Probing osmotic effects on invertase with L-(−)-sucrose†‡

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L-(−)-Sucrose was efficiently synthesized using intramolecular aglycon delivery and used to elucidate osmotic effects on the activity of invertase, which catalyzes the hydrolysis of D-(+)-sucrose. The osmotic effect imposed by L-sucrose was responsible for more than 30% of the activity loss ascribed otherwise to "substrate inhibition."

Introduction

The diverse roles of carbohydrates in nature can be divided into two broad categories: those defined by molecule-specific recognition such as metabolism and protein-based signaling pathways, and those derived from physicochemical properties such as biomechanical stability and colligative effects. Although the latter is often thought to be nonspecific, molecular fine structure can have a strong influence on the solution properties of sugars. This makes it challenging to differentiate recognition processes from nonspecific effects in the biological functions of carbohydrates.

Here we deconvolute solute effects from substrate recognition by evaluating the activity of a sugar-processing enzyme in the presence of a mirror-image carbohydrate. This approach is predicated on the condition that such molecules are not recognized as substrates, but are otherwise physicochemically identical to the natural enantiomer. In this study we examine the inhibitory effects of sucrose on yeast invertase (β-fructofuranosidase; E.C. 3.2.1.26), and its association with the phenomenon known as substrate inhibition.[1,2] By using L-(−)-sucrose (**1**) as the dominant solute, we show that osmotic effects contribute significantly toward the apparent substrate inhibition of invertase. In addition, we find the k_{cat} and K_M values of invertase to be sensitive to osmolytes such as L-sucrose, even at relatively low concentrations.

The invertase family has a long and cherished history in enzymology and biotechnology, having served as the classic model of Michaelis–Menten kinetics at low substrate concentrations,[3] and also as a confectionery agent for increasing sweetness and forestalling sucrose crystallization by the *in situ* generation of fructose.[4] The decrease in invertase activity with increasing sucrose concentration is well known, and has been ascribed to the simultaneous occupancy of two sucrose molecules in the active site (Fig. 1b).[2,5] This popular model for substrate inhibition can be expressed as a modified form of the Michaelis–Menten equation [eqn (1)]:[1,6]

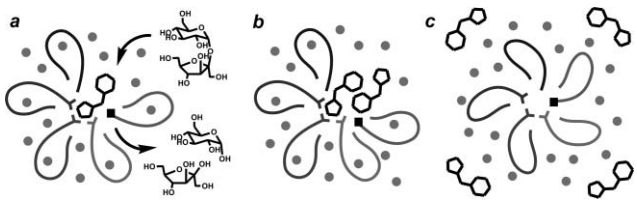

Fig. 1 Models of sucrose-induced inhibition of invertase. (*a*) Catalytic hydrolysis of sucrose by invertase (dark square = active site; dots = H_2O). (*b*) Substrate inhibition with active site blocked by second molecule S [*cf.* eqn (1)]. (*c*) Lower conformational mobility due to reduction in water activity, based on the preferential exclusion principle.

$$v = \frac{k_{cat}[E_0][S]}{K_M + [S] + [S]^2 / K'_S} \quad (1)$$

where v, k_{cat}, $[E_0]$, and K_M are the velocity, forward rate constant, total enzyme concentration, and Michaelis constant respectively, and K'_S is the dissociation constant of the inactivated complex. However, the substrate inhibition model does not actually fit well with the kinetics of invertase activity (Fig. 2). The relevance of substrate inhibition is further challenged by a recent crystal structure of an invertase–sucrose complex, which suggests a well-defined binding domain in the active site for the fructofuranoside ring, but no comparable points of contact for the accompanying α-glucopyranoside.[7] This, coupled with glucose's poor ability to act as a competitive inhibitor, indicates that the active site of invertase does not encourage the occupancy of a second sucrose molecule by glucopyranose recognition.

