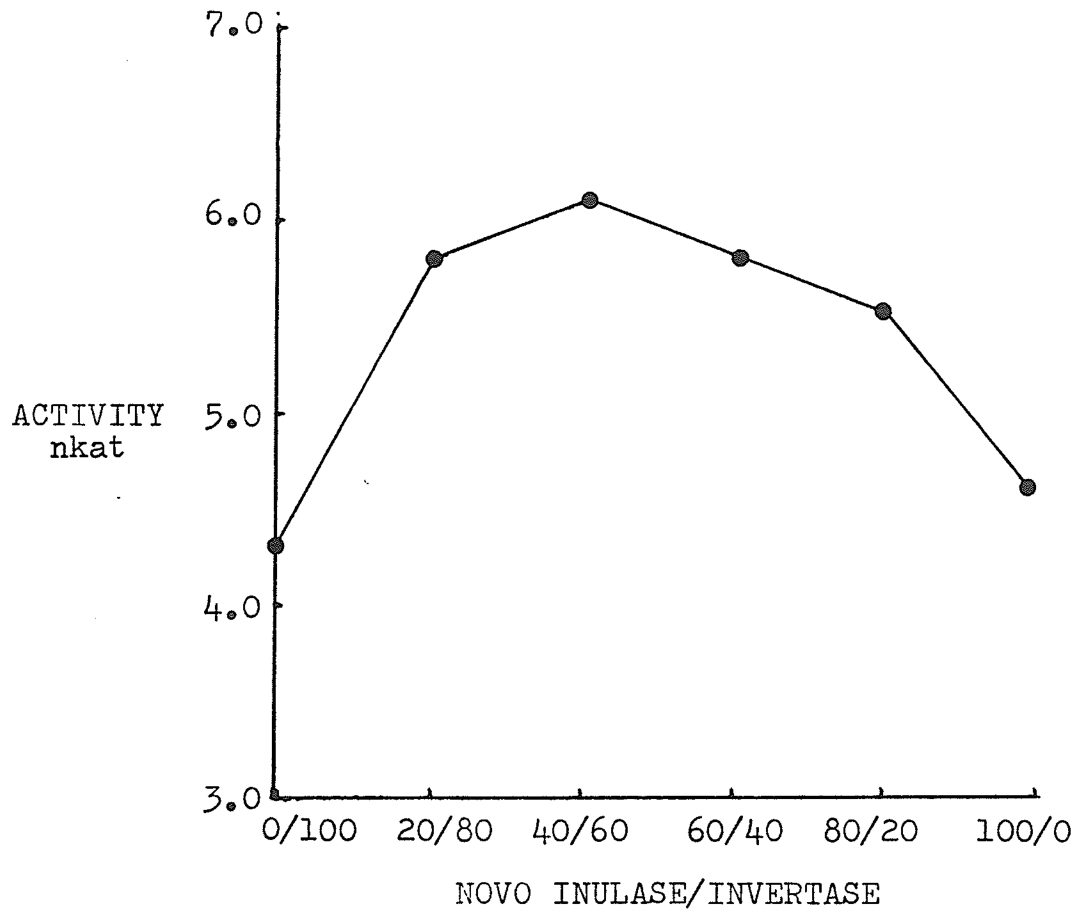


sucrose is reflected in slightly higher glucose and fructose levels. It is the complementary hydrolysis of sucrose by the invertase, thus freeing the inulase to hydrolyse larger polymers, that accounts for the increased activity of these mixtures. When hydrolysis occurs with invertase, (0/100) the initially present sucrose is hydrolysed and there is no subsequent build up. But due to the poor ability of invertase to hydrolyse the larger polymers, the amount of fructose released is reduced by about 25%. The glucose content is similar to the other two patterns. An important observation however is that the fructose level increases only slightly from 30-60 minutes. This is reflected in the F/G ratio. Whereas the F/G ratio for the other two patterns increases from 30-60 minutes F/G (100/0)=+.28, F/G (60/40)=+.26), the F/G ratio for the invertase remains essentially unchanged, (+.02) It is evident that hydrolysis with invertase alone would take a long time, while hydrolysis with inulase alone would result in a net build up in sucrose till other polymers had been hydrolysed. A mixture of the two enzymes appears to make the most efficient use of the substrate.

The Novo inulase and the invertase, with balanced activities curve were used in varying ratios on a substrate of 2.0%. Jerusalem artichoke extract. The results illustrated in figure 10 indicate that mixtures of the two enzymes results in higher activity than either enzyme alone. The highest activity recorded was for a ratio of 40/60, (Novo inulase/invertase) and represents approximately a 30% increase in activity. The lower optimum ratio of 40/60, (compared with 80/20 on 2% inulin) reflects the lower

FIGURE 10

ACTIVITY[1] OF VARYING RATIOS OF INULASE/INVERTASE ON 2.0% JERUSALEM ARTICHOKE EXTRACT



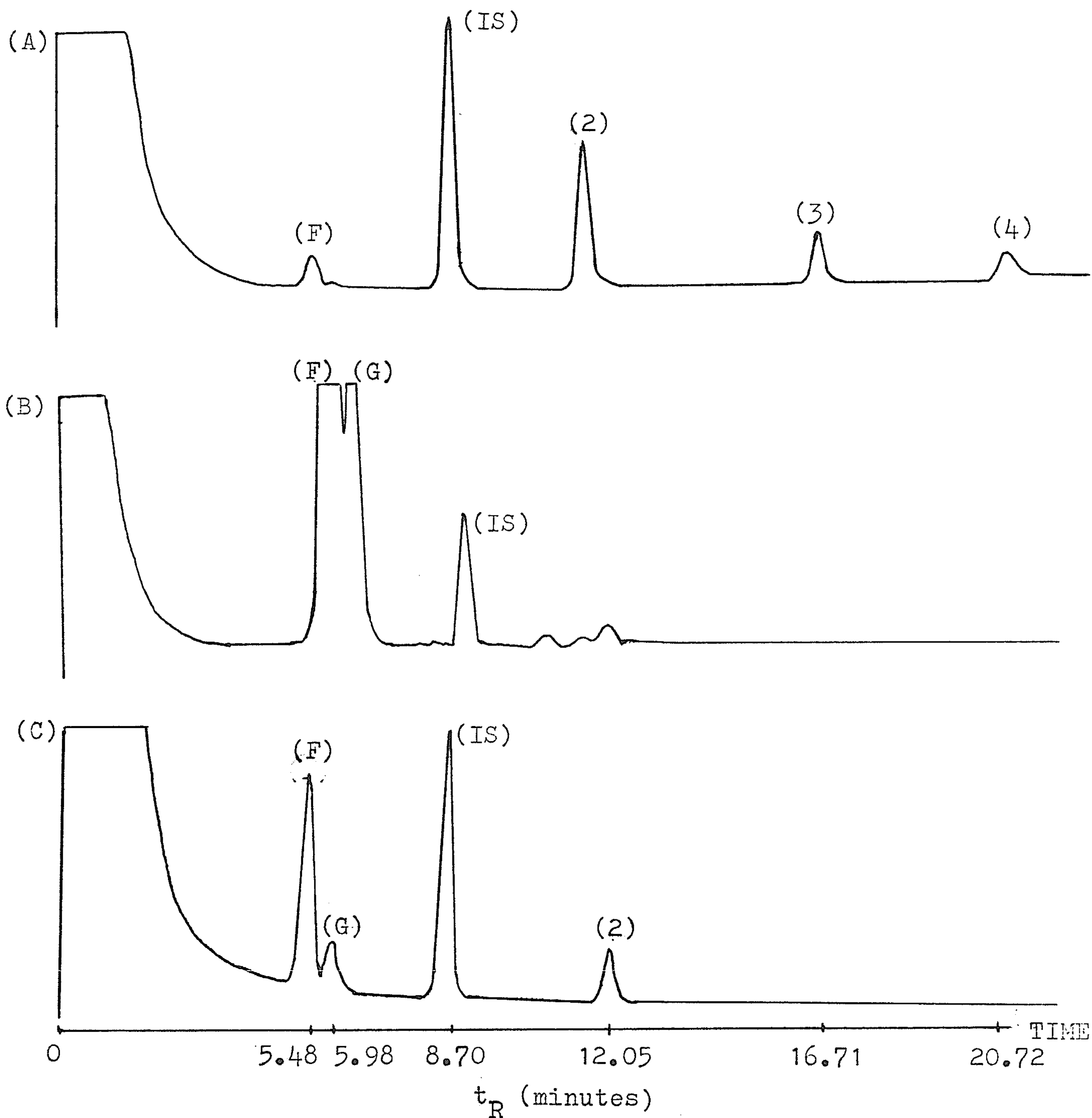
[1] activity after one hour, average of duplicate experiments

average DP of this substrate, (DP 4) and particularly, the abundance of sucrose and lower polymers. Gas chromatography was used to examine the production of different sugars as a result of hydrolysis. Figure 11 depicts chromatograms of: (A) unhydrolysed 2% Jerusalem artichoke extract, (B) acid hydrolysed extract, and (C) enzyme hydrolysed extract, (100/0 Novo inulase). The chromatogram of the unhydrolysed sample indicates the presence of fructose, sucrose, DP 3 and DP 4. The absence of glucose is kikely due to conversion of fructose and glucose to sucrose by tuberal enzymes via the uridine diphosphogluceric acid cycle. The chromatogram of the acid hydrolysed sample reveals the presence of fructose, glucose in a ratio of about 3:1, there was no sucrose present. In addition, there were three unidentified peaks at 10.43, 11.22 and 11.79 minutes. The similarity of these peaks to those on the chromatogram of acid hydrolysed 2% inulin further indicates their origin as being byproducts of acid hydrolysis. The chromatogram of the enzyme hydrolysed sample, (100/0 Novo inulase) revealed fructose and glucose present in the ratio of about 5:1. There was a small amount of sucrose present and no higher polymers were detected. The unidentified peaks of the acid hydrolysed sample were also absent.

