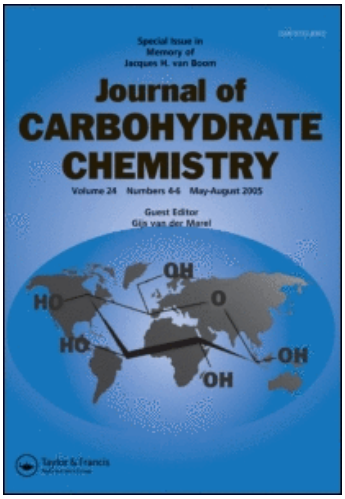

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Enzymic Formation of β-D-Fructofuranosides from Sucrose: Activity and Selectivity of Inventase in Mixtures of Water and Alcohol

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ENZYMIC FORMATION OF β-D-FRUCTOFURANOSIDES FROM SUCROSE: ACTIVITY AND SELECTIVITY OF INVERTASE IN MIXTURES OF WATER AND ALCOHOL

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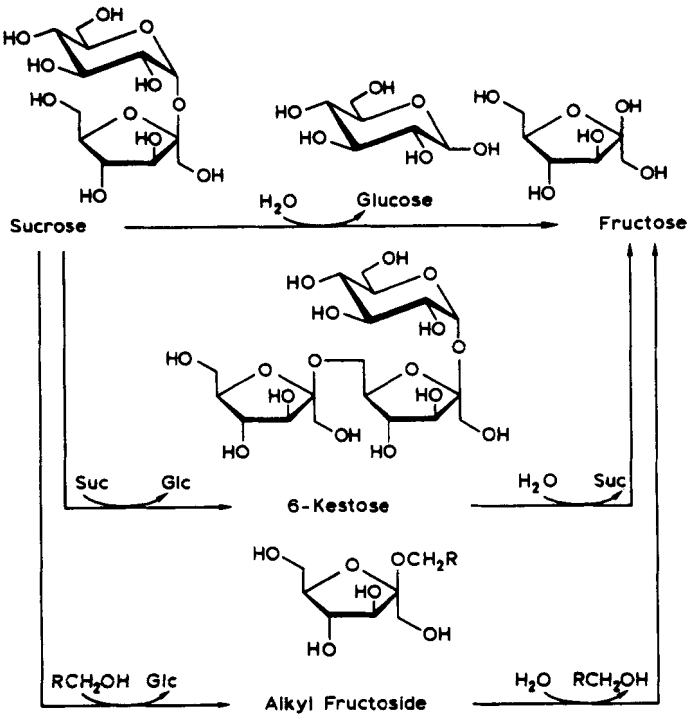
ABSTRACT

Invertase-catalysed alcoholysis of sucrose in water - primary alcohol mixtures containing up to 70% organic solvent shows 5-40% alkyl β-D-fructofuranoside formation. No formation of fructosides was observed under anhydrous conditions. The kinetics of the competing reactions of water and primary alcohol as fructosyl acceptors with sucrose as fructosyl donor were studied in order to establish the scope and limitations of the method. On a molar basis, water was a relatively unreactive acceptor. 6-Kestose formation due to sucrose itself as a fructosyl acceptor was suppressed by the presence of aliphatic alcohols. The results have been explained by assuming an nonspecific non-polar binding site to be present in the enzyme cavity near the specific polar binding site for the β-D-fructofuranosyl group of the substrate.

INTRODUCTION

The utilisation of protecting and activating groups allows the selective formation of numerous types of glycosidic bonds. The reaction of unprotected carbohydrates with alcohols, however, in the presence of glycosyl transferases (EC 2.4) or glycoside



SCHEME 1. Invertase-catalysed reactions in solutions of sucrose in aqueous primary alcohol.

hydrolases (EC 3.2) is much more straightforward. These two classes of enzymes have been called collectively glycosylases by Hehre et al.,[1] on account of the similar reaction mechanism of alcoholysis and hydrolysis, and the occurrence of many enzymes showing the characteristics of both classes. For instance, invertase (β-D-fructofuranosidase, EC 3.2.1.26) from yeast (Saccharomyces cerevisiae), which catalyses the hydrolysis of β-D-fructofuranosides, is also capable of catalysing alcoholysis with primary alcohols.[2,3] Thus, by converting sucrose in water - alcohol mixtures in the presence of invertase, hydrolysis and alcoholysis are parallel reactions and several alkyl β-D-fructofuranosides (up to octyl) have been prepared.[4-6] Since fructosyl transfer also occurs to the primary alcohol groups of

sucrose, D-fructose, and D-glucose, small amounts of oligosaccharides may be formed in addition.[2] The trisaccharide 6-kestose, which is formed by fructosyl transfer to HO-6^f of sucrose, is the main transfructosylation product in concentrated solutions of sucrose.[7] In the presence of water the fructosides formed will be subject to hydrolysis. The invertase-catalysed reactions of sucrose in mixtures of water and primary alcohol are summarised in Scheme 1.

Optimisation of the preparation of fructosides from sucrose requires knowledge of the effect of high concentrations of alcohols on the selectivity of the reaction, i.e. the rates of hydrolysis and alcoholysis. As an extension of our study[7] on the kinetics of invertase-catalysed reactions in concentrated solutions of sucrose, we have investigated the scope and limitations of the use of invertase for the preparation of alkyl β-D-fructofuranosides in mixtures of water and organic solvents (including reactive primary alcohols and non-reactive solvents). Also some experiments have been performed in anhydrous solvents.

RESULTS AND DISCUSSION

Invertase Activity in Anhydrous Solvents

The activity of invertase in the absence of water was tested using both lyophilised and phosphorous pentoxide-dried invertase at 25 and 45°C (ref. 8, cf. ref. 9-10). No conversion of sucrose was detected in suspensions in anhydrous 1-butanol, 1-octanol, or mixtures thereof as the medium. The lack of conversion of sucrose might partly be due to the low solubility of sucrose in these alcohols (e.g. the solubility of sucrose in butanol at 80°C is 0.12 g/100 g[11]). Upon addition of 10 volumes of water the formulations in 1-octanol and 1-butanol showed complete conversion of sucrose. Thus, no complete irreversible inactivation of invertase occurs in 1-octanol or 1-butanol. In anhydrous pyridine, which is a much better solvent for sucrose, no sucrose conversion

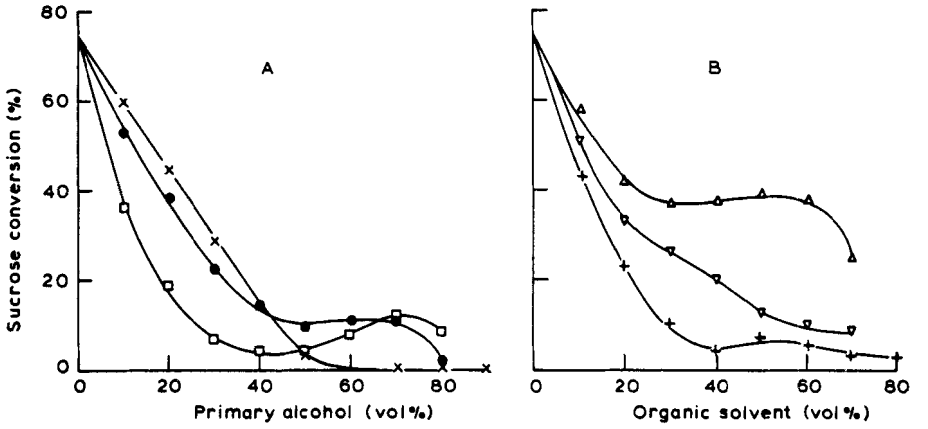
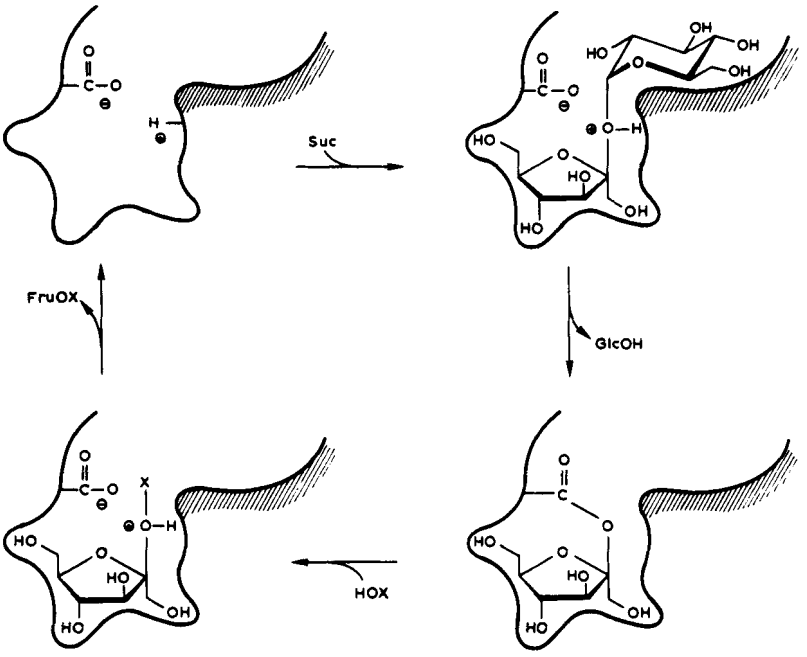


FIG. 1. Sucrose conversion by invertase after 5 min reaction (0.1 M sucrose, 100 mg/L invertase, pH 4.8, 25°C). A. In mixtures of water and methanol (x), ethanol (●), or allyl alcohol (□). B. In mixtures of water and tert-butanol (Δ), acetone (▽), or 1,4-dioxane (+).

was detected in the presence of 5% 1-butanol. Upon addition of 10 volumes of water to the pyridine suspension sucrose was hydrolysed at a very low rate. The basic pyridine will affect the state of protonation of the active site of invertase, thus decreasing its activity. A sharp decrease in invertase activity was also observed upon addition of 10% pyridine to aqueous sucrose.

Invertase Activity in Water - Organic Solvent Mixtures

Since no formation of fructosides was observed under essentially anhydrous conditions, we have studied the activity of invertase in mixtures of water and organic solvents up to compositions allowing complete dissolution of 0.1 M sucrose at 25°C. In the case of reactive primary alcohols (Fig. 1A) the conversion rate decreases with increasing concentration of alcohol, with a relative maximum at high concentrations of ethanol or allyl alcohol. A related behaviour was observed for the non-reactive cosolvents dioxane and tert-butanol (Fig. 1B). Thus, the maxima do not originate from alcoholysis occurring at high concentration of primary alcohol and



SCHEME 2. Model of the active site of invertase during hydrolysis (HOX = water) or alcoholysis (HOX = primary alcohol) of sucrose. The non-polar part of the aglycon site is shaded.

indicate the complex nature of the effect of organic solvents on the activity of invertase. Up to 70% v/v organic solvent (50% for methanol), invertase activity is still sufficient for preparative purposes.

Active Site and Mode of Action of Invertase

Invertase is a glycoprotein with a polysaccharide content exceeding 50%. The primary structures of the polysaccharide branches[12] and the protein backbone[13] have been elucidated, but the structure of the active site of invertase is not known. Some important information has been obtained from hydrolysis studies (cf. Scheme 2). No β-D-fructofuranoside is known that is not hydrolysed by invertase. Upon minor modification of the β-D-fructofuranosyl moiety, however, hydrolysis has never been

observed.[14-17] Thus, invertase has a very specific fructosyl binding site and a rather unspecific aglycon binding site. The aglycon site seems to have a somewhat non-polar character, since we observed that butyl β-D-fructofuranoside (K_m = 9.4 mM) showed a higher affinity for invertase than sucrose (K_m = 38 mM).[18] The non-polar character of the aglycon site is in accordance with the weak binding of raffinose (K_m = 240 mM)[19] and the very weak inhibition by D-glucose (partial non-competitive inhibition, K_I = 410 mM)[18].