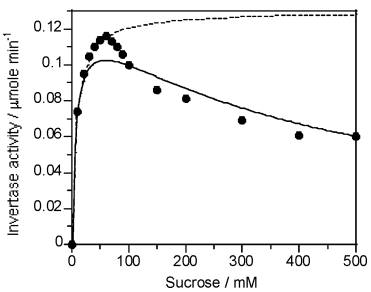

Fig. 2 Substrate inhibition of yeast invertase by D-(+)-sucrose. Experimental data (•) compared with Michaelis–Menten kinetics (—; K_M = 7.76 mM, $V_{max} = k_{cat}[E_0] = 0.13$ μmol min^{-1}) and least-squares fit according to eqn (1) (—; $K'_S = 436 \pm 45$ mM).

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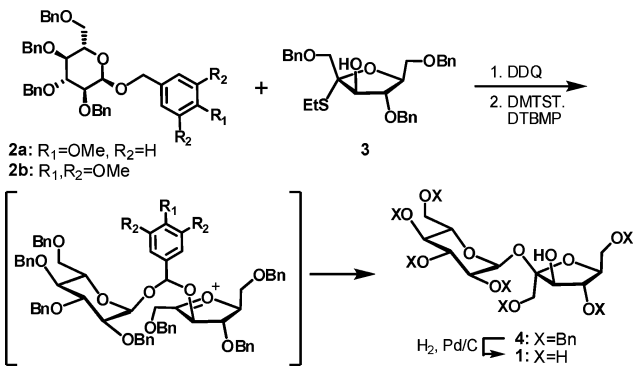
† The IUPAC designation of D- and L-sugars is specifically valid for monosaccharides. However, extension of this designation toward common disaccharides is both convenient and in widespread practice.

‡ Electronic supplementary information (ESI) available: Experimental procedures and NMR characterization related to the synthesis of L-sucrose, and invertase purification and kinetics. See DOI: 10.1039/b810158b

Colligative effects, which are often nonlinear with respect to solute fraction,[8,9] may be responsible for the discrepancies in enzyme kinetics derived from eqn (1). In particular, sucrose is an effective osmolyte and can reduce water activity to a greater extent than most neutral solutes.[10,11] The role of osmotic effects in substrate inhibition is likely driven by the preferential exclusion principle, introduced by Lee and Timasheff (Fig. 1c).[12] The preferential exclusion of sucrose from protein surfaces is thought to provide some protection against thermal denaturation by increasing the surface tension of the macromolecular cavity and reinforcing the hydration shell, forcing the protein to adopt a more compact conformation.[13] The same effect may also reduce the conformational mobility of enzymes, with a subsequent decrease in k_{cat}. In the case of invertase, experimental verification of an osmotic effect by sucrose is complicated by its dual role as osmolyte and substrate. We address this problem by using biochemically inert L-sucrose as a surrogate osmolyte.

Results and discussion

An expedient synthesis of L-sucrose **1** was developed based on intramolecular aglycone delivery (IAD),[14–16] using the oxidative coupling of L-glucoside **2b** and L-fructofuranosyl donor **3** to form mixed acetals (Scheme 1). This strategy has been used in the synthesis of other mirror-image disaccharides such as L-trehalose,[17] as well as the coupling of β-D-fructofuranosides to *p*-methoxybenzyl (PMB) ethers.[16] However, PMB α-L-glucoside **2a** could not be coupled with **3**, possibly due to the short lifetime of the intermediate quinone methide. The coupling efficiency was improved by replacing **2a** with 3,4,5-trimethoxybenzyl L-glucoside **2b**, whose lower oxidation potential enabled the stereoselective formation of L-sucrose derivative **4** in 41% yield, followed by hydrogenation to afford **1** in sufficient amounts for our studies.[18]



Scheme 1 Synthesis of L-(−)-sucrose **1** using the IAD strategy. DDQ = 2,3-dichloro-5,6-dicyanoquinone; DMTST = dimethyl(methylthio) sulfonium triflate; DTBMP = 2,6-di-*tert*-butyl-4-methylpyridine.

L-Sucrose was tested as a substrate against purified yeast invertase, and found to be inert to enzymatic hydrolysis.[18] The osmotic effect of sucrose on invertase was then evaluated by adding increasing amounts of L-sucrose to solutions of invertase with 60 mM D-sucrose, the optimal concentration for enzymatic activity (Fig. 3a). The relative decrease in activity at the highest D/L-sucrose concentrations used (0.5 M) was more than 1/3 of the activity loss ascribed otherwise to canonical substrate inhibition.