It was observed that the optimum ratio of enzymes was widely different for these two substrates and that the average DP of these substrates also differed widely, (inulin average DP 19, Jerusalem artichoke extract average DP 4). To determine whether the position of the optimum blend was dependent on the average DP of the substrate, inulin and the artichoke extract were mixed in

FIGURE 11

GAS LIQUID CHROMATOGRAM OF 2% JERUSALEM ARTICHOKE EXTRACT (A) UNHYDROLYSED, (B) ACID HYDROLYSED, (C) ENZYME HYDROLYSED, (IS)=INTERNAL STANDARD, (F)=FRUCTOSE, (G)=GLUCOSE, (S)=SUCROSE, (3)=DP3, (4)=DP4.



different proportions, (total concentration 2%) to create substrates of different average DP's.

On a substrate of 1% inulin and 1% Jerusalem artichoke extract, (average DP 11.5) a similar trend in activity, as on 2% inulin, was found, (figure 12). Here, the maximum level of activity is found between 60/40 and 80/20, (Novo inulase/invertase), and represents an increase in activity of about 30%. All combinations of the two enzymes are higher in activity than either enzyme alone.

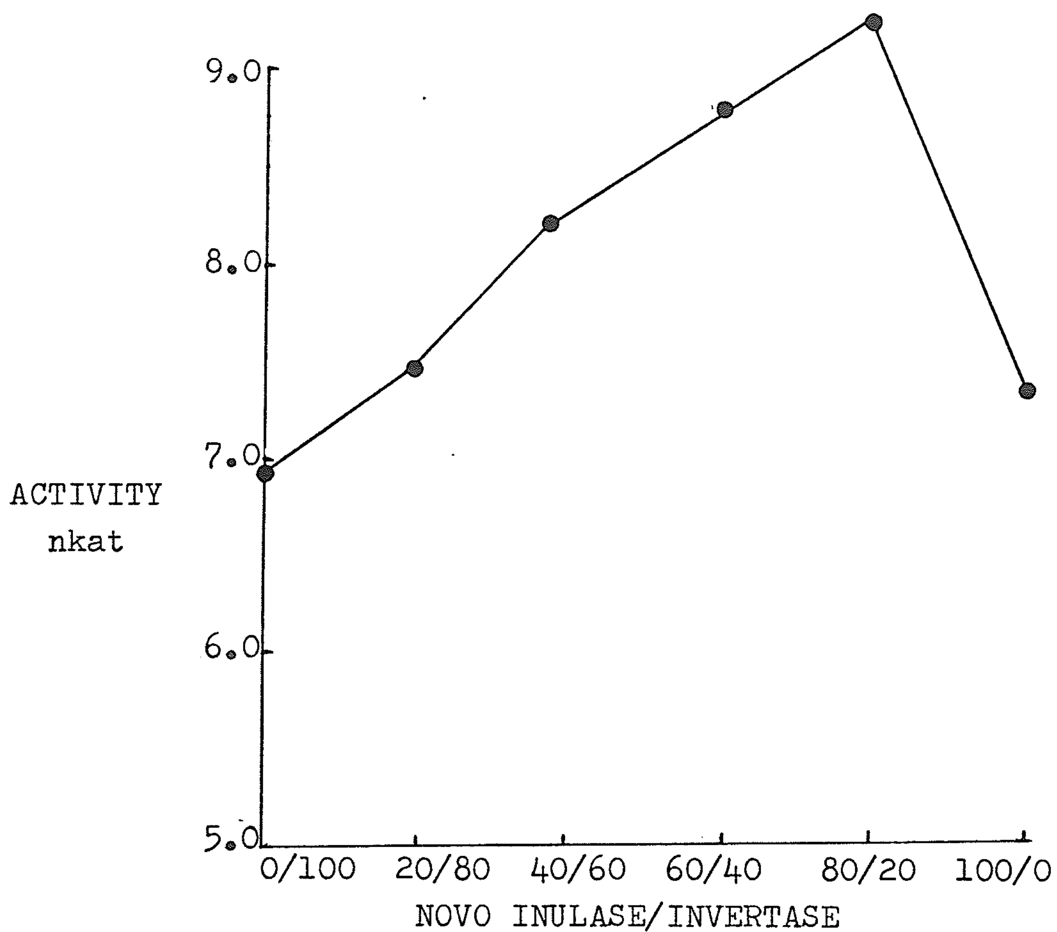
On a substrate consisting of 1.5% Jerusalem artichoke extract and 0.5% inulin, (average DP 7) a similar bell-shaped activity curve is observed, (figure 13). On this substrate the maximum level of activity lies between 40/60 and 60/40, (Novo inulase/invertase). The increase in activity was recorded to be about 25%.

The effect of the average DP of the substrate on the position of optimim ratio can be further examined by plotting the results from the above four experiments, (figure 14 a). Generally, the optimum ratio increases with the average DP of the substrate. As the average DP of the substrate increases, a mixture of enzymes with a higher proportion of inulase becomes more suitable. As is also evident from the graph, when the average DP of the substrate reaches 11.5 or higher, the position of the optimum ratio becomes constant. A quadratic curve can also be fitted to this data, (figure 14 b). Because this data was determined by intervals of 20%, the two lines are only an approximation of this relationship.

As was mentioned earlier, the 40/60 ratio was the optimum

FIGURE 12

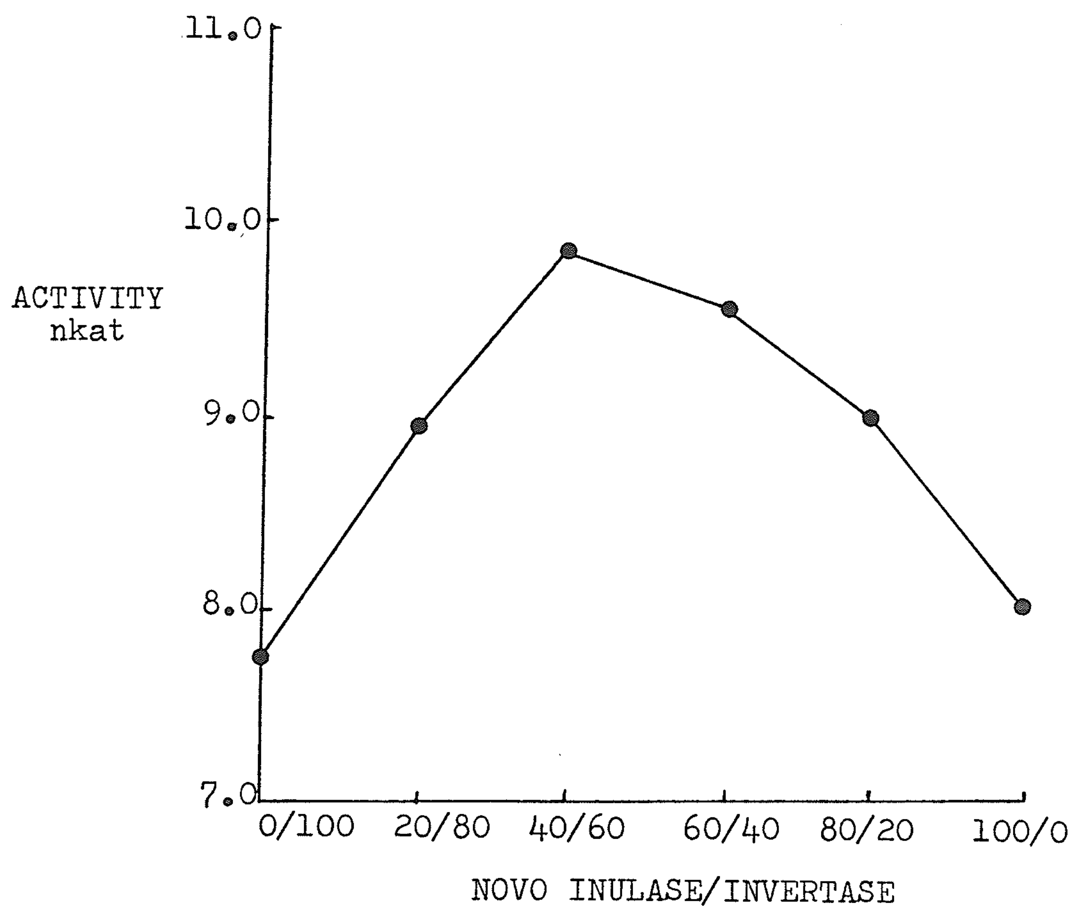
ACTIVITY[1] OF VARYING RATIOS OF INULASE/INVERTASE ON (1% INULIN + 1% JERUSALEM ARTICHOKE EXTRACT)



