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Note

Inhibitory effect of diglucosylamines on two β-glucosidases ☆

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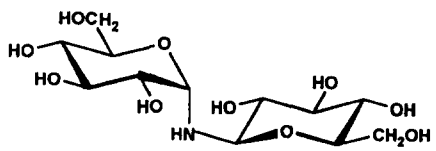
Glycosylamines are compounds of interest for the enzymology of carbohydrates due to their ability to act as competitive inhibitors for glycosidases [1,2]. They usually bind more tightly with the enzymes than their neutral counterparts, provided that the basic character of their nitrogen atom is not diminished [2]. A main drawback of glycosylamines, however, is their tendency to hydrolyze [3]. Diglycosylamines may be expected to be more stable than glycosylamines because the combined electron-withdrawing effect of the two-ring oxygens may decrease the basicity of the glycosylamine nitrogen. This paper deals with an investigation of the utility of diglucosylamines as inhibitors for β-glycosidases from *Trichoderma reesei* and from sweet almonds.

Two anomers of diