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SUCROSE HYDROLYSIS BY INVERTASE. CHARACTERIZATION OF PRODUCTS AND SUBSTRATE INHIBITION

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ABSTRACT

In the hydrolysis of highly concentrated sucrose solutions with invertase, D-fructose was shown to be a competitive inhibitor, and D-glucose a partial noncompetitive inhibitor. The equation developed by combining both inhibitory effects allows the mathematical description of batch hydrolysis of dilute sucrose solutions. The decrease in invertase activity observed for increasing sucrose concentration does not depend on the medium viscosity, or on a modification of the enzyme properties, but on a modification of the structure of the substrate intra- and intermolecular hydrogen bonds. This observation led to the development of an original model describing the influence of sucrose concentration on the initial rate of the hydrolysis reaction.

INTRODUCTION

The production of liquid sugar solutions from starch by use of amylolytic enzymes combined with D-glucose isomerase[1] has become very attractive and has created a very important impact on the sugar industry. Similarly, mixtures of D-glucose and D-fructose may be obtained from the hydrolysis of sucrose. In this field, enzymic hydrolysis with invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) avoids the production of colored oxidation compounds that are the by-products of acid hydrolysis and require a decoloration treatment[2]. From an economy point of view, the industrial-scale enzymic hydrolysis must be achieved with highly concentrated sucrose solutions (up to 1 kg/L) and avoid any dilution and further concentration operations. This raises the problem of invertase action with high substrate- and product-concentrations and low-water activity. These conditions are, therefore, far from the usual ones employed for characterization of initial rate enzyme activity in dilute solutions. In order to develop a mathematical description of invertase action under such conditions, it was necessary to precisely characterize the inhibitory effects of both product and substrate. Although many publications have described invertase activity since the original work of Michaelis and Menten[3], no precise data are available, to our knowledge, on the inhibitory ef-

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fect of D-fructose and D-glucose[4], and several models have been proposed to describe the inhibitory effect of an excess of sucrose[5-7]. These models do not account, however, for the presence of anomalies on the experimental curves; these were recently attributed to a modification of the sucrose structure in concentrated solutions[8].

EXPERIMENTAL

Enzyme. — Invertase from baker's yeast (Sigma Chem. Co., grade VI) was used without previous purification.

Invertase activity assay. — The reaction was started by the addition of a 0.2% invertase solution in 0.1M acetate buffer, pH 4.5 (0.2 mL) to a sucrose solution in the same buffer (20 mL). The variation in concentration of reducing sugars resulting from invertase activity was measured by the 2,4-dinitrosalicylic acid method[9], as a function of time, to determine the initial reaction-rate. Standardization was obtained with different concentrations of an equimolar mixture of D-glucose and D-fructose, in 0.1M acetate buffer, pH 4.5. One unit of invertase activity corresponds to the amount of enzyme that catalyzes the hydrolysis of 1 mmol of sucrose per min under the conditions of assay.

Products inhibition. — The initial rate of reaction was determined at 40° for various sucrose concentrations (25, 50, 100, and 200mM), without and with addition of either D-fructose (0.1, 0.2, and 0.4M) or D-glucose (0.05, 0.1, 0.2, and 0.4M).

Batch hydrolysis of sucrose solutions. — The hydrolysis kinetics of either dilute (0.2M) or concentrated (0.8M) sucrose solutions were determined by monitoring the amount of reducing sugars produced, as a function of time at 40°, in a reaction mixture containing a sucrose solution in 0.1M acetate buffer, pH 4.5 (20 mL) and 0.2% invertase solution in the same buffer (0.2 mL).

Computer analysis of experimental data. — The optimal value of the parameters was determined by successive iterative calculation in order to minimize the error e, as defined in Eq. *1*

$$e = \sum_{i=1}^{i=n} (i) \quad 1 - \frac{Y_{C(i)}}{Y_{D(i)}} \qquad (1)$$

where i is the point index, n the total number of points, $Y_{C(i)}$ the calculated value of point i, and $Y_{D(i)}$ the given value of point i.

Effect of viscosity. — The influence of viscosity on invertase action was determined by measuring, at 53°, the initial rate of hydrolysis of a 0.4M sucrose solution containing either carboxymethylcellulose (Prolabo) or polyacrylamide (Prolabo). Carboxymethylcellulose was used at 1.72% concentration in order to give, at 53°, the same viscosity as a 2.67M sucrose solution at the same temperature, *i.e.*, 43 mPa · s. Polyacrylamide was added at various concentrations to obtain various viscosity values ranging from 1 to 106 mPa · s.

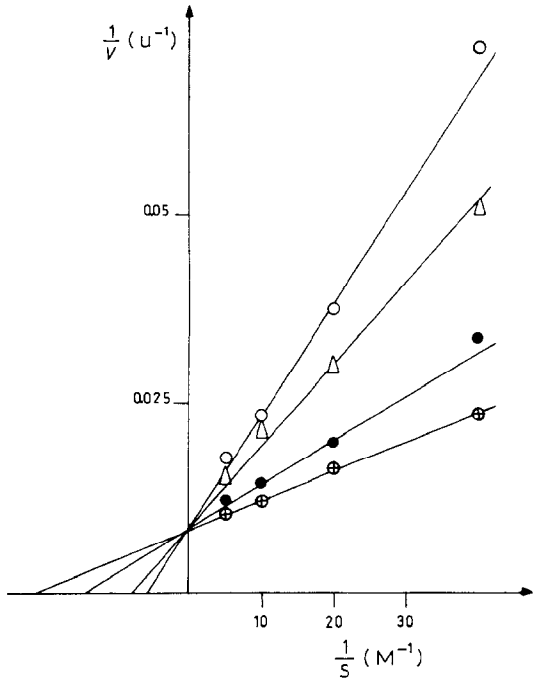

Fig. 1. D-Fructose inhibition. Effect of sucrose concentration on invertase activity. The invertase activity was determined at 40° and pH 4.5; a 0.2% invertase solution (0.2 mL) was added to the sugar solution (20 mL) with various initial concentrations of D-fructose. None (—⊕—), 0.1 (—•—), 0.2 (—△—), and 0.4M (—○—).

Effect of water activity. — The effect of compounds known to deeply modify the activity of water was determined, at 25°, by adding either 0.1M magnesium chloride (Prolabo) or 1% poly(1,2-ethanediol) (Prolabo, M_r 4000), to the enzyme-assay mixture.

RESULTS

Product inhibition. — The inhibitory effect of D-fructose and D-glucose was characterized for a concentration range of sucrose at which sucrose inhibition may be neglected. For D-fructose inhibition, the reciprocal plot of the results obtained for various initial D-fructose-concentrations gave straight lines (Fig. 1) showing a common intercept with the 1/V axis, and an increasing slope for an increasing D-fructose concentration. The corresponding values of the apparent Michaelis constant, K'_m, determined from the intercept with the 1/S axis, are given in Table I. These K'_m values gave a linear relationship with initial D-fructose concentrations. Thus, these results agree with a competitive inhibition of invertase activity by D-fructose, according to the classical equation for competitive inhibition *2*,

$$V = \frac{V_{max}(S)}{K'_m + (S)} = \frac{V_{max}(S)}{K_m(1 + (F)/K_F) + (S)} \quad (2)$$

where V is the initial reaction-rate, V_{max} the maximal, initial reaction-rate, (S) the initial concentration of sucrose, (F) the initial concentration of D-fructose, K_m the Michaelis constant, and K_F the inhibition constant of D-fructose. From the experimental data, the following values were calculated: $V_{max} = 127$ U, $K_m = 49$mM, and $K_F = 128$ mM.

For D-glucose inhibition, a linear plot was obtained for each initial D-glucose concentration when applying the reciprocal representation to the experimental data (Fig. 2), with an increasing slope when the concentration of D-glucose was enhanced, and a common intercept with the 1/S axis. However, the values of the apparent, maximal, initial reaction rate (V_{max}), determined from the intercept of the $1/V$ axis (Table II), do not fit with the classical equation for noncompetitive inhibition[10] 3,

$$V = \frac{V'_{max}(S)}{K_m + (S)} = \frac{V_{max}}{(1 + (G)/K_G)} \cdot \frac{(S)}{K_m + (S)} \quad (3)$$

TABLE I

EFFECT OF INITIAL CONCENTRATION OF D-FRUCTOSE ON THE VALUE OF THE APPARENT MICHAELIS CONSTANT K'_m

Concentration of D-*fructose* (M)	K'_m (mM)
0	49
0.1	78
0.2	138
0.4	205

TABLE II

EFFECT OF INITIAL CONCENTRATION OF D-GLUCOSE ON THE VALUE OF THE APPARENT MAXIMAL, INITIAL-REACTION RATE V'_{max}

Concentration of D-*glucose* (M)	V'_{max} (U)
0	127
0.05	113
0.1	101
0.2	81
0.4	69

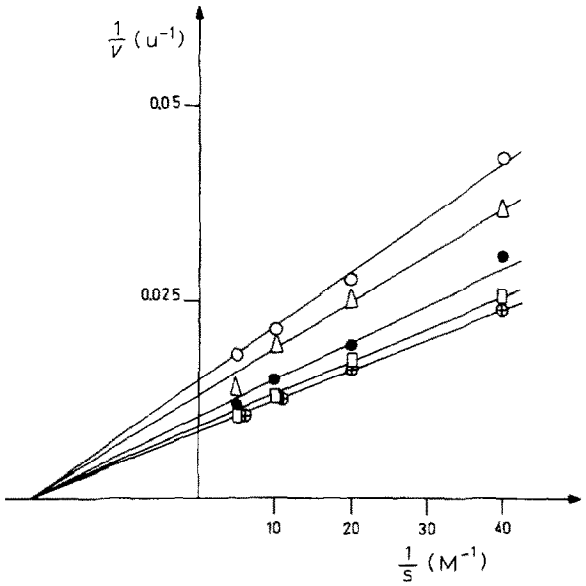

Fig. 2. D-Glucose inhibition. Effect of sucrose concentration on invertase activity. The invertase activity was determined at 40° and pH 4.5; a 0.2% invertase solution (0.2 mL) was added to the sugar solution (20 mL) with various initial D-glucose concentrations. None (—⊕—), 0.05 (—□—), 0.1 (—●—), 0.2 (—△—), and 0.4M (—○—).

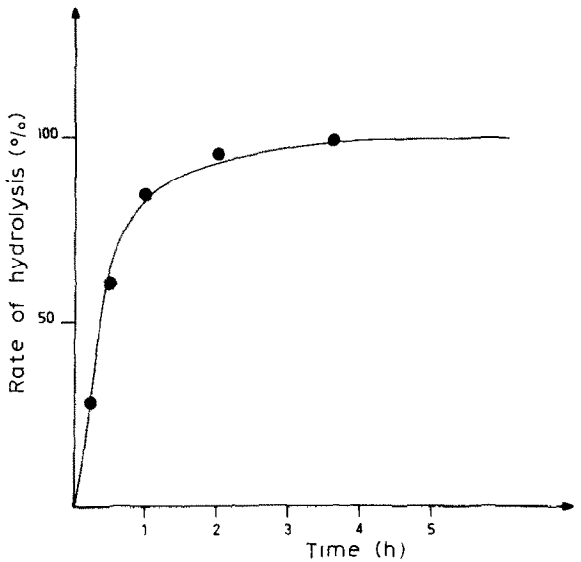

Fig. 3. Effect of time on the rate of hydrolysis of a 0.2M sucrose solution. The hydrolysis was determined at 40° and pH 4.5; a 0.2% invertase solution (0.2 mL) was added to the sucrose solution (20 mL). Experimental data (●); and calculated plot (——) from values given by Eq. 5.

where G is the initial concentration of D-glucose, and K_G the inhibition constant of D-glucose. However, the values of V'_{max} are consistent with the characteristic relation *4* of a partial noncompetitive inhibition[10].

$$V'_{max} = V_{max} \frac{[1 + \beta/K_G\,(G)]}{[1 + (G)/K_F]} \qquad (4)$$

where β is a constant. The following values were calculated from the experimental data: $K_G = 0.27$M, and $\beta = 0.23$.

In the study of the kinetics of batch hydrolysis by invertase of diluted sucrose solutions, a 0.2M sucrose solution was used (Fig. 3). Under such experimental conditions, the inhibitory effect of sucrose may be neglected. The derivative at each point of the experimental curve is therefore the hydrolysis rate. We have attempted to describe the experimental results with a kinetic model that combines the competitive inhibitory effect of D-fructose and the partial noncompetitive effect of D-glucose. The combination of Eqs. *2*, *3*, and *4* led to Eq. *5*.

$$V = \frac{V_{max}\,[1 + \beta/K_G\,(G)]}{[1 + (G)/K_G]} \cdot \frac{(S)}{K_m\,[1 + (F)/K_F] + (S)} \qquad (5)$$

For testing the validity of this model the experimental values (V_{max} 127 U and K_m 49mM) were assigned to the parameters V_{max} and K_m. The values of the others parameters (K_F, K_G, and β) corresponding to the optimal fitting with experimental data were determined by computer analysis: $K_F = 0.23$M, $K_G = 0.41$M, and $\beta = 0.3$. The calculated curve obtained with these parameter values is given in Fig. 3, showing an excellent correlation with experimental data.

Substrate inhibition. — In the study of the effect of viscosity, the addition of a 1.72% solution of carboxymethylcellulose to a 0.4M sucrose solution did not significantly modify the invertase activity at various pH values (Table III). At 53°, this CMC concentration gave a viscosity equal to that of a 2.67M sucrose solution at the same temperature. However, at such a high sucrose concentration, invertase activ-

TABLE III

EFFECT OF pH AND CARBOXYMETHYLCELLULOSE ON INVERTASE ACTIVITY[a]

Sucrose concentration (M)	*Carboxymethyl-cellulose concentration* (%)	*pH* 4.0	4.5	5.0	5.5
0.4	0	130	170	170	155
0.4	1.72	145	190	165	165
2.67	0	65	55	45	50

[a]The invertase activity is expressed as initial rate of reaction (*U*)

TABLE IV

EFFECT OF POLYACRYLAMIDE CONCENTRATION ON INVERTASE ACTIVITY

Polyacrylamide concentration (%)	*Viscosity (mPa · s)*	*Initial reaction rate (U)*
0	1	165
0.25	3.4	160
0.75	18	190
1.0	34	170
1.5	106	160

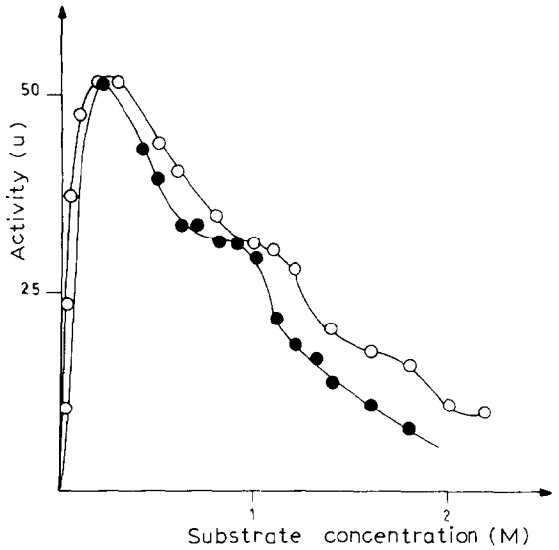

Fig. 4. Effect of magnesium chloride on invertase activity. The effect of sucrose concentration on invertase activity was determined at 25° and pH 4.5; a 0.2% invertase solution (0.2 mL) was added to the assay mixture (20 mL) with (—●—) or without (—○—) 0.1M magnesium chloride.

ity is greatly decreased (Table III). Thus, this decrease may not be attributed to the effect of the viscosity of the medium. The same conclusion may be drawn from the study of the effect of polyacrylamide concentration, at pH 4.5, on invertase activity (Table IV); a variation in viscosity from 1 to 106 mPa · s did not cause a significant change in invertase activity.

In the study of the effect of water activity, the addition of 0.1M magnesium chloride to the reaction medium increased the inhibition of invertase for sucrose concentrations above 0.4M (Fig. 4), and modified the position of the plateaus that are obtained without the addition of magnesium chloride. The addition of 1% of poly(1,2-ethanediol) to the reaction mixture modified the position of the plateaus in a way similar to that obtained by the addition of magnesium chloride (Fig. 5).

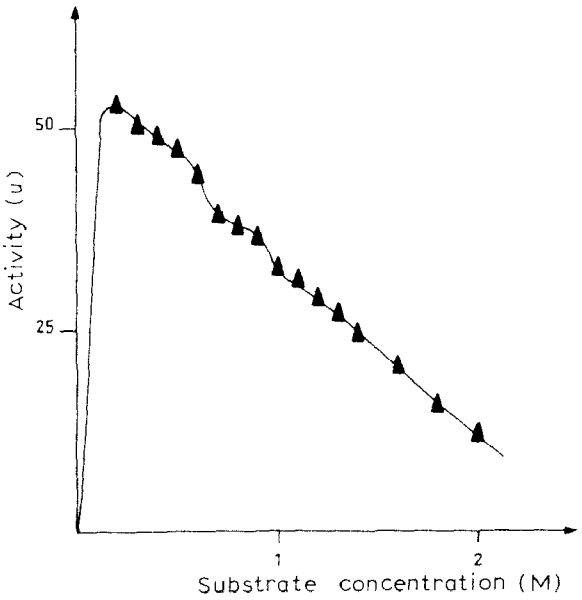

Fig. 5. Effect of poly(1,2-ethanediol) on invertase activity The effect of sucrose concentration on invertase activity was determined at 25° and pH 4.5; a 0.2% invertase solution (0 2 mL) was added to the assay mixture (20 mL) with 1% of poly(1.2-ethanediol)

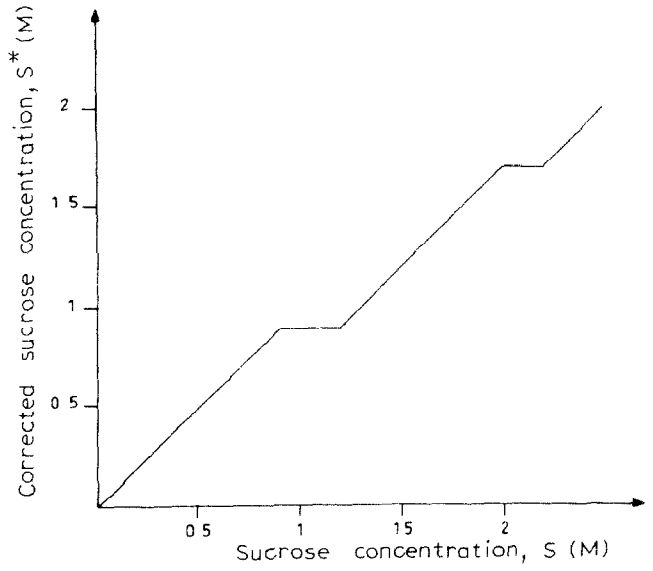

Fig. 6. Effect of initial sucrose concentration (S) on the corrected sucrose concentration value (S*). (S*) values were calculated from experimental data given in Fig. 4 (invertase activity without addition of magnesium chloride).

The results obtained with either magnesium chloride or poly(1,2-ethanediol) emphasize the direct connection between water and invertase activity for concentrated sucrose solutions. It has been suggested that the presence of plateau irregularities in the kinetic curves obtained with high sucrose concentrations may be due to a two-step folding of the sucrose molecule[11]. This folding results from the formation of one or two intramolecular hydrogen bonds as a consequence of a decrease in water activity[8]. If it is assumed that the folded structures of sucrose are not susceptible to invertase hydrolysis, it is possible to introduce a modified sucrose concentration (S*). This (S*) parameter takes into account the observation that, at the position of the plateau, an increase in sucrose concentration does not result in a modification of invertase activity (Fig. 4). The relation between (S*) and (S) is given in Fig. 6. Furthermore, when the concentration of sucrose was increased, intermolecular hydrogen bonds appeared[12], which resulted in substrate aggregation. In order to describe this phenomenon, the simplified scheme including Eqs. *6–10* may be proposed,

$$E + S \leftrightharpoons ES \rightarrow E + P \tag{6}$$

$$ES + S \leftrightharpoons ESS \tag{7}$$

$$ES + S\text{–}S \leftrightharpoons ESSS \tag{8}$$

$$K_S = \frac{(ES) \cdot (S)}{(ESS)} \tag{9}$$

$$K'_S = \frac{(ES) \cdot (S - S)}{(ESSS)} . \tag{10}$$

where S–S is the sucrose dimer resulting from aggregation, and ESS and ESSS are inactive forms. The combination of the formation of both intra- and inter-molecular hydrogen bonds leads to Eq. *11*.

$$V = \frac{V_{max}\,(S^*)}{K_m + (S^*) + (S^*)^2/K_S + (S^*)^3/K'_S} \tag{11}$$

This equation has been tested for the mathematical description of the experimental data given in Fig. 4 (results obtained without magnesium chloride addition). For this purpose, values obtained from initial reaction rates corresponding to concentrations of sucrose lower than 0.4M were assigned to V_{max} and K_m values, V_{max} 70 U, and K_m 42mM. A computing analysis gave, for the other parameters, K_S = 1.56M and K'_S = 1.33M. The calculated curve obtained with these parameter values is given in Fig. 7, and compared to the experimental data.

The batch hydrolysis of a 0.8M sucrose solution by invertase was investigated

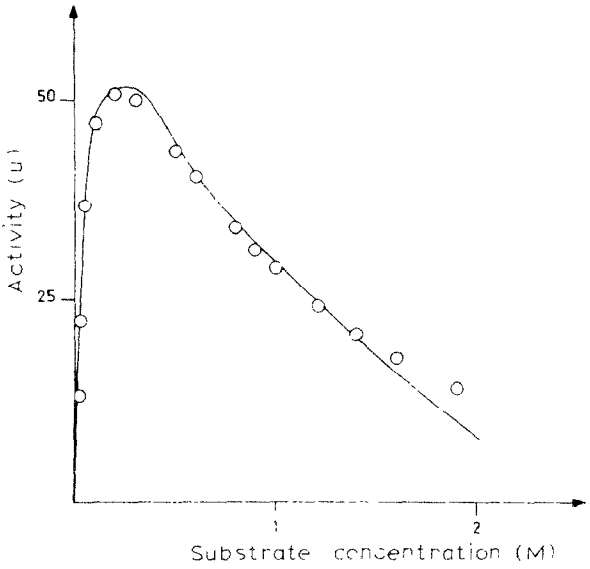

Fig 7. Effect of corrected sucrose concentration (S^*) on invertase activity. The invertase activity was determined at 25° and pH 4.5, a 0.2% invertase solution (0.2 mL) was added to the sucrose solution (20 mL). Experimental data (○), and calculated plot (——) from values given by Eq *11*

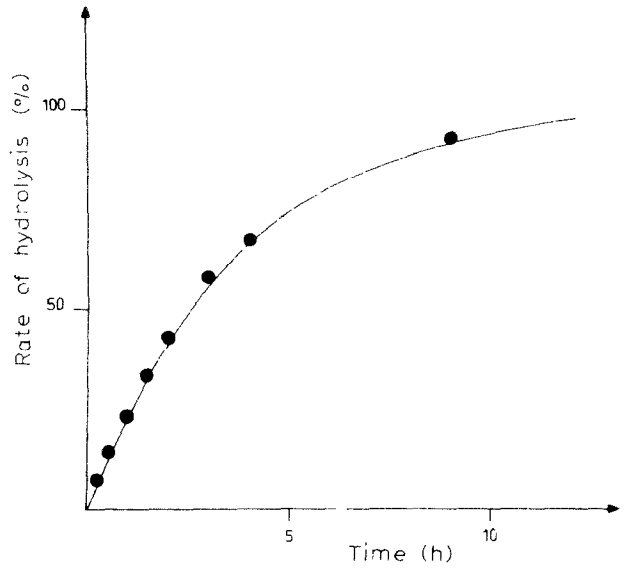

Fig 8. Effect of time on the rate of hydrolysis of a 0.8M sucrose solution. The hydrolysis was determined at 40° and pH 4.5, a 0.2% invertase solution (0.2 mL) was added to the sucrose solution (20 mL). Experimental data (●), and calculated plot (——) from values given by Eq *12*

(Fig. 8). Under such conditions, all the inhibition phenomena had to be taken into consideration: inhibition by D-fructose, D-glucose, and sucrose. In an attempt to describe the experimental results, Eqs. *5* and *11* were combined to give Eq. *12*.

$$V = \frac{V_{max}\,[1 + \beta/K_G\,(G)]}{[1 + (G)/K_G]} \cdot \frac{(S)}{K_m\,[1 + (F)/K_F] + (S) + (S)^2/K_S + (S)^3/K'_S} \quad (12)$$

For computer analysis, the following values were assigned: K_m = 49mM, K_G = 270mM, K_F = 128mM, and β = 0.23. For the other parameters, the optimal fitting was obtained with the following values: V_{max} = 177 U, K_S = 2.0M, and K'_S = 1.5M.

DISCUSSION

The aim of this work was to characterize the inhibition phenomena that affect invertase when concentrated sucrose solutions are hydrolyzed to produce liquid sugar solutions. Under such conditions, invertase is simultaneously subjected to inhibition by D-fructose, D-glucose, and sucrose. The absence of information on the inhibition by the products of invertase[4,13] required a characterization of both D-fructose and D-glucose effects. D-Fructose shows the behavior of a competitive inhibitor (Fig. 1), which is consistent with the mechanism proposed for invertase action involving a covalent D-fructosyl–enzyme intermediate[14]. D-Glucose was shown to be a partial noncompetitive inhibitor (Fig. 2).

The combination of the competitive inhibitory effect of D-fructose and the partial noncompetitive inhibitory effect of D-glucose gave Eq. *5*, which was tested to mathematically describe the batch hydrolysis of a dilute (0.2M) solution of sucrose. Under such conditions, inhibition by sucrose may be neglected, and invertase was only subjected to product inhibition. From the variation of the rate of sucrose conversion as a function of time (Fig. 3), the rate of hydrolysis was deduced. The rate of production of D-glucose and D-fructose is the time derivative at each point of this curve, and the rate of hydrolysis of sucrose was calculated from the values thus obtained. The value of two parameters of Eq. *5* (V_{max} and K_m) was assigned to their previously determined experimental value. A computer analysis was used to calculate the best fitting value of the three other parameters (K_F, K_G, and β). The values obtained are in good agreement with the values previously determined for these parameters from the separate study of inhibition by D-fructose (Fig. 1), and inhibition by D-glucose (Fig. 2). Eq. *5* was used to calculate again the value of the reaction rate value as a function of sucrose concentration and, from this, the variation of sucrose conversion rate as a function of time. The calculated curve that resulted is in excellent agreement with experimental data, as shown in Fig. 3. Thus, the combination of both inhibitory effects of D-fructose and D-glucose allows a very satisfactory description of the kinetics of batch hydrolysis of a dilute solution of sucrose.

When the initial reaction rates of sucrose hydrolysis by invertase are considered, the classical model of substrate inhibition does not allow the description of the decrease in efficiency of invertase, observed when the concentration of sucrose is increased[6,7,11]. Various explanations of this difference have been suggested. MacLaren[5] took into consideration the effect of medium viscosity in the expression of the reaction rate given by the Michaelis–Menten equation by adding a correction factor related to the ratio of water to sucrose solution viscosity. MacLaren's equation allows a satisfactory description of the experimental data for low concentrations of sucrose, but does not fit well for high concentrations of sucrose. A similar equation was tested by Bowski *et al.* [6] who concluded that the ratio of water to sucrose solution viscosity is not adequate to describe the decrease of invertase activity. Furthermore, Kertesz[15] has shown, in 1935, that the viscosity increase resulting from the addition of citrus pectins to reaction mixtures containing invertase had no noticeable effect on the activity of the enzyme. For these reasons, we determined the influence of two viscosity-producing reagents on the activity of invertase. Firstly, the viscosity of a concentrated (2.67M) solution of sucrose was reproduced by adding carboxymethylcellulose to a dilute (0.4M) solution of sucrose. The results (see Table III) show that the decrease in invertase efficiency observed when increasing the concentration of sucrose from 0.4 to 2.67M may not be attributed to a pure effect of increase in viscosity. The effect of pH change on invertase activity was greatly diminished (Table III) for a concentrated (2.67M) solution of sucrose. Furthermore, increasing the medium viscosity from 1 to 106 mPa · s by addition of polyacrylamide to a 0.4M solution of sucrose had no significant effect on invertase activity (Table IV). These experiments clearly demonstrate that the decrease in invertase efficiency observed for increasing concentration of sucrose cannot be attributed to a pure effect of viscosity.