[1] activity after one hour, average of duplicate experiments

FIGURE 13

ACTIVITY[1] OF VARYING RATIOS OF INULASE/INVERTASE ON (1.5% INULIN + 0.5% JERUSALEM ARTICHOKE EXTRACT)

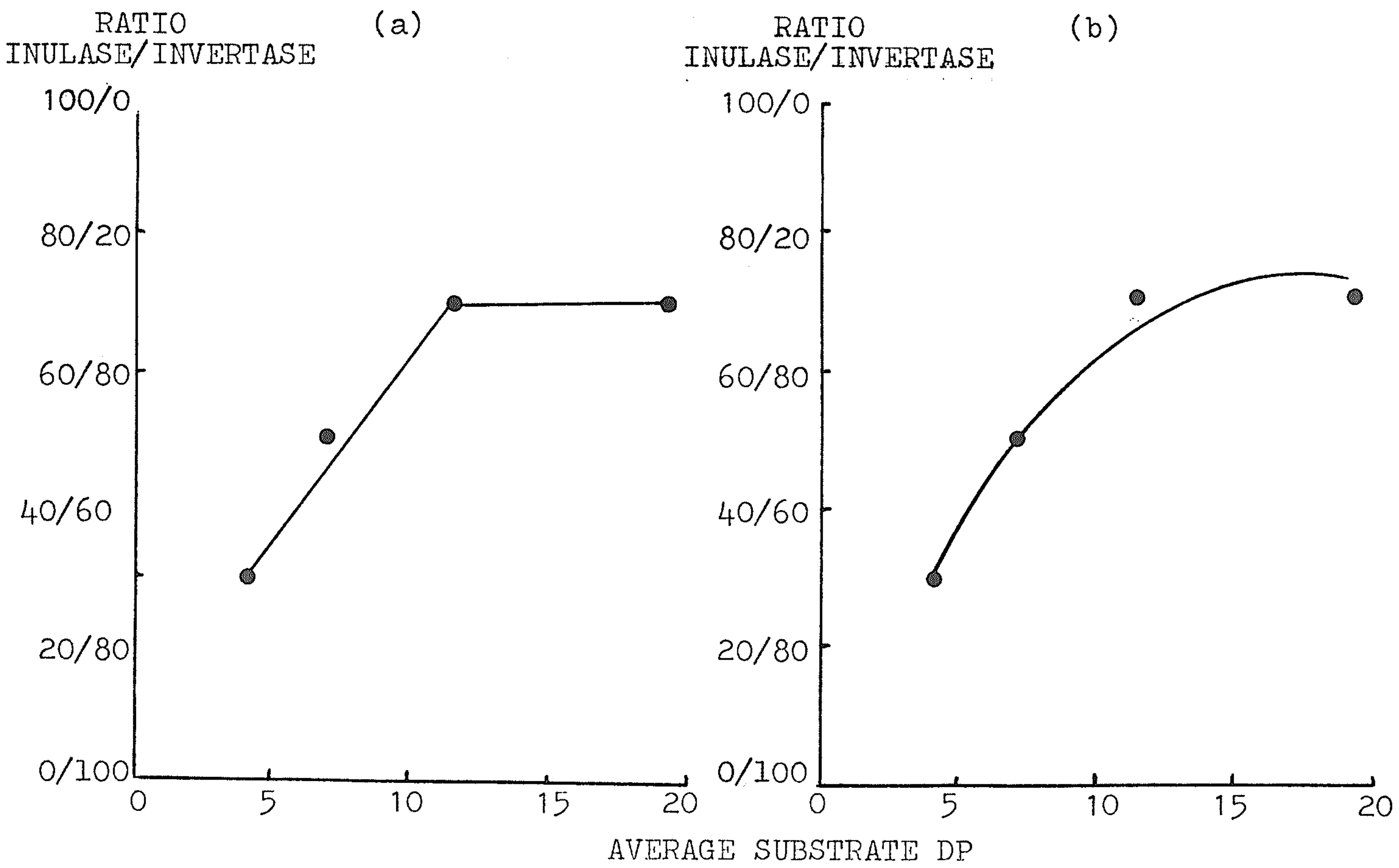


[1] activity after one hour, average of duplicate experiments

FIGURE 14

CHANGE IN OPTIMUM RATIO OF ENZYMES OVER VARYING AVERAGE SUBSTRATE DP

(a) linear approximation (b) quadratic approximation.

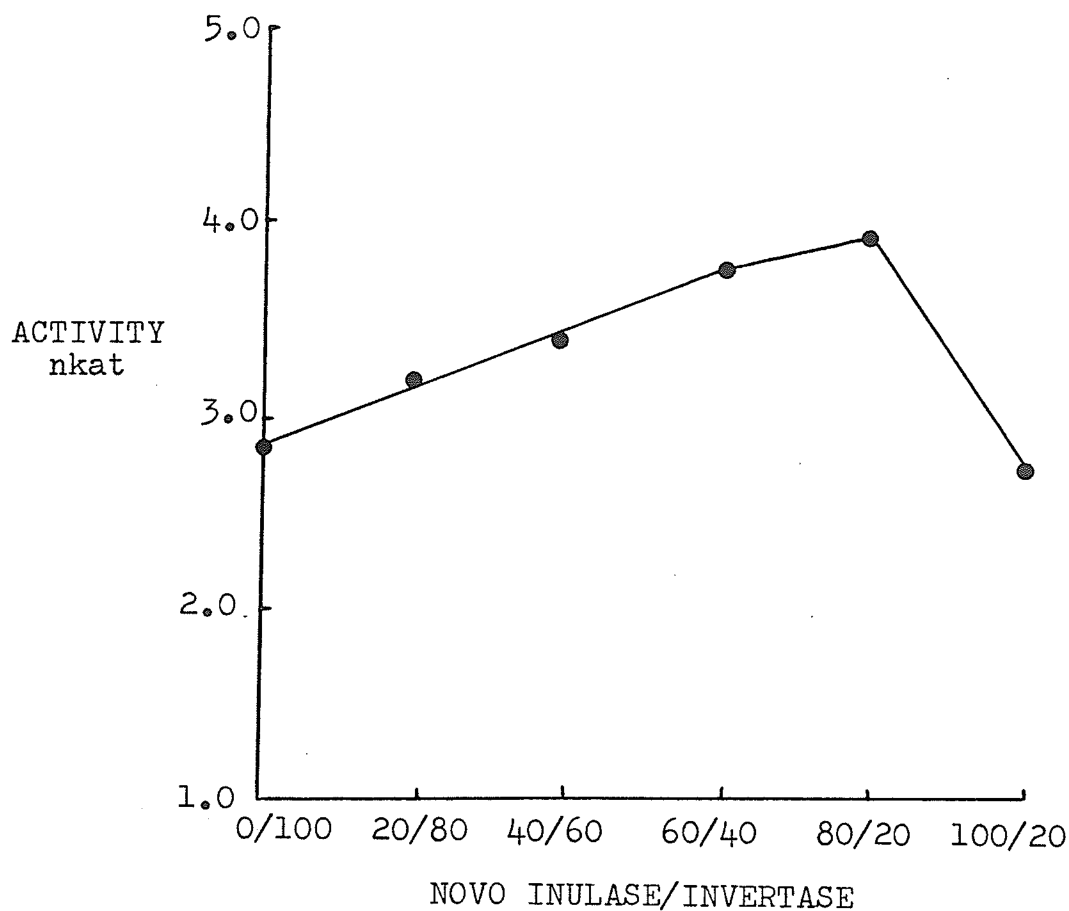


ratio on a substrate of 2.0% Jerusalem artichoke extract. However, on a substrate of 0.7% Jerusalem artichoke extract, the 80/20 ratio was recorded as the optimum ratio at one hour. Figure 15 illustrates hydrolysis of 0.7% Jerusalem artichoke extract by varying ratios of enzymes. The trend is similar to that for other substrates. At the maximum point of activity there is an increase in activity of about 40%. The major difference is the change in position of the optimum ratio. This change in optimum ratio, from 40/60 to 80/20, (a change to a larger proportion of inulase) possibly indicates that when there is a shortage of substrate the inulase and invertase begin to compete for the substrate. It is apparent then that the inulase competes successfully, (thus a shift to more inulase in the optimum ratio). This feature is reflected in the Km's of these two enzymes. From the information in table V, it is apparent that inulase Km's for sucrose are generally five times lower than invertase Km's for sucrose.