The catalytic site of invertase is assumed to contain an imidazolium and a carboxylate group.[21] Upon protonation of the glycosidic oxygen atom of sucrose by the imidazolium group, cleavage of the bond between this atom and fructosyl C-2 occurs. On the analogy of the mechanistically related enzyme levansucrase,[20] an ester bond between C-2 and the carboxylate group of the enzyme will be formed. Formation of a β-D-fructofuranosyl ester is most probable because inversion at fructosyl C-2 is sterically unfavourable.[23] α-D-Glucopyranose is liberated from the active site and the cleavage of the ester bond by water or a primary alcohol yields β-D-fructofuranose[24] or alkyl β-D-fructofuranoside, respectively. All reactions thus are supposed to proceed with retention of configuration (Scheme 2).

Invertase Selectivity in Water - Alcohol Mixtures

The aglycon site of invertase will show hydrophobic interactions with aliphatic alcohols, without much specificity towards the shape of the molecule. Sucrose, that has been shown to be a weak substrate inhibitor,[7,20] will probably bind in a rather nonspecific manner at the aglycon site of invertase or the invertase-fructosyl complex. Thus, hydrolysis was retarded,[20] but sucrolysis was retarded even more.[7] Non-reactive binding of sucrose close to the aglycon site (Scheme 3, I and II) at high sucrose concentration can explain these results. According to the substrate inhibition observed, the reaction I→II is slower than IV→V.

The nature of these inhibition phenomena might be established by studying the action of invertase in the presence of alcohols that form more specific complexes at the aglycon site. Thus, the relative importance of hydrolysis, alcoholysis, and sucrolysis has been studied in 1 M aqueous tert-butanol and in 1.5 M aqueous allyl alcohol (10% v/v in both cases) at variable concentration of sucrose. Use has been made of initial rates in order to eliminate any effect of product inhibition and hydrolysis of newly formed fructosides. In Fig. 2 these rates are compared to the initial rates in the absence of organic solvents.[7]

tert-Butanol only decreases the rate of hydrolysis if [Sucrose] < 0.4 M, which is due to a higher apparent K_m of sucrose in 10% tert-butanol (K_m = ~270 mM) than in water (K_m = 38 mM). This means that tert-butanol behaves as a weak competitive inhibitor (K_I = ~170 mM) because of complexation at the non-polar part of the aglycon site of invertase (Scheme 3, VII). Sucrose will form a stronger complex (IV) because of its affinity to the fructosyl site, but after liberation of glucose (IV→V) complexation of tert-butanol at the aglycon site of the enzyme-fructosyl complex will occur (V→VIII) without much competition by sucrose. This complexation hardly affects the reaction rate of the enzyme-fructosyl complex with water, according to the similar shapes of the curves of water and 10% tert-butanol at [Sucrose] > 0.4 M in Fig. 2A. Thus, the rate of VIII→IX is comparable to the rate of V→VI. According to the corresponding curves in Fig. 2B, however, the reaction rate of the enzyme-substrate complex V with the large sucrose molecule is reduced by a factor 3 in the presence of tert-butanol. Formation of VIII inhibits the formation of II with subsequent reaction to III. tert-Butanol seems to block the catalytic site for the large sucrose molecule, whereas the entrance of water to the catalytic site is not inhibited.

Allyl alcohol will bind at the aglycon site in a manner comparable to tert-butanol in complex VII, but proves to be a more efficient inhibitor. Binding of allyl alcohol to the enzyme-fructosyl complex will result in allyl fructoside formation, but

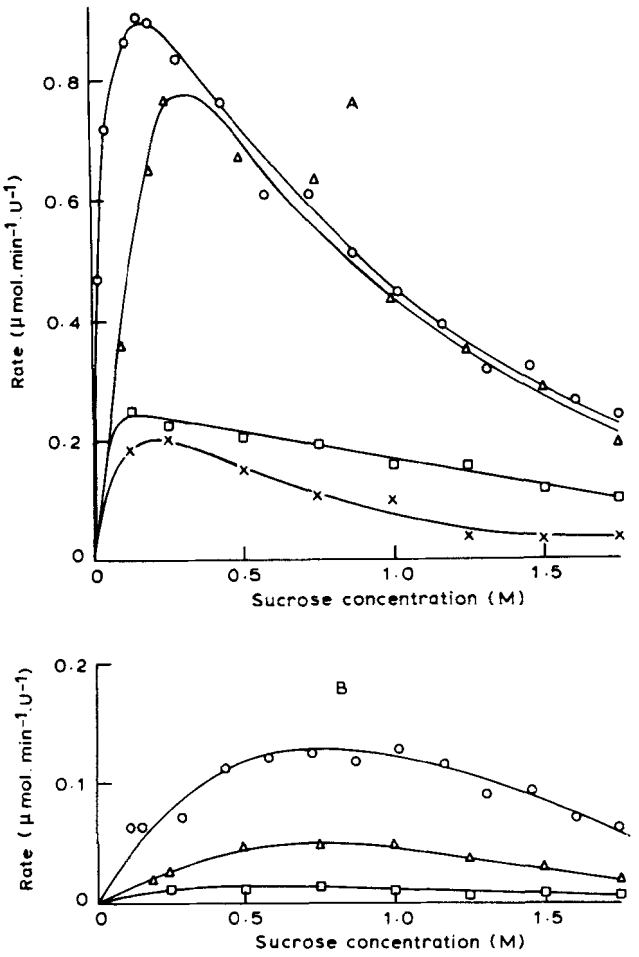
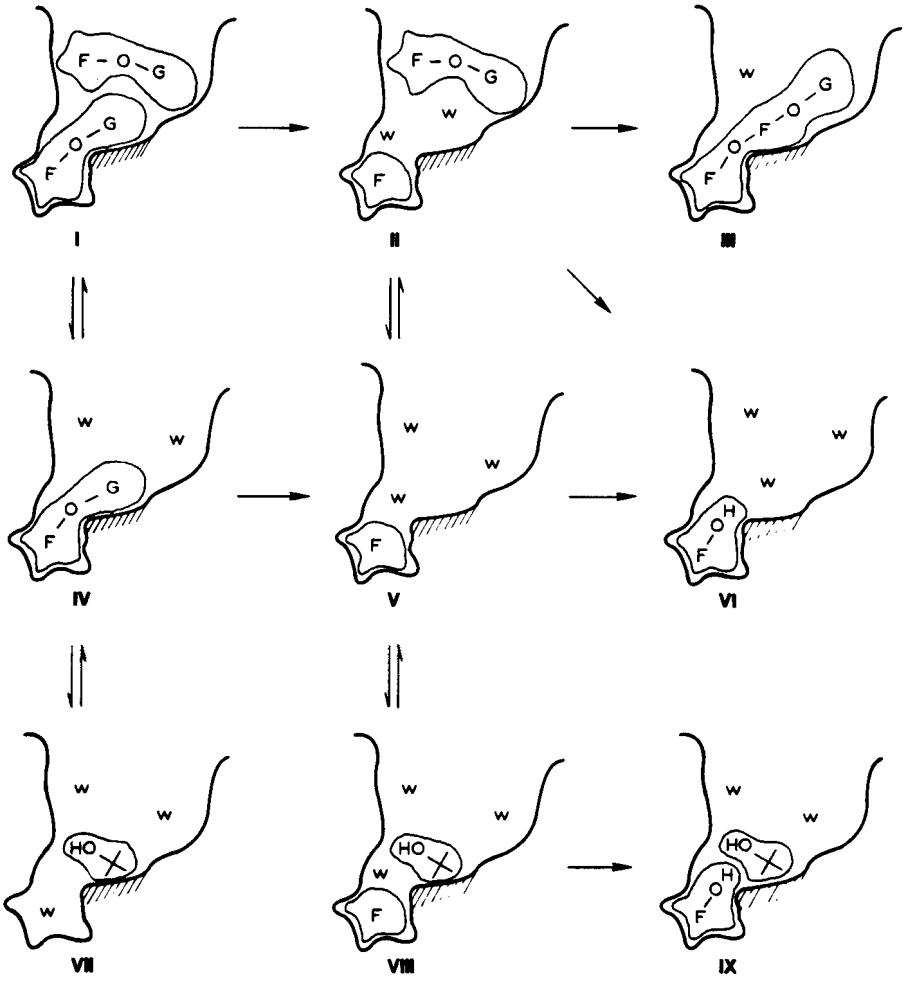


FIG. 2. Initial rates of hydrolysis and alcoholysis of sucrose (pH 4.8, 25°C). A. Hydrolysis in water (o) and in 10% tert-butanol (Δ); Hydrolysis (□) and allylolysis (x) in 10% allyl alcohol. B. Sucrolysis in water (o), 10% tert-butanol (Δ), and in 10% allyl alcohol (□).

additional inhibition may occur by reverse orientation of the allyl alcohol molecule at the non-polar site (i.e. its hydroxyl group pointing away from the fructosyl group).

