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Note

Invertase-catalysed fructosyl transfer in concentrated solutions of sucrose

ADRIE J. J. STRAATHOF, ANTONIUS P. G. KIEBOOM, AND HERMAN VAN BEKKUM
Laboratory of Organic Chemistry, Delft University of Technology, Julianalaan 136, 2628 BL Delft (The Netherlands)

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Invertase (β-D-fructofuranosidase, EC 3.2.1.26) was used by Michaelis and Menten[1] in their classic kinetic study of the enzymic hydrolysis of sucrose. Interest in the action of invertase in concentrated solutions has been aroused by the commercial production of invert sugar at concentrations up to 3M sucrose, using invertase from yeast *(Saccharomyces cerevisiae)*. At concentrations exceeding 0.2M, invertase activity was shown to deviate from Michaelis–Menten kinetics since the rate of hydrolysis gradually decreased. This effect has been attributed to the decrease of the concentration of water[2–4], substrate inhibition[3–5], and substrate aggregation[5,6]. It does not depend on the viscosity of the medium[3,5,7].

A factor which has been neglected in this context is the transfructosylating capability of invertase[8–10]. In the presence of primary alcohols, transfer of β-D-fructofuranosyl groups to such alcohols competes with transfer to water. During the hydrolysis of sucrose, fructosyl transfer to the primary hydroxyl groups of sucrose yields small proportions of the non-reducing trisaccharides 1-kestose, 6-kestose, and neokestose by fructosyl transfer to HO-1[f], HO-6[f], and HO-6[g], respectively. Also, reducing disaccharides are formed during the reaction by fructosyl transfer to the primary hydroxyl groups of D-glucose and D-fructose. Eventually, all oligosaccharides are hydrolysed to D-glucose and D-fructose.

A quantitative study[10] showed that the formation of oligosaccharides should not be neglected in 0.29M sucrose. We assumed that the formation of oligosaccharides would be even more pronounced at increased concentrations of sucrose and consequently would decrease the rate of hydrolysis of sucrose. Therefore, we have studied the action of invertase using concentrations of sucrose up to 2.34M.

H.p.l.c. allowed quantitative analysis of mixtures of D-glucose, D-fructose, 6-kestose, inulobiose, and sucrose; the last peak was shown to contain a small proportion of 6-*O*-β-D-fructofuranosyl-D-glucose. 1-Kestose and neokestose were not detected, indicating that these trisaccharides were present at a concentration far below that of 6-kestose[8].

All reactions were performed at 25° in 0.01M acetate buffer (pH 4.8). Fig. 1 shows a typical example of the course of the conversion. 6-Kestose was formed

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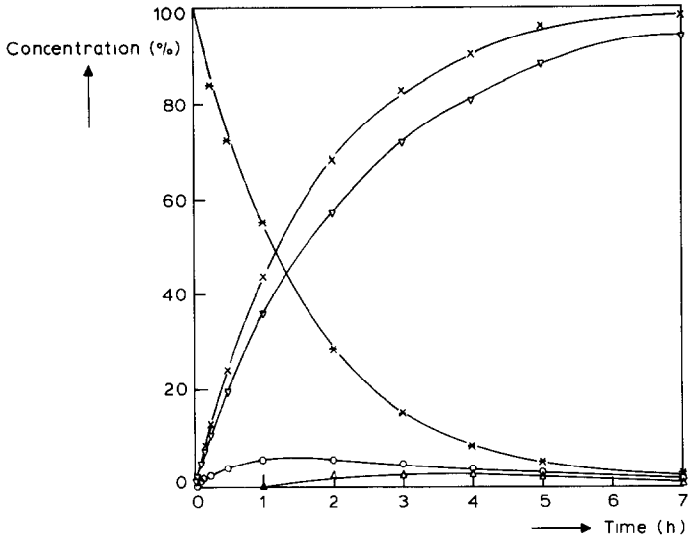

Fig. 1. Conversion of 1.75M sucrose by invertase (48,000 U/L) in 0.01M acetate buffer (pH 4.8) at 25°; ⋆, sucrose and 6-*O*-β-D-fructofuranosyl-D-glucose; ×, D-glucose; ▽, fructose; ○, 6-kestose; △, inulobiose. Concentrations are relative to the initial molar concentration of sucrose.

during the initial stage of the reaction, but inulobiose was not present in significant amounts before 20% of the sucrose had been converted and reached its maximum concentration at ~90% conversion of sucrose, whereas the amount of 6-kestose was at a maximum at ~60% conversion, irrespective of the initial concentration of sucrose.

The maximum concentration of these oligosaccharides relative to the initial concentration of sucrose did not increase up to 2.34M of the latter (Fig. 2). 6-Kestose showed a maximum (7.1 mol%) at 1.3M sucrose, whereas inulobiose reached its maximum (2.6 mol%) at 1.75M sucrose.

The observation that higher concentrations of sucrose did not lead to an increase in the concentration of oligosaccharides reflects a change in selectivity of either the formation or hydrolysis of these oligosaccharides relative to the hydrolysis of sucrose. Initial rate studies provided an opportunity for a more detailed investigation of this phenomenon. These rates were determined for concentrations of sucrose in the range 0.02–2.34M (Fig. 3). The rate of formation of D-fructose was used to calculate the rate of hydrolysis of sucrose. All previous studies concerning invertase kinetics used the rate of formation of D-glucose or reducing sugar, assuming equal concentrations of D-glucose and D-fructose. The present results show that, for concentrated solutions of sucrose, this leads to errors up to 25%, since D-glucose is liberated by both the hydrolysis of sucrose and the formation of kestose.

The initial rate of hydrolysis (r_h), as calculated from the formation of D-

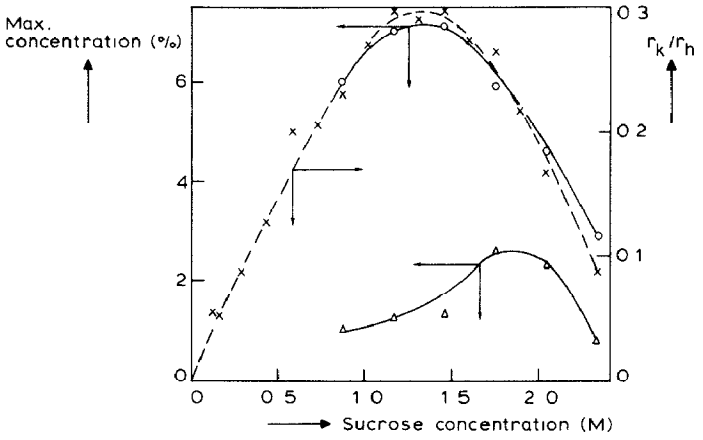

Fig. 2. Left axis: maximum concentration of 6-kestose (○) and inulobiose (△) relative to the corresponding initial molar concentration of sucrose. Right axis: ratio (×) between the initial rate of formation of kestose and the initial rate of hydrolysis of sucrose.

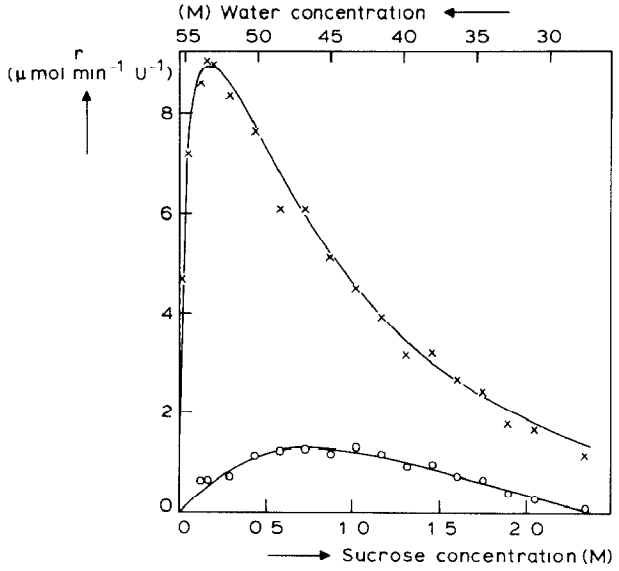

Fig. 3 Initial rates of hydrolysis of sucrose (×) and formation of kestose (○) as a function of the concentrations of sucrose and water. The curves are calculated with the use of equations *4* and *5*.

fructose, qualitatively shows the behaviour reported before[2–7]. Thus, an increase according to the Michaelis–Menten model up to 0.2M sucrose was followed by a gradual decrease at higher concentrations. The initial rate of formation of kestose (r_k) showed a much flatter and lower maximum, at ~0.8M sucrose.

The ratio r_k/r_h increased up to 0.3 with increasing concentration of sucrose up to 1.3M, but decreased at higher concentrations (Fig. 2). This behaviour is in harmony with the pattern of the maximum concentration of 6-kestose, as shown in

Fig. 2. It is tempting to ascribe the reduced maximum amount of 6-kestose at concentrations of sucrose exceeding 1.3M to the decrease in the formation of kestose relative to the hydrolysis of sucrose, and to assume that the ratio of hydrolyses of kestose and sucrose undergoes no pronounced change.

The formation of both 6-kestose and D-fructose will involve one common enzyme–fructosyl complex that can react with sucrose (rate constant k_k) or water (rate constant k_h), respectively[11]. Thus, the ratio of reaction rates (r_k/r_h) is expected to be constant after correction for the molar concentration of sucrose ([S]) and water ([W]) present (equation *1*). Fig. 4, however, shows that this ratio decreased linearly for concentrations of sucrose exceeding 0.4M, according to equation *2*.

$$\frac{r_k}{r_h} \cdot \frac{[W]}{[S]} = \frac{k_k}{k_h} \qquad (1)$$

$$\frac{r_k}{r_h} \cdot \frac{[W]}{[S]} = 20.6 - 8.86\,[S] \qquad (2)$$

The negative term in equation *2* originates from the non-ideality of concentrated solutions of sucrose. At concentrations up to 2.34M, the molar ratio of water to sucrose decreases to 12, and intra- and inter-molecular hydrogen-bonding of sucrose thus will occur[12–14]. It is assumed that these hydrogen bonds may affect the reactivity of sucrose as a fructosyl acceptor if HO-6^f is involved. The intramolecular hydrogen-bonding, the extent of which is not agreed[12,13], does not seem[13] to involve HO-6^f. Therefore, intermolecular hydrogen-bonding, leading to association of sucrose molecules, is thought to be largely responsible for the reduction in rate. For steric reasons, the HO-6^f groups involved in hydrogen-bonding are considered to become less susceptible to attack by the enzyme–fructosyl complex. Although no quantitative data are available, the extent of this association will be approximately proportional to $[S]^2$. The fraction (α) of sucrose that shows reactivity towards formation of kestose is thus given by equation *3*. Substitution of $\alpha[S]$ for [S] in equation *1* fully meets the experimental results of equation *2* for $k_k/k_h = 20.6$ and $c = 0.43\text{M}^{-1}$.

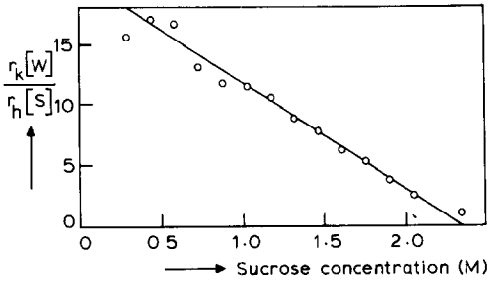

Fig. 4. Ratio between the initial rates of formation of kestose and hydrolysis of sucrose corrected for the concentrations of sucrose and water, as a function of the concentration of sucrose (equation *2*).

$$\alpha[S] = [S] - c \cdot [S]^2 \quad (3)$$

Combes and Monsan[5] introduced the inhibition terms $[S]^2/K_s$ and $[S]^3/K'_s$ in the Michaelis–Menten equation to account for the decrease in the rate of hydrolysis of sucrose at concentrations exceeding 0.2M. They explained these terms by substrate inhibition of the enzyme–sucrose complex by a second molecule of sucrose and a dimer of sucrose, resulting from intermolecular hydrogen-bonding.