Another important consideration is whether the increase in activity of the blends of enzymes, as observed at one hour, will actually result in faster, complete hydrolysis of the substrate. Jerusalem artichoke extract, (0.7%) was hydrolysed by various blends of Novo inulase and invertase, (balanced activities). The degree of hydrolysis was measured over time. As can be seen from figure 16, all mixtures eventually reached 100% hydrolysis. After one hour the rate of hydrolysis by 0/100, (invertase) slows considerably. Extrapolation of this line indicates complete hydrolysis would be reached after six hours. This decrease in

FIGURE 15

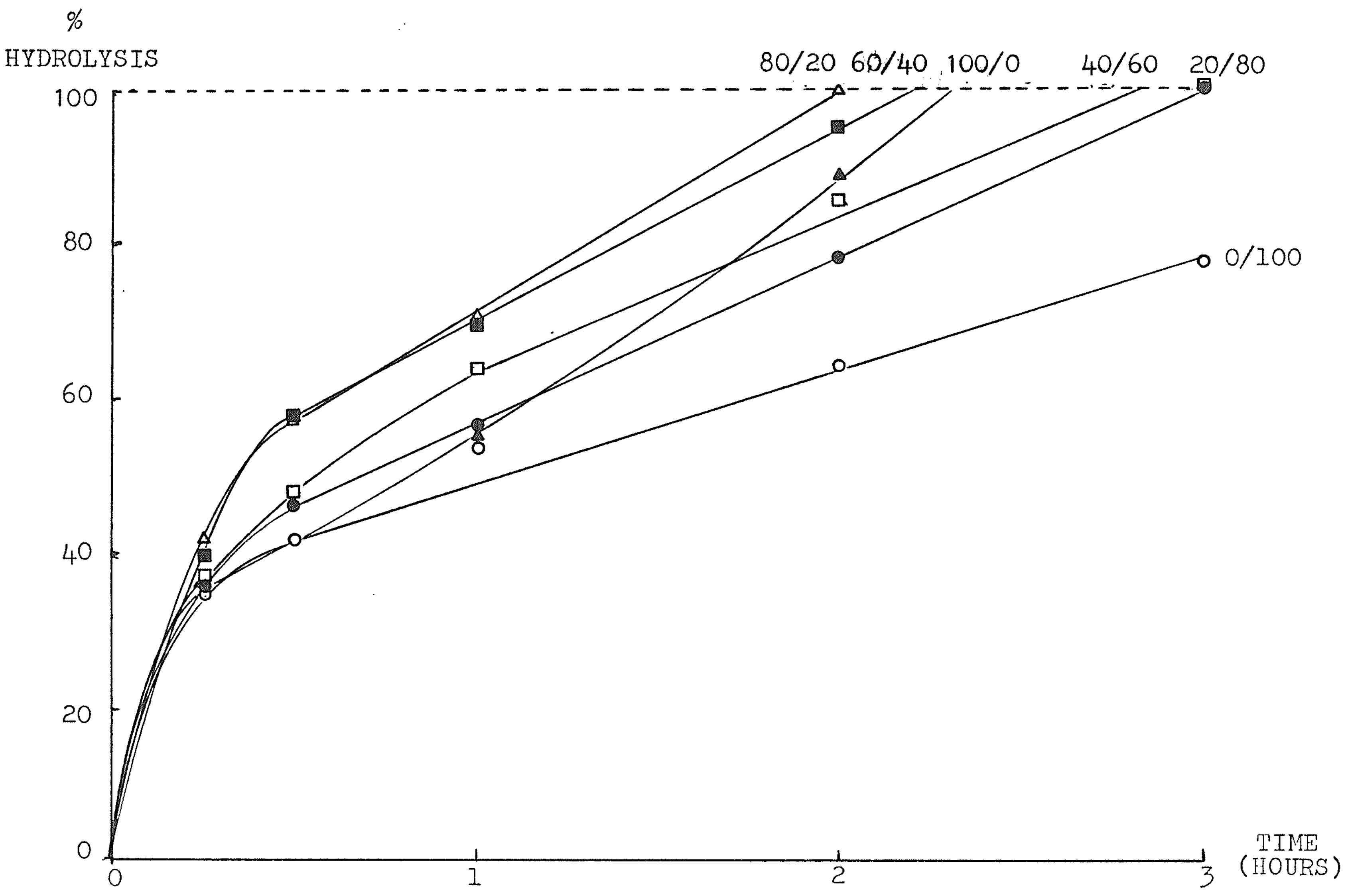
ACTIVITY[1] OF VARYING RATIOS OF INULASE/INVERTASE ON 0.7% JERUSALEM ARTICHOKE EXTRACT



[1] activity after one hour, average of duplicate experiments

FIGURE 16

DEGREE OF HYDROLYSIS OF JERUSALEM ARTICHOKE EXTRACT BY NOVO INULASE/INVERTASEE RATIOS OVER TIME.

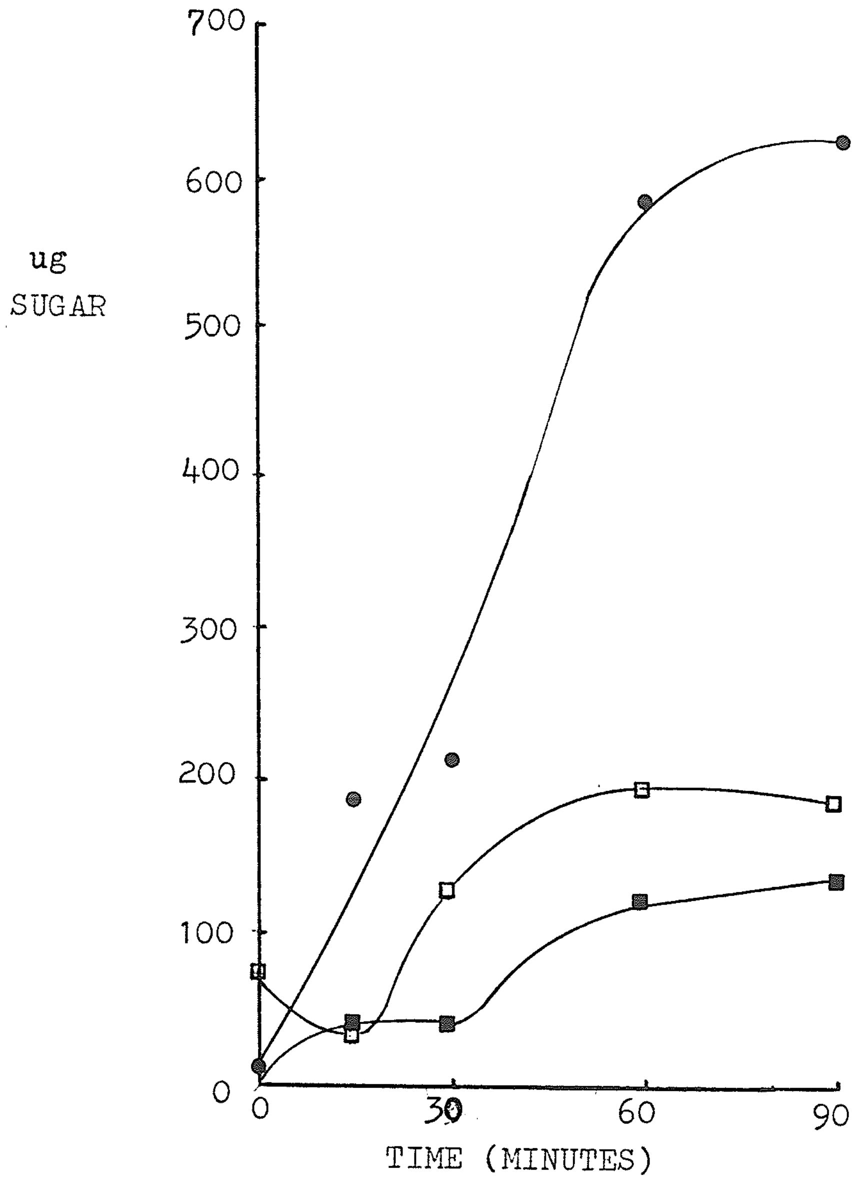


activity is likely due to exhaustion of sucrose and other smaller polymers which are easily hydrolysed by the invertase. The optimum ratio at one hour is the 80/20 mixture which is also the mixture to completely hydrolyse the substrate the fastest, followed by mixtures with decreasing proportions of inulase. The Novo inulase, (100/0) completely hydrolyses the substrate faster than those mixtures with a lower proportion of inulase than invertase, (40/60, 20/80). The degree of hydrolysis with the 100/0 mixture is greater than the 40/60 or 20/80 mixtures, presumably because, by the time 50% of the substrate has been hydrolysed, (one hour) there are insufficient levels of sucrose and small polymers to sustain invertase activity. The small amounts of sucrose produced by the action of the inulase is competed for by the inulase and invertase. The inulase, by virtue of its higher affinity for the substrate in comparison to invertase, (lower average Km) succeeds.