The inhibiting effect of tert-butanol and allyl alcohol on the sucrolysis as determined by these initial rate studies, is reflected by the maximum amount of 6-kestose observed in the course of the reaction (Fig. 3).



SCHEME 3. Effect of sucrose (G-O-F), tert-butanol (+OH), and water (w) on the formation of fructose (F-OH) and 6-kestose (F-O-F-O-G) in the active site of invertase. The non-polar part of the aglycon site is shaded.

A comparison of the effect of various alcohols on the different reaction rates can be made after correction of the ratio of the initial rate of alcoholysis (r_a) to hydrolysis (r_h) for the molar ratio of water to alcohol ([W]/[ROH]). The molar selectivity towards alcoholysis ($S_{ROH/W}$) thus defined is a measure of the

$$S_{ROH/W} = \frac{r_a}{r_h} \cdot \frac{[W]}{[ROH]} \qquad (1)$$

reactivity of sucrose towards primary alcohols relative to water, in the presence of invertase (equation 1).

Fig. 4 shows that $S_{ROH/W}$ has not a fixed value for either sucrose or allyl alcohol, but it is clear that these alcohols are better fructosyl acceptors than water ($S_{ROH/W}>1$). Although additional effects, like variation in water, sucrose, and alcohol activities[25] and differences in solvent composition in the microdomain of invertase relative to the bulk solvent[26-27] will be of importance as well, the decrease in $S_{ROH/W}$ observed upon addition of sucrose, allyl alcohol, or tert-butanol is assumed to be largely the result of interactions in the enzyme cavity according to the picture given above.

With increasing concentration of aliphatic alcohol, saturation of the aglycon site will occur, and the ratio of reaction rates of alcoholysis and hydrolysis will show only a small increase. The selectivity, which is calculated after correction of the concentrations, will decrease. Thus, the selectivity of ethanolysis $S_{EtOH/W}$ in 0.44 M sucrose in the presence of 10, 30, 50, and 70% aqueous ethanol was 7, 3, 2, and 0.5, respectively. As

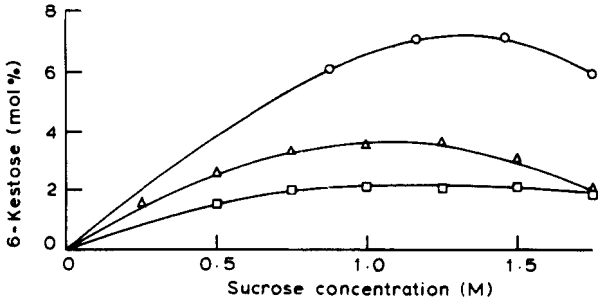


FIG. 3. Maximum concentration of 6-kestose relative to the corresponding initial concentration of sucrose in the absence of organic solvent (o), in 10% tert-butanol (Δ), and in 10% allyl alcohol (□) (pH 4.8, 25°C).

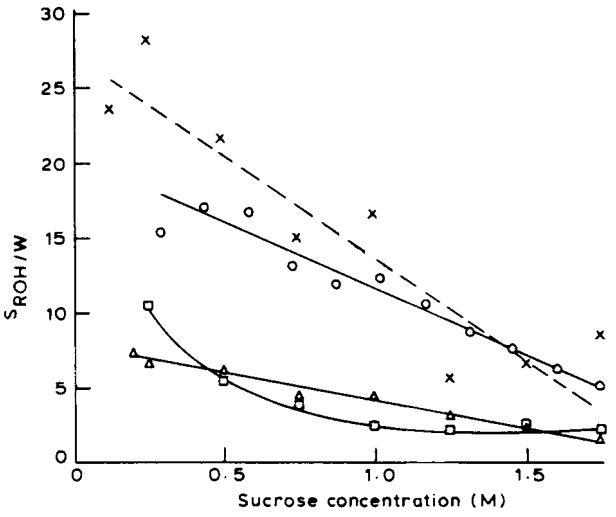


FIG. 4. Selectivity towards sucrolysis in the absence of organic solvent (o), in 10% tert-butanol (Δ), and in 10% allyl alcohol (□); selectivity towards allylolysis in 10% allyl alcohol (x).

a consequence, the maximum amount of ethyl fructoside formed was 9, 9, 13, and 10%, respectively. Thus, the decreasing relative molar selectivity towards alcoholysis opposes the more favourable molar ratio of alcohol to water.