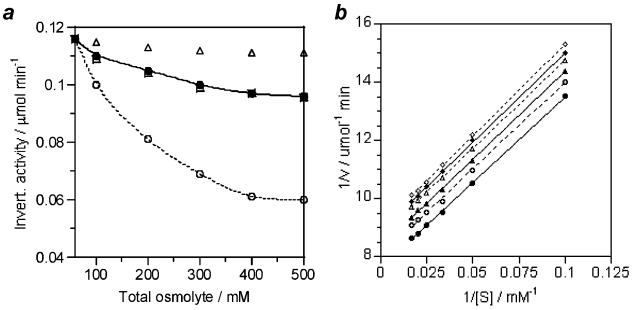


Fig. 3 (a) Osmotic effects on invertase activity, using L-sucrose (•), D-trehalose (□), and lactose (△). Invertase activity as a function of pure D-sucrose (◦) is shown for comparison. Total amount of osmolyte includes the initial concentration of D-sucrose ([S_0] = 60 mM). (b) Lineweaver–Burk plot illustrating the osmotic effect (using trehalose) at low D-sucrose concentrations (10–60 mM). Trehalose concentrations: 0 mM (•); 40 mM (◦); 140 mM (▲); 240 mM (△); 340 mM (♦); 440 mM (◇).

The nonspecific osmotic effect of L-sucrose on invertase was established in two ways. First, a parallel study with the disaccharide D-trehalose, a constitutional isomer of sucrose and a well known osmolyte, revealed a very similar reduction in the catalytic rate of invertase, whereas the weak osmolyte D-lactose had nearly no effect on enzyme activity (Fig. 3a). A Lineweaver–Burk analysis of invertase activity as a function of trehalose concentration reveals a modest decrease in K_M and V_{max} reminiscent of uncompetitive inhibition (Fig. 3b),[6,18] but the large inhibition constant (K_i = 1.6 ± 0.2 M) implies that the enzyme–osmolyte interactions are nonspecific.

Second, competitive inhibition assays with D-fructose, L-fructose, and glycerol were run to address the role of chiral recognition in substrate inhibition. The loss of fructofuranosidase activity in the presence of D-fructose (K_i = 7 mM) was 47% at 100 mM, whereas inhibition by glycerol and L-fructose at 100 mM were only 5% and 2% respectively, establishing that competitive inhibition is chirospecific.[18] By comparison, the decelerating effect of L-sucrose at 100 mM was 17%, indicating that chiral recognition is not relevant in osmotic pressure effects.

Evaluation of other crowding agents revealed a general reduction in invertase activity with increasing solute concentration.[19–21] Interestingly, both small achiral polyols (glycerol, mw 92) and macromolecular solutes such as dextran (40 kd) and polyethylene glycol (PEG; 5 kd) had similar decelerating effects as the disaccharide osmolytes, but with variable impact on K_M and catalytic efficiency k_{cat}/K_M (Fig. 4).[18] Dextran and PEG enhanced the catalytic efficiency of invertase with increasing solute fraction, in accord with a recent study on enzyme kinetics under crowded conditions.[20] In contrast, a slight decrease in k_{cat}/K_M was observed for D-trehalose and glycerol. We presume L-sucrose to have the same effect on catalytic efficiency as the other osmolytes, although our limited supply precluded a parallel study.

These experiments indicate that crowding agents decelerate invertase kinetics by limiting conformational mobility.[19] In the case of small, neutral osmolytes such as sucrose, preferential exclusion of solute increases the surface tension of the enzyme's hydration cavity, raising the activation barrier between conformational states. In the case of macromolecular solutes, a greater frequency of repulsive steric interactions drives proteins toward more compact conformations.[22] Experimental evidence for crowding-induced