The evolvement of different sugars was measured by gas chromatography, as a result of hydrolysis of 0.7% Jerusalem artichoke extract by the optimum mixture of 80/20, (Novo inulase/ invertase). As can been seen from figure 17, there is a large initial increase in fructose, after which the rate of increase in fructose slows and will eventually plateau, as all the inulin is hydrolysed. Sucrose content initially drops then increases at a decreasing rate. The initial drop in sucrose concentration is due to hydrolysis of initially present sucrose. As other higher polymers are hydrolysed to the dimer, (sucrose) sucrose content increases. Eventually the rate of production of sucrose will

FIGURE 17

EVOLVEMENT OF SUGARS OVER TIME BY HYDROLYSIS OF 0.7% JERUSALEM ARTICHOKE EXTRACT WITH 80/20, (NOVO INULASE/INVERTASE), FRUCTOSE (●), SUCROSE (◻), GLUCOSE (■).



decrease and there will be a net decrease in sucrose content. Glucose content is dependent upon hydrolysis of sucrose. As would be expected glucose content increases rapidly at first then slows down but still increases as long as there is sucrose to be hydrolysed.

4.6 COMPUTER MODEL: POTENTIAL ECONOMICAL ASPECTS OF ARTICHOKE EXTRACT HYDROLYSIS BY BLENDS OF INULASE AND INVERTASE ENZYMES.

During storage of Jerusalem artichoke tubers from fall to spring, changes occur in the inulin and related fructosan components. Generally, the high molecular weight fraction decreases, thus increasing the low molecular weight fraction. This is reflected in a decrease in the F/G ratio from a value of 9:1 in the fall, to a value of 3:1 in the spring. The F/G ratio and therefore, the average DP of the fructosans found in artichoke extracts will depend on storage time. This study has shown that extracts can be hydrolysed more efficiently with blends of inulases and invertase than with inulases alone. The optimal ratio of the enzyme blend changes with the average DP of the fructosans.

The following computer model serves to illustrate the relationship between the use of enzyme blends for the hydrolysis of artichoke extracts and the economical aspect of hydrolysis. Programs used to generate the data are listed in appendix 5. In order to facilitate implementation of the model, the following assumptions were made. The model is that of a batch procedure for the production of high fructose syrups by enzymic hydrolysis of artichoke extracts. Hydrolysis must be completed in a constant time period, therefore the variable factor is the dose of enzyme

needed, (DOSE). The optimum ratio of enzymes, (OPT) for any given substrate with a characteristic average DP is defined according to figure 14(a) where 1) RATIO = 5.4(DP)+9.6, for $0 < DP \leq 11.5$ and 2) RATIO = 70, for $DP > 11.5$. The ratio of inulase/invertase used, (RATIO) is based on units of activity and not on grams of preparation or cost units. It was further assumed that the maximum increase in activity possible, by using OPT as compared to 100% inulase is an increase of 50%.

There are three factors which affect the economics of the hydrolysis. They are: 1) the average DP of the substrate, 2) the ratio of inulase/invertase, (RATIO), 3) the cost ratio, (CR) between inulase and invertase, (that is the cost of inulase per unit activity/cost of invertase per unit activity).

The first step in analysis of this model is to examine the effect of substrate DP and enzyme RATIO on DOSE. As is illustrated by the response surface in figure 18, the lowest DOSE of enzyme is found at the optimum ratio, (OPT) for any given substrate DP. For example on a substrate of average DP=6 and a ratio of 80 (80% inulase/invertase) the DOSE=132.76. For the same DP=6 and the optimum ratio of 42.2, DOSE=100, the least value of any other RATIO.

The second step of analysis is to examine the cost of hydrolysis, (COST). COST depends upon two factors, the DOSE of enzymes, (figure 18) and the COST ratio, (CR) between inulase and invertase. This relationship is demonstrated for the case of an extract with an average DP of 6, (figure 19).

When CR between the ratios is equal to 1, the optimum ratio

FIGURE 18

RESPONSE SURFACE OF ENZYME DOSE AS A FUNCTION OF ENZYME RATIO AND SUBSTRATE DP.

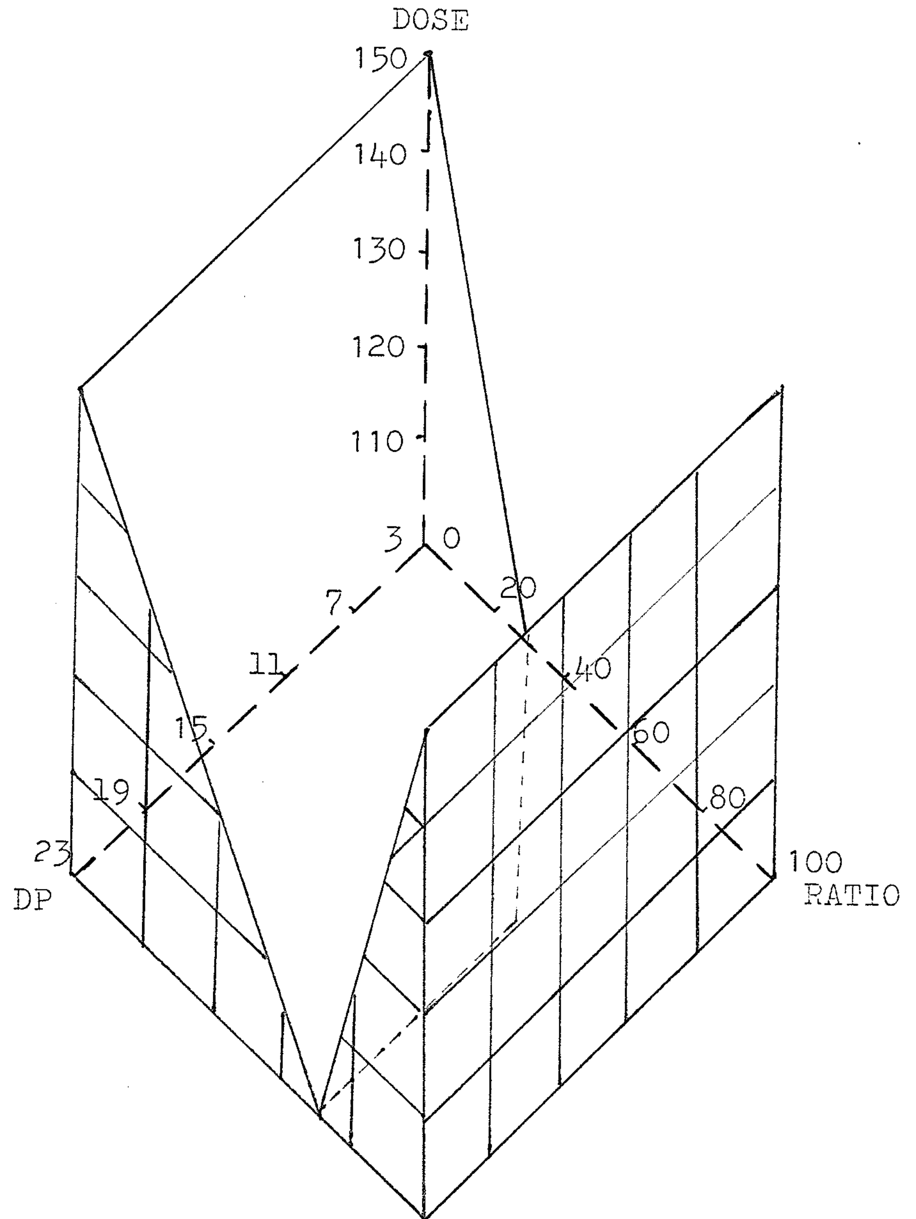
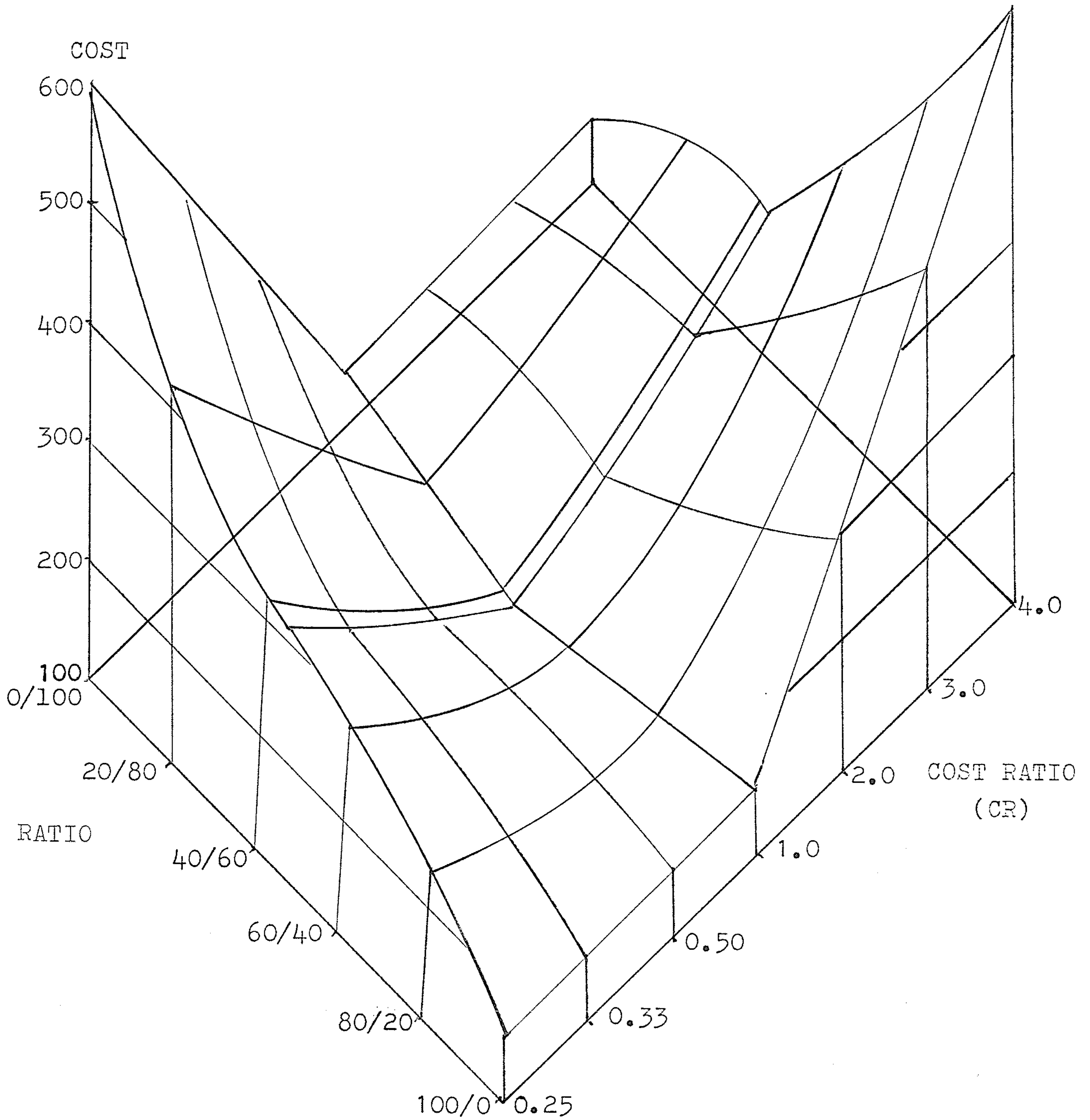