This result applies to all alcoholysis reactions studied by us. Optimal formation of fructosides occurs therefore at relatively low concentration of sucrose (Fig. 4) together with intermediate concentrations of alcohol (40-75% v/v, Fig. 5). In general, the maximal amount of alkyl β-D-fructofuranoside observed decreases with increasing size of the fructosyl acceptor.

The present work and interpretation may be of relevance for the enzymic preparation of α-[28] and β-[28-29]galactosides, α-[30] and β-[29-31]glucosides, α-mannosides,[28] and α-maltosides[32] by alcoholysis of glycosides, which have also been performed at a remarkably low concentration of alcohol (~10-60% v/v). This low alcohol content will probably have favoured the solubility of the substrates and the stability of the enzymes applied. Initial reaction rates of alcoholysis and hydrolysis, which might reveal

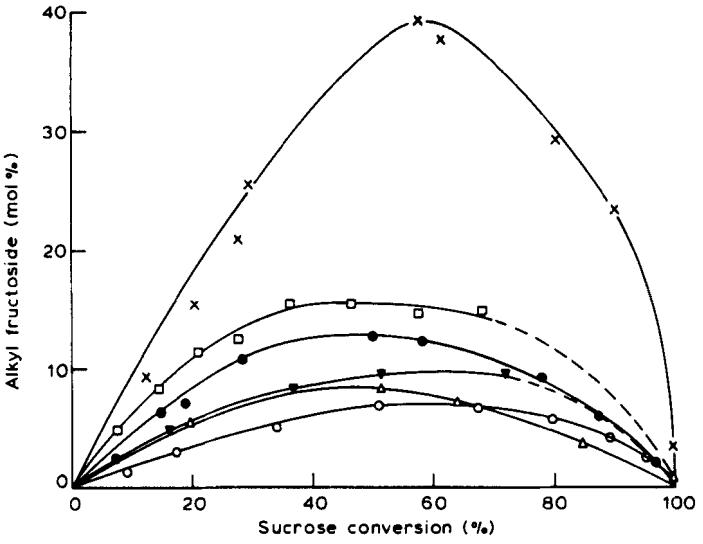


FIG. 5. Course of the alkyl β-D-fructofuranoside concentration (relative to the initial sucrose concentration) at the optimum conditions observed for fructoside formation in the presence of invertase at 25°C, pH 4.8: x, methyl fructoside (40% v/v MeOH, 150 g/L sucrose); ●, ethyl fructoside (50% v/v EtOH, 150 g/L sucrose); ▼, propyl fructoside (55% v/v PrOH, 150 g/L sucrose); Δ, butyl fructoside (50% v/v BuOH, 100 g/L sucrose, heterogeneous); □, allyl fructoside (75% v/v AllOH, 100 g/L sucrose); o, 6-kestose (500 g/L sucrose).

some general negative effect of high alcohol concentration on the selectivity of glycosylases towards alcoholysis, were, however, not reported.

The enzymic formation of alkyl fructofuranosides (this work) appears to be less favourable than that of alkyl aldopyranosides.[28-31] This is due to both kinetic and thermodynamic reasons. The steric hindrance for alcohols (relative to water) to react with an enzyme-fructofuranosyl complex will be much larger than with e.g. an enzyme-glucopyranosyl complex, because of the bulky fructosyl 1-CH_2OH group. Therefore, secondary and tertiary alcohols do not react with the fructosyl-invertase complex, whereas their enzymic conversion to alkyl aldopyranosides is well-known.[28-31] Furthermore, it must be noted that differences

in hydrophobic nature and in bulkiness of the alcohols might strongly influence their ability to bind at the aglycon site and, consequently, their relative apparent reactivity. In addition, fructofuranosides are in aqueous solution thermodynamically less stable than glucopyranosides.[33] Thus, in concentrated aqueous solutions of D-glucose, enzymic formation of disaccharides by reversion reactions occurs,[34] but equilibration of concentrated aqueous solutions of D-fructose (or invert sugar) in the presence of invertase does not show any disaccharide formation.