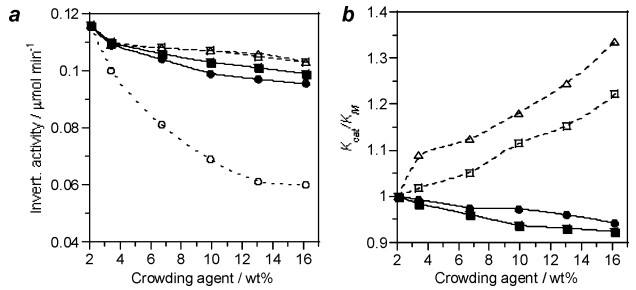


Fig. 4 (a) Influence of crowding agents on invertase activity as a function of solute fraction: trehalose (•); glycerol (■); 40 kd dextran (□); 5 kd PEG (△). [S_0]= 60 mM or 2 wt%; invertase activity as a function of pure D-sucrose (◦) is shown for comparison. (b) Influence of macromolecular crowding agents *versus* osmolytes on catalytic efficiency (k_{cat}/K_M). Lines are drawn to guide the eye.

changes in protein conformation has been reported in both cases.[20,23] Therefore, despite their different mechanisms, both osmotic pressure and entropic steric repulsion can modulate enzyme activity by reducing its effective free volume.[24]

The association and rate constants for invertase are also sensitive to osmotic pressure. Kinetic analyses are typically performed under the assumption of ideal solution conditions, with constant K_M and k_{cat}. However, these rate constants cannot be extrapolated toward studies involving osmolytes. In our case, we find that K_M and k_{cat} values are affected by sugar concentrations as low as 100 mM, well below the levels typically associated with nonlinear colligative effects. We find that K_M and k_{cat} are best described as log functions with respect to osmolyte concentration (see Fig. 5).[18]

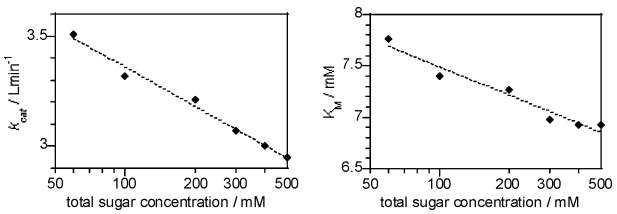


Fig. 5 Semilogarithmic plots of k_{cat} (*left*) and K_M (*right*) *versus* total sugar concentration, up to 0.5 M ([S_0] = 10–60 mM). $k_{cat}(S) = 4.539–0.590$ log[S] (in L min^{-1}); $K_M(S) = 9.318–0.913$ log[S] (in mM).

The issue of substrate inhibition was revisited after subtracting the osmotic effects of added L-sucrose (*cf.* Fig. 3a) from the activity profile of invertase with D-sucrose. Applying k_{cat} and K_M as log[S] functions (*cf.* Fig. 5) produced a good least-squares fit of the corrected data to eqn (1) with a K'_S of 1.03 ± 0.10 M (Fig. 6). With osmotic effects taken into account, the substrate inhibition constant is more than double the earlier value. The revised analysis reveals that true substrate inhibition has considerably less influence on invertase activity than previously thought.[25]

Conclusions

Our study demonstrates that the physicochemical influence of substrates on enzymes may be distinguished from activities involving enantioselective recognition by introducing mirror-image molecules as co-solutes. In the case of invertase, the application of L-sucrose enabled the separation of osmotic effects from true substrate inhibition, and provided a benchmark for evaluating

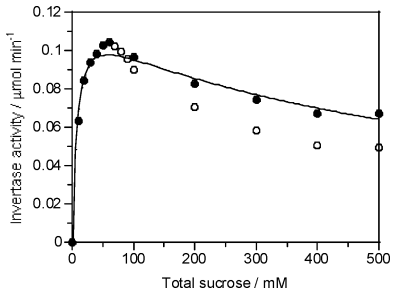


Fig. 6 Substrate inhibition by D-sucrose corrected for osmotic effects (•), and least-squares fit according to eqn (1) (—) using K_M and k_{cat} as functions of log[S] (*cf.* Fig. 5). Original data (◦) is shown for comparison.

crowding effects on invertase activity by other osmolytes and macromolecular solutes. The strong osmotic effect on invertase may have implications on its native functions in plant physiology: recent developments suggest possible osmoregulatory roles associated with root and stem elongation.[26–28] It is remarkable that relatively low levels of osmolytes can cause k_{cat} and K_M values to deviate significantly from those measured under ideal solution conditions. Such environmental effects should be taken into account when measuring enzyme kinetics with high solute concentrations or in physiologically relevant environments.