FIGURE 19

RESPONSE SURFACE OF COST OF HYDROLYSIS FOR VARIOUS RATIOS OF ENZYME, (INULASE/INVERTASE) AT DIFFERENT COST RATIOS.



(42% inulase/58% invertase) is clearly the most economical ratio as well. When CR is less than 1, although the OPT ratio is the ratio with least DOSE, the benefit is not great enough to offset the cost of the proportion of the more expensive enzyme. Therefore by the time CR$<$0.50 the most economical ratio is the 100% inulase. When CR is greater than 1, the reverse is true. As CR increases the benefit of decreased DOSE is offset by rising cost, due to the increasing proportion of the more expensive inulase. When CR is less than or equal to 2.0, the economical optimum is the same as OPT. When CR is greater than 2.0 some ratio less than OPT will be the most economical. For this model, if the invertase were capable of completely hydrolysing inulin, the economical optimum would be 100% invertase.

It is of interest to note that as CR increases above 1, although the cost of operating at OPT increases, the COST relative to 100% inulase decreases according to the equation $COST=(1 - 2^{-CR})$. This implies that there is a point when further increase in CR has a negligible effect on the cost of operating at OPT. For this model, when CR$\doteq$ 7, the COST at OPT increases by less than 1%.

CHAPTER 5 CONCLUSIONS AND RECOMMENDATIONS

The results of this study indicated that the Irgazyme M-10 preparation contained inulase activity. Activity of this preparation was determined to be 0.17 nkat/mg. This is low in comparison to a commercial inulase preparation developed by Novo Industries Ltd. with an activity of 50 nkat/mg. Although the activity of the Irgazyme M-10 preparation was partially increased by purification, it became apparent that more than a one-step procedure was necessary to achieve satisfactory levels of activity. In addition to both being derived from *Aspergillus* sp. other similarities existed. Both contain more than one inulase with slightly differing properties. The pH optimum was found to be nearer 4.5 than 6.0, while the highest activity was recorded at 55°C. It is possible that the Novo inulase preparation is actually a purified form of the Irgazyme M-10 preparation.

These parameters of temperature and pH optima indicate that the inulase enzymes from Irgazyme M-10 or the Novo preparation would be compatible with the processing technique of Hoehn *et al.* (1981), for the production of high fructose syrups by enzymatic hydrolysis of inulin from Jerusalem artichoke tubers. The enzymes were able to completely hydrolyse inulin and related fructosans without development of certain breakdown products characteristic of acid hydrolysis, as revealed by gas chromatography. No off colour problems were observed with enzyme hydrolysis. In addition, the inulases would be prevented from contaminating the final product. The molecular weights of

inulases ($>$50,000) is far in excess of the cut off of 1000, characteristic of the ultrafiltration membrane used in this process.

It was observed that mixtures of either of these inulase preparations and invertase demonstrated greater hydrolytic activity than the same concentration of either enzyme alone. Furthermore, it is apparent that the mixture of enzymes with the highest activity, was dependant on the average DP of the substrate. Generally as the average DP increases, the proportion of inulase in the optimum ratio also increases. Substrate concentration also has an effect on the formulation of the optimum ratio. The extent of impact of this variable on enzyme activity has not been completely investigated. It is therefore recommended that further investigations be conducted into the effect of substrate concentration on the activity of the inulase/ invertase system. This is of importance considering that industrial applications of these enzymes to inulin hydrolysis will occur at substrate limiting concentrations, by virtue of the poor solubility of inulin at lower temperatures. It is further recommended that investigation be conducted to determine the effect of polymer distribution within a substrate, on the activity of these enzyme mixtures. For example, a substrate with an average DP of 10 may be composed of a normal distribution of polymers about the mean, either a narrow or wide distribution, or an imbalance of polymers resulting in a skewed distribution. Such a characterization of polymer distribution, although difficult, could be obtained in part through the use of high pressure liquid chromatography.

In addition, it should be noted that although this study has investigated the interaction between invertase and inulase preparation per se, the inulase preparation is actually a mixture of an endo and an exo inulase. It is likely that the average DP of a substrate will affect the optimum proportion of these two inulases. For example, if a substrate was comprised mostly of polymer lengths larger than the preferred range of the exo inulase, a mixture of inulases favoring the endo inulase could possibly be more active than the reverse mixture.

From studies carried out on the economic potential of this system, it appeared there was economic benefit in using a mixture of inulase/invertase to hydrolyse inulin. The correct application of this system is dependant upon knowledge of the average DP of the substrate and the cost ratio between the two enzymes.

The mechanism that accounts for this complementary hydrolysis between inulase and invertase is open to speculation. An examination of some of the known and observed data, does provide some understanding. It has been known that inulases exhibit a preference for certain polymer lengths as substrates. An endo-inulase from Kluyveromyces fragilis was reported to exhibit preference for polymers of lower DP, especially sucrose, (Nahm and Byun, 1977). In contrast however, Nakamura et al. (1978), reporting on an extracellular endo-inulase from Aspergillus niger, found this enzyme to be inactive on sucrose and scarely active on polymers smaller than the pentasaccharide. Zittan (1981) reports that the Novo inulase, prepared from Aspergillus sp. was

capable of hydrolysing all polymer lengths encountered in a substrate of commercial inulin. The S/I ratio was determined for the Novo inulase during the course of the study and was found to be 2.9. The Novo inulase preparation, consisting of an exo and an endo-inulase, hydrolysis sucrose three times faster than commercial inulin. Based on information available about inulase, it can be deduced that the Novo inulase exhibited a preference for fructosans of lower DP > sucrose > fructosans of higher DP. This also seems to be suggested by the data given in figure 9. In the example of hydrolysis of inulin with 100% Novo inulase there is a near constant amount of sucrose present during the first 60 minutes of hydrolysis. This indicated that although sucrose hydrolysis occured, polymers of higher DP were hydrolysed faster, indicating preferential hydrolysis. Since the Novo inulase is capable of hydrolysing inulin, the sucrose would be hydrolysed last. Due to the presence of the exo-inulase in this preparation, all polymers of higher DP, DP's within the preferred range for hydrolysis. In the example where both invertase and the Novo inulase are present, the sucrose is rapidly hydrolysed. Thus, the two enzymes complement one another by hydrolysing preferred substrates.