When similar inhibition terms were incorporated in equations *4* and *5*, there was good correlation with the experimental results for K_m 37.8mM, K_i 1.2M, K'_i 3.3M^2, k_k 4.7×10^{-7} min^{-1}.U^{-1}, and k_h 2.3×10^{-8} min^{-1}.U^{-1} (see Fig. 3).

$$r_k = \frac{k_k \cdot \alpha[S] \cdot [S]}{K_m + [S] + [S]^2/K_i + [S]^3/K'_i} \quad (4)$$

$$r_h = \frac{k_h \cdot [W] \cdot [S]}{K_m + [S] + [S]^2/K_i + [S]^3/K'_i} \quad (5)$$

More information about substrate inhibition and hydrogen-bonding in concentrated solutions of sucrose is required for a more detailed understanding of the phenomena.

EXPERIMENTAL

Invertase was a kind gift of Gist-brocades (Delft, The Netherlands). The specific activity was 240 U (μmol of fructosyl units transferred per min per mg) with 0.2M sucrose.

The conversion of sucrose was started by addition of a buffer solution (5 mL, 0.08M sodium acetate, pH 4.8) of invertase to aqueous sucrose (35 mL) at 25°, yielding an acetate buffer (0.01M, 40 mL) of 720–120,000 U/L of invertase and 0.02–2.34M sucrose. Samples (2 mL) were added to aqueous silver nitrate to stop the reaction (invertase was inactive in 0.01M silver nitrate at 25°) and ethylene glycol was added as internal standard for h.p.l.c.

The h.p.l.c. system used has been described in detail elsewhere[15]. The Aminex HPX 87 C column (Ca^{2+} form) at 60° was eluted with water at 0.6 mL/min. Base-line separation of seven components was achieved. Retention times (min): *A* 6.10, *B* 7.47, sucrose 8.93, glucose 11.2, *C* 13.2, fructose 15.6, ethylene glycol (internal standard) 19.2. Fractions corresponding to these peaks were collected for identification.

T.l.c.[16] was performed on silica gel 60 F254 (Merck) with water-saturated 1-butanol–ethanol (100:40) and detection with aniline–diphenylamine–acetone–85% phosphoric acid (4 mL:4 g:200 mL:30 mL), at 100° for 10 min.

The amount of *A* was too small for identification. *B* was identified as 6-kestose [β-D-Fru*f*-(2→6)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*] since its eluate yielded D-

glucose and D-fructose in the ratio 1:2 on incubation with invertase. In addition, t.l.c. showed a spot, R_{Suc} 0.38, corresponding to 6-kestose [R_{Suc} 0.41[16], *cf.* 1-kestose (0.51) and neokestose (0.75)[16]]. The blue colour of this spot indicated the absence of a reducing fructosyl residue. *C* was assigned to 1-*O*-β-D-fructofuranosyl-D-fructose (inulobiose) since the mixture of oligofructosides obtained by partial acid hydrolysis of inulin[17] showed the same peak in h.p.l.c. and the same red spot at R_{Suc} 0.68 in t.l.c. Moreover, increased formation of *C* was observed upon addition of D-fructose to the inverting solution of sucrose. The h.p.l.c. eluates of D-glucose and D-fructose showed no additional spots, but sucrose contained a contaminant, R_{Suc} 0.60. The blue colour of this small spot and its increased formation on the addition of D-glucose to the inverting solution of sucrose indicated it to be 6-*O*-β-D-fructofuranosyl-D-glucose.

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