EXPERIMENTAL

Reaction Procedure. Sample preparation was essentially the same as reported before.[7] A buffer solution (5 mL, 0.08 M sodium acetate, pH 4.8) of invertase (Maxinvert powder, 240 U/mg,[7] Gist-brocades, Delft) was added to a solution (35 mL) of sucrose in aqueous alcohol at 25°C, yielding a reaction mixture of the correct composition. Samples were added to aqueous silver nitrate. Ethylene glycol or D-glucitol was added as internal standard for HPLC and the solvent was evaporated as far as required. Hydrolysis of butyl β-D-fructofuranoside was performed on a 4 mL scale starting with 20, 50, and 100 mM aqueous solutions of this compound. For initial rate determinations five samples at <10% conversion were analysed.

HPLC Analysis. HPLC of alkyl fructosides was performed using a Waters Ass. M45-pump, a cartridge packed with 3-aminopropyl-triethoxysilane-modified silica contained in a Waters Ass. RCM 100 module, and a Waters Ass. R401 differential refractometer. The flow of acetonitrile–water (85:15) was 1 mL/min at 25°C. Retention times (min): Alkyl β-D-fructofuranosides: butyl 5.12, propyl 5.90, ethyl 6.36, methyl 7.98; D-fructose 10.6, D-glucose 12.5, sucrose 22.1. More reproducible results were obtained using ion-moderated partitioning chromatography. An Aminex HPX 87C column at 60°C was eluted with water at 0.6 mL/min.[7,35] The alkyl β-D-

fructofuranosides, however, were not base-line separated from glucose or fructose. Allyl β-D-fructofuranoside had the same retention time as D-glucose and was therefore determined by a molar balance: [AllFru] = [Suc] - [Fru] - 2[6-kestose]. HPLC peaks of alkyl β-D-fructofuranosides were assigned upon comparing the chromatograms of anomeric mixtures of alkyl D-fructosides (prepared according to the Fischer-method[36]) before and after incubation with invertase in aqueous solution.

Butyl β-D-Fructofuranoside. A solution of sucrose (60 g) in acetate buffer (200 mL, 0.02 M, pH 4.8) and 1-butanol (200 mL) was incubated with invertase (200 mg) at 25°C during 15 min. Sodium carbonate was added up to pH 10 and the solvents were removed in vacuo. The syrup thus obtained was extracted with boiling ethanol. Ethanol was removed in vacuo and the extraction was repeated until a syrup (6.6 g) was obtained which was enriched in butyl β-D-fructofuranoside. Chromatography over Silica Gel 60 (300 cm^3, Merck) with ethyl acetate (150 mL), ethanol-ethyl acetate 1:9 (300 mL), and 1:4 (200 mL), and collection of the fractions between 400 and 640 mL, yielded 0.80 g butyl β-D-fructofuranoside as a syrup, which was pure according to HPLC, and was completely hydrolysed to fructose and butanol upon incubation with invertase.

Test of Invertase Activity under Anhydrous Conditions. Anhydrous invertase was prepared by lyophilisation from 0.5 M sodium citrate buffer (pH 4.7) or by drying to constant weight at 15 torr in the presence of an excess of either zeolite NaA or phosphorous pentoxide. Microcrystalline sucrose (0.25 g, particle size <100 μm, dried on phosphorous pentoxide) was suspended in 1-octanol, 1-butanol, or pyridine (5 mL, dried on zeolite NaA). 1-Butanol (0.25 mL), containing anhydrous sodium acetate (0.1 M) and acetic acid (0.1 M), was added. After addition of dried invertase (0.1 g) the suspension was stirred vigorously for 24 h. TLC was performed on silica gel 60 F254 (Merck) with chloroform-methanol-water (60:35:6) and detection with sodium metaperiodate-tolidine.[37] The limit of detection was <1% sucrose conversion.

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