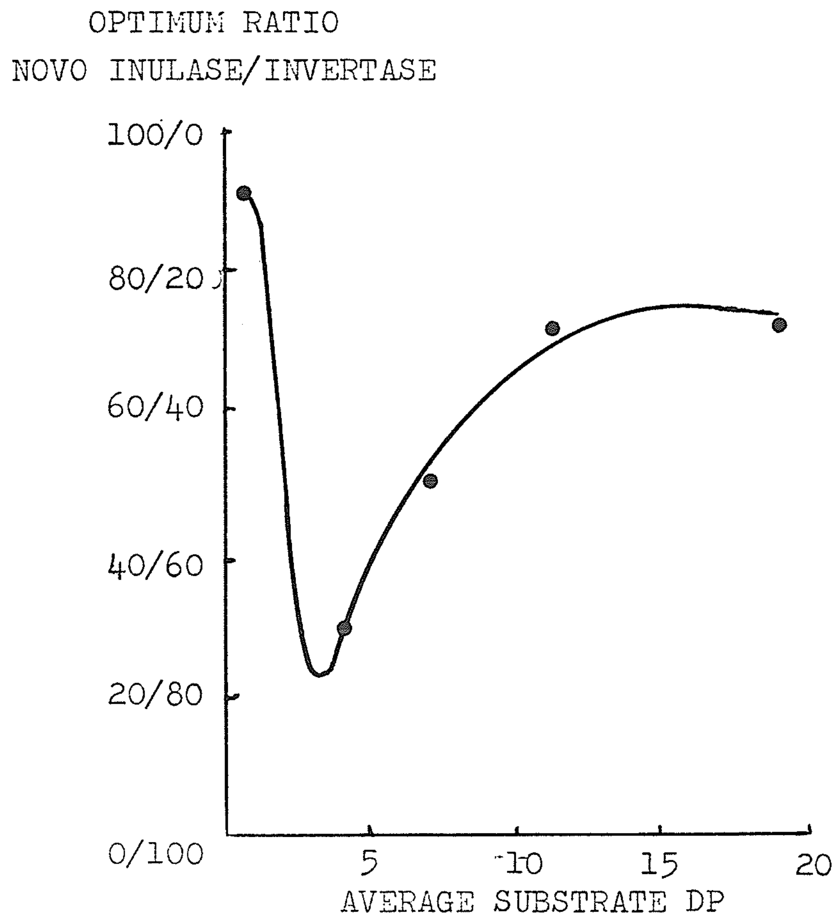
An interesting situation arises when hydrolysis proceeds to the point where the substrate concentrations are very low and mostly sucrose predominates. As is indicated by the hydrolysis of 0.7% Jerusalem artichoke extract, (figure 16) complete hydrolysis is reached sooner by the 100% inulase then by mixtures with lower proportions of Novo inulase then invertase. The

change in order, from that predicted by figure 15, occurs just after one hour has elapsed. By this time about 50-60% hydrolysis has occured. Presumably the bulk of polymers have been hydrolysed leaving mostly sucrose. Those with lower DP are hydrolysed by preference while those with higher DP are cleaved by the exo-inulase, thereby ensuring their hydrolysis ahead of sucrose. The 100% Novo inulase, completing hydrolysis ahead of the 40/60 or 20/80 mixtures implies that the Novo inulase is faster at hydrolysing sucrose than the corresponding component of invertase in the other mixtures. This could also explain why lowering the concentration of artichoke extract from 2.0% to 0.7% results in an increase in proportion of Novo inulase in the optimum ratio. At 0.7% substrate concentration, there is a considerable amount of inulase not "occupied" with hydrolysis of inulin. Since there is a large proportion of sucrose present initially, the invertase is out-competed for sucrose as substrate, by the Novo inulase. The implication then, is that the Novo inulase Km for sucrose is lower than that of the invertase. This is possibly suggested by the data given in table V, which indicated inulase Km's are generally five times lower for sucrose than the invertase Km's. Figure 13 predicted that the optimum ratio for a substrate of average DP=2, (sucrose) was in the range 0/100-20/80, Novo inulase/invertase. If it is true that the Novo inulase has a lower Km for sucrose than the invertase, then an optimum ratio in the range 80/20-100/0 would have to be expected. The relationship between DP and ratio of enzymes would then be best described by a quadratic polynomial as

indicated in figure 20. A determination of the optimum ratio on sucrose (DP=2) and other substrates of similar DP (ie. DP=2.3, 2.5, 3.0) would be necessary to confirm this, as would a comparative study of Km's.

FIGURE 20

THEORETICAL RESPONSE OF OPTIMUM RATIO OF ENZYMES TO AVERAGE SUBSTRATE DP.



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APPENDIX 1 SAMPLE CALCULATIONS

APPENDIX 1(a) CALCULATION OF RESPONSE FACTORS

mg sugar	Mean peak area	Standard deviation
5.0 mg Fructose	2.6×10^5	±0.1
5.0 mg Glucose	2.6×10^5	±0.1
5.0 mg Sucrose	1.9×10^5	±0.1
6.0 mg IS	1.8×10^5	±0.1

$$RF_S = (As/Ws)(WIs/AIs)$$

$$RF_{Fructose} = (2.6 \times 10^5/5 \text{ mg})(6.0 \text{ mg}/1.8 \times 10^5) = 1.733$$

$$RF_{Glucose} = (2.6 \times 10^5/5 \text{ mg})(6.0 \text{ mg}/1.8 \times 10^5) = 1.733$$

$$RF_{Sucrose} = (1.9 \times 10^5/5 \text{ mg})(6.0 \text{ mg}/1.8 \times 10^5) = 1.267$$

APPENDIX 1(b) CALCULATION OF MG SUGAR BY GLC

$$W_S = (As/RFs)(WIs/AIs)$$

$$W_{Fructose} = (2.9 \times 10^5/1.733)(2.1 \text{ mg}/9.0 \times 10^4) = 3.90 \text{ mg}$$

APPENDIX 1(c) CALCULATION OF ENZYME ACTIVITY

ug/ml Sugar Corrected = ug/ml test - ug/ml enzyme blank - ug/ml substrate blank

Katals = MOLES/SEC

Corrected ug/ml = 972.1 -312.0 - 2.2 = 657.9 ug

657.9 ug = 0.658×10^{-3} g

6.58×10^{-5}g/180 = 3.66×10^{-7} moles

3.66×10^{-7} moles/3600 sec = 1.015×10^{-10} KATALS
= 0.10 nKATALS

APPENDIX 1(d) CALCULATION OF REJECTION COEFFICIENT

$$R = \frac{\ln(Cf/Co)}{\ln(Vo/Vf)}$$

where Cf = final macrosolute concentration in the retentate

Co = initial macrosolute concentration

Vo = initial sample volume

Vf = final retentate volume

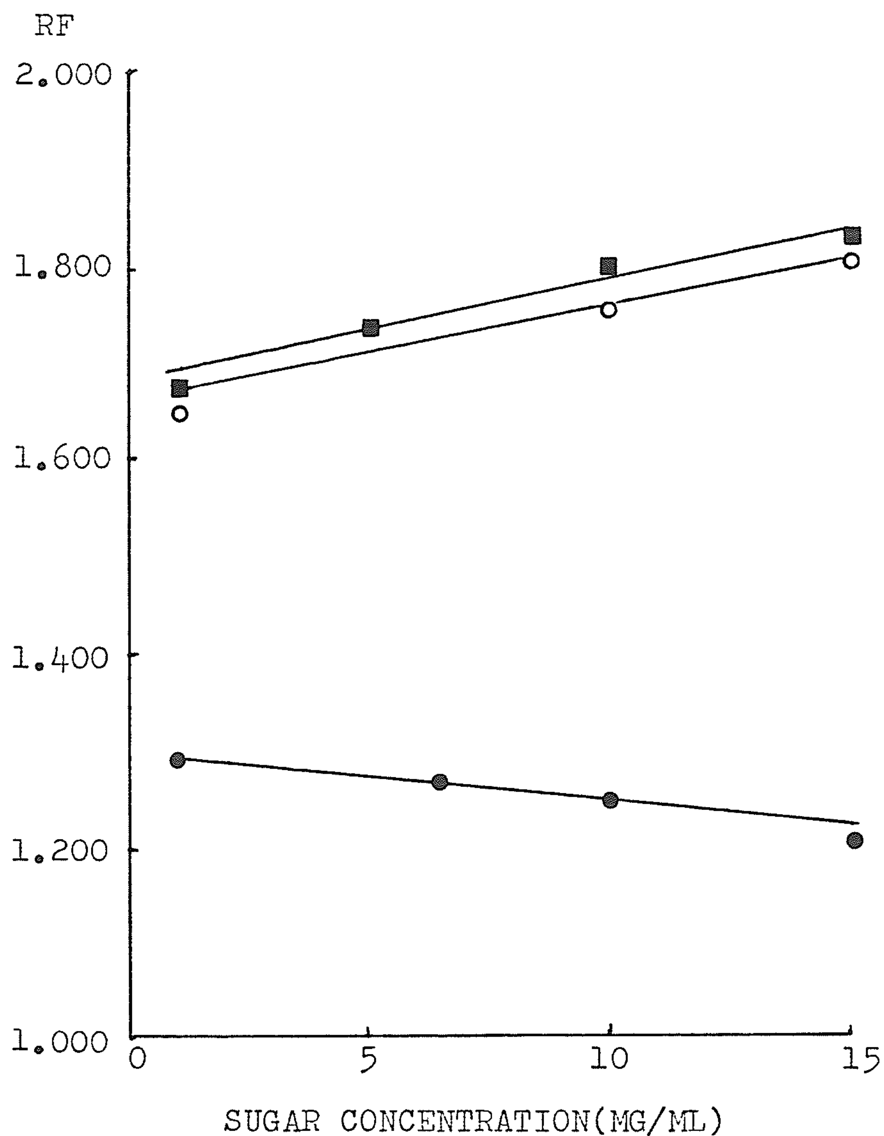
eg. purification of inulase activity in Irgazyme M-10 with XM 50

test 1 R protein = $\frac{\ln(28.357 \text{ mg/ml}/8.140 \text{ mg/ml})}{\ln(60 \text{ ml}/12.5 \text{ ml})} = 0.79$

test 2 R protein = $\frac{\ln(55.964 \text{ mg/ml}/8.140 \text{ mg/ml})}{\ln(60 \text{ ml}/6\text{ml})} = 0.83$

APPENDIX 2

RESPONSE FACTORS (RF) OF SUCROSE(●), FRUCTOSE(○) and GLUCOSE(■), AT VARIOUS CONCENTRATIONS.