Another approach for explaining the kinetic behavior of invertase in concentrated solutions of sucrose was to consider the effect of water concentration alone, or in combination with inhibition by an excess of substrate[6]. For this purpose, the classical equation for inhibition by an excess of substrate was corrected by the ratio of free to total water concentration. However, the resulting equation was shown to be inadequate for describing the experimental results reported by Besserdich *et al.*[7]. Although data are available for the hydration of D-glucose, D-fructose, and sucrose, we did not select water activity as a parameter, because these data vary as widely as 7 molecules of water per molecule of sucrose according to Einstein[16], but between 2.4 and 10.4 molecules depending on the method of determination, according to Allen *et al.*[17]. From data on the activity of water for sucrose solutions at 25°, Akhumov[18] determined the number of molecules of hydration of the sucrose molecule; at a sucrose concentration < 1.8M, the number was constant and equal to 5, and at concentrations > 1.8M, this value decreased with the increase in sucrose concentration to reach 4.8 for a 2.5M concentration. Furthermore, a detailed kinetic study of the effect of sucrose concentration on the initial hydrolysis-rate showed, in the decreasing part of the curve, the presence of two flat levels[11].

The positions of these flat levels, which begin at 1 and 1.8M at 25°, respectively, correspond to the positions of the discontinuities observed in Laser–Raman[12] studies of solute–solvent interactions in aqueous solutions of sucrose. Thus, the decrease in invertase efficiency resulting from the decrease in sucrose concentration may result from a modification of the distribution of intra- and inter-molecular hydrogen bonds, and follows the concomitant decrease in water activity of the reaction mixture[11]. To confirm this hypothesis, we determined the effect of compounds, such as magnesium chloride and poly(1,2-ethanediol) that are known to modify the activity of water as a consequence of their water affinity, and which have no effect on invertase activity. The addition of 0.1M magnesium chloride gave a significant decrease in invertase activity for sucrose concentrations > 0.4M (Fig. 4). Furthermore, the position of the plateau in the decreasing part of the curve was shifted towards lower sucrose concentrations. As magnesium ions have a very high affinity for water molecules[19], this result may be interpreted by a decrease of the water activity by magnesium ions, and, as a consequence, a modification of the distribution of intra- and inter-molecular hydrogen bonds within sucrose molecules at lower sucrose concentrations. A similar effect was observed when poly(1,2-ethanediol) was added to the reaction mixture (Fig. 5). On the other hand, an increase in the reaction temperature, which involves an increase in sucrose solubility, shifted the position of the plateaus towards higher concentrations of sucrose[20]. Thus, the decrease in invertase activity observed for sucrose concentrations > 0.4M seems to depend on inhibition by excess substrate and on modification of water–sucrose and sucrose–sucrose interactions, which result in the folding of the sucrose molecule by formation of one or two intramolecular hydrogen bonds to give sucrose clusters. The recent discussion of the intramolecular hydrogen-bonding of sucrose by Bock and Lemieux[21] should also be mentioned. These structural modifications were considered in an attempt to achieve a modeling of the invertase kinetic data. At first, it was assumed that the folded molecules of sucrose may not be susceptible to invertase hydrolysis and a modified sucrose concentration (S*) was introduced. This eliminated the portions corresponding to the flat levels in the processing of the experimental data. Secondly, the sucrose clusters resulting from sucrose aggregation were assumed to combine with invertase to give inactive complexes[6], as in the case of inhibition by excess substrate[7]. For the modeling, only clusters resulting from the aggregation of two molecules of sucrose were taken into account, but, obviously, it is possible to consider higher-degree clusters. This would increase the corresponding polynomial degree of the denominator of Eq. *11* and, thus, increase the precision of the fitting. As shown in Fig. 7 the resulting Eq. *11* allows an excellent mathematical description of the curve $V = f(S^*)$.

Finally, a description of the kinetic data from the total batch hydrolysis of a concentrated (0.8M) sucrose solution was attempted. Under such conditions, inhibition by both products and substrate is to be considered, and the equations developed to describe the inhibition by D-fructose, D-glucose and sucrose were combined, Eqs. *5* and *11* giving Eq. *12*. As the initial concentration (0.8M) of sucrose

is lower than that where the structure of sucrose is modified, (S^*) was, in this case, equal to (S). As shown in Fig. 8 Eq. *12* gives a satisfactory description of the experimental data, the calculated curve of the variation of sucrose conversion as a function of time being in excellent agreement with the experimental data. In conclusion, the mathematical description of the hydrolysis by invertase of concentrated solutions of sucrose requires simultaneous consideration of the inhibition by D-fructose, D-glucose, and sucrose. The decrease in invertase efficiency observed with high concentration of sucrose seems to be directly related to the modification of the structure of the molecule of sucrose under such conditions, which result from a decrease in water activity

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