Experimental

Synthesis of L-sucrose

Substituted α-benzyl L-glucosides **1a–e** were prepared from commercial sources of L-glucose or from L-glucal as previously described.[17,29] L-Fructose was prepared in gram quantities from L-sorbose using a protocol reported by Zhao and Shi,[30] and converted into α-thioethyl derivative **2** by adapting previously reported methods developed with the natural enantiomer.[16]

A mixture of DDQ (330 mg, 1.44 mmol) and 4 Å molecular sieves in CH_2Cl_2 (4 mL) at 0 °C was treated with a solution of **2** (400 mg, 0.80 mmol) and **1e** (760 mg, 1.12 mmol) in CH_2Cl_2 (4 mL). The mixture was warmed to RT and stirred for 6 h, then quenched with an aqueous solution of 0.7% ascorbic acid, 1.3% citric acid, and 0.9% NaOH (12 mL). The reaction mixture, which formed a yellow suspension upon standing, was diluted with EtOAc (50 mL) and filtered through Celite prior to aqueous workup. The crude acetal was concentrated to a brown oil, dried by azeotropic distillation with toluene and placed under high vacuum for 1 h, then dissolved in anhydrous CH_2Cl_2 (10 mL) and transferred to a flask containing DTBMP (920 mg, 4.4 mmol) and 4 Å molecular sieves (200 mg). The mixture was stirred for 15 min at RT, treated with DMTST (88 mg, 4.0 mmol), then stirred for 16 h. The mixture was diluted with EtOAc (50 mL) and filtered through Celite prior to aqueous workup and silica gel chromatography (10% EtOAc in hexanes), which yielded heptabenzyl L-sucrose **3** as a light yellow oil (520 mg, 41%).

Heptabenzyl derivative **3** (600 mg, 0.6 mmol) was dissolved in MeOH (25 mL) and treated with $Pd(OH)_2$ on carbon (250 mg), then stirred under an atmosphere of H_2 for 24 h. The reaction mixture was filtered through a pad of prewashed Celite and concentrated to yield L-sucrose **4** as a waxy solid (200 mg, 98%). IR (neat) 3312, 1605, 1420, 1054; δ_H (500 MHz; CD_3OD) 5.36

(d, 1H, $J = 4$ Hz), 4.07 (d, 1H, $J = 9.3$ Hz), 4.08–3.99 (m, 1H), 3.80–3.64 (m, 7H), 3.57 (d, 1H, $J = 3.3$ Hz), 3.41–3.33 (m, 4H); δ_C (125 MHz; 10% D_2O in CD_3OD) 103.84, 92.18, 82.20, 77.62, 74.18, 72.88, 71.69, 69.76, 62.41, 61.99, 60.64; HRMS(ESI): calcd for $C_{12}H_{22}O_{11}Na$ 365.1060 $[M + Na]^+$, found 365.1062; $[a]_D^{20} = -67$ ($c = 1$, H_2O).

Invertase kinetics with L-sucrose and other crowding agents

Activity measurements were performed at optimum substrate concentrations ($[S_0] = 60$ mM) in the presence of crowding agents, using a microscale version of the dinitrosalicylate (DNS) assay for reducing sugars.[31] k_{cat} and K_M values were obtained using low substrate concentrations ($[S_0] = 10$–60 mM) at constant solute fraction, based on the combined wt% of D-sucrose and crowding agent. In a typical assay, a 40 μL aqueous solution containing D-sucrose (120 mM) and L-sucrose (0–880 mM) was diluted in a test tube with 30 μL of 0.05 M sodium acetate (pH 4.7) buffer and heated to 55 °C. 10 μL of a yeast invertase stock solution (0.3 μM) was added and the reaction mixture was incubated for 10 min at 55 °C, then quenched by the addition of DNS reagent (1000 μL) and heated to near reflux for 5 min. The solution was quickly cooled to RT and diluted twofold, followed by an absorbance measurement at 540 nm.

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