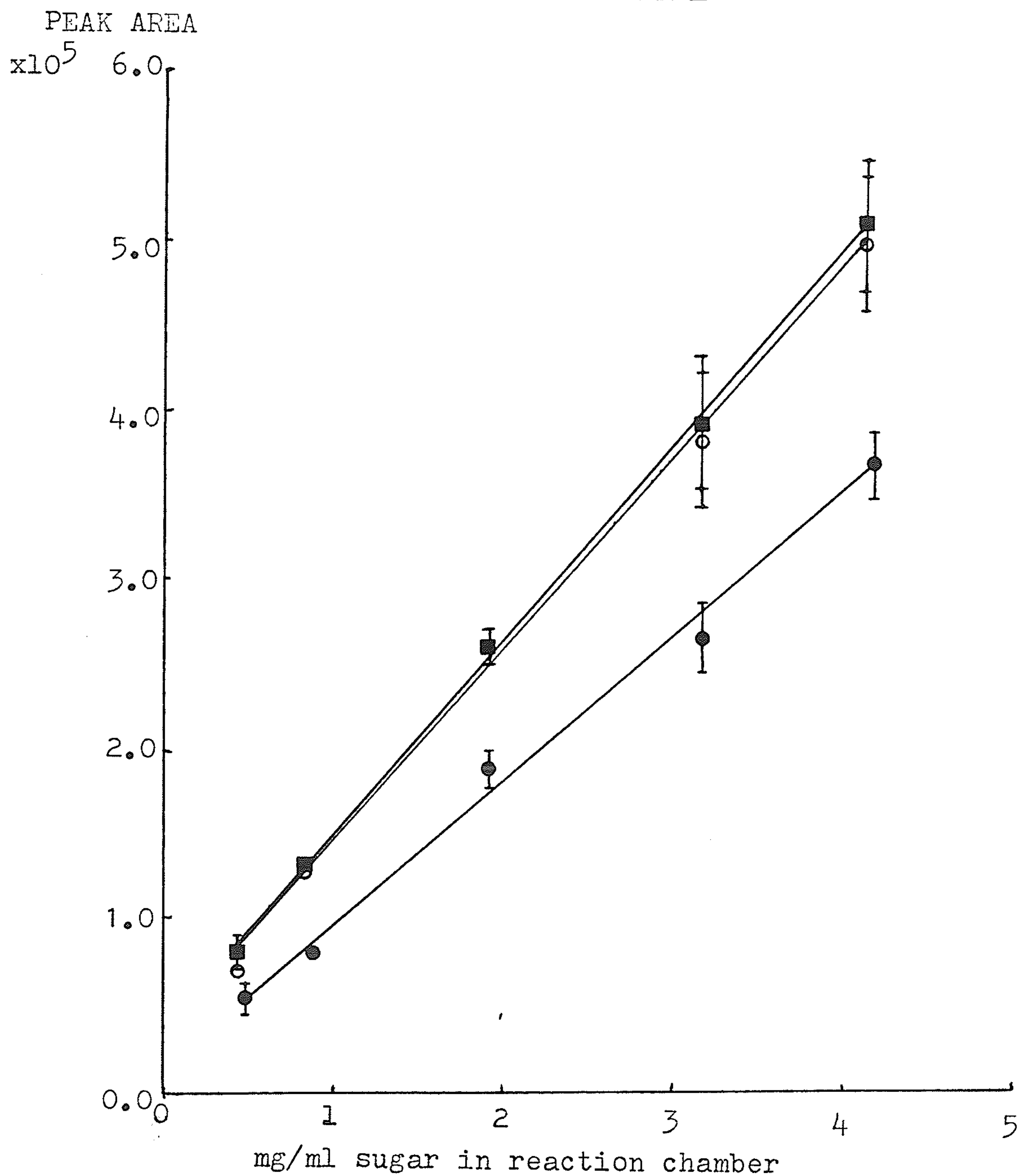


APPENDIX 3 TABLE OF RESPONSE FACTORS

	SAMPLE WEIGHT(mg)				
SUGAR	1.0	2.0	5.0	10.0	15.0
FRUCTOSE	1.65	1.76	1.73	1.75	1.80
GLUCOSE	1.67	1.84	1.73	1.80	1.84
SUCROSE	1.27	1.10	1.27	1.25	1.22

APPENDIX 4

PEAK AREA RESPONSE OF SUCROSE(●), FRUCTOSE(■) AND GLUCOSE(○) AT DIFFERENT CONCENTRATIONS.



APPENDIX 5 PROGRAMS FOR COMPUTER MODEL

APPENDIX 5(a) Determination of DOSE units for ratios of inulase/invertase on substrates with average DP's 3 to 17.

```
1           INTEGER J
2           REAL DP,RATIO,OPT,DOSE
3           PRINT 4
4           OPT = 70.00;RATIO = 0.0
      C
6           DP=3.0
7           DO 6 J=1,8
8              IF(DP.LE.11.5) OPT = 5.4*DP+9.6
9              WHILE(RATIO.LE.100.0) DO
10                CALL LEVEL(OPT,RATIO,DOSE)
11                PRINT 14,DP,RATIO,100.0-RATIO,DOSE
12                RATIO = RATIO + 5.0
13             END WHILE
14             DP = DP+2.0
15             RATIO = 0.0
16     6    CONTINUE
17          STOP
18     4    FORMAT('0',T10,'SUBSTRATE DP', T30,'RATIO INU/INV',
          *       T50,'TOTAL DOSE (UNITS)')
19     14   FORMAT(' ',T14,F6.2,T30,F6.2,F6.2,T62,F6.2)
20          END
      C
21          SUBROUTINE LEVEL(OPT,RATIO,DOSE)
22             REAL OPT,RATIO,DOSE
23             DOSE = 100.0
24             IF(RATIO.LE.OPT)DOSE=DOSE+(OPT-RATIO)/OPT*50.00
25             IF(RATIO.GT.OPT)DOSE=DOSE+(RATIO-OPT)/(100.0-OPT)*50.00
26          RETURN
26          END
```

APPENDIX 5(b) Determination of COST of hydrolysis for ratios of inulase/invertase, at a given Cost Ratios for a substrate of average DP=6.0.

```
1          REAL COST, OPT, DP, RATIO, PINU, PINV, DOSE
2          READ 2, DP, PINU, PINV
3          OPT = 70.00; RATIO=0.0; COST=0.0
6          IF(DP.LE.11.5)OPT=5.4*DP+9.6
7          PRINT 14, DP, OPT, 100.0-OPT
8          PRINT 24, PINU, PINV
9          PRINT 34
   C
10         WHILE(RATIO.LE.100.0) DO
11            CALL LEVEL(RATIO, OPT, DOSE)
12            COST=(RATIO/100.0*DOSE*PINU)+(100.0-RATIO)100.0*DOSE*
                    PINV)
13            PRINT 44, RATIO, 100.0-RATIO, DOSE, COST
14            RATIO = RATIO + 1.0
15         END WHILE
16         STOP
17    2    FORMAT(3F4.2)
18    24   FORMAT('O',T10,'WHEN THE COST OF INULASE IS',F4.2, UNITS,
        *      ' AND THE COST OF INVERTASE IS ',F4.2,' UNITS,',///)
19    14   FORMAT('O',T10,'THE OPTIMUM RATIO FOR HYDROLYSIS ON',
        *      'THIS SUBSTRATE OF DP = ',F6.2,
        *      ' IS ',F6.2,' % INULASE NAD ',F6.2,' % INVERTASE.')
20    34   FORMAT('O',T10,'RATIO OF INU/INV',T30,'TOTAL DOSE (UNITS)'.
        *      T50,'TOTAL ENZYME COST (UNITS')
21    44   FORMAT(' ',T13,F6.2,F6.2,T36,F6.2,F6.2,F7.2)
22         END
   C
23         SUBROUTINE LEVEL(RATIO,OPT,DOSE)
24         REAL OPT,RATIO,DOSE
25            DOSE = 100.0
26            IF(RATIO.LT.OPT)DOSE=DOSE+(OPT-RATIO)/OPT*50.00
27            IF(RATIO.GT.OPT)DOSE=DOSE+(RATIO-OPT)/(100.0-OPT)*50.0
28          RETURN
29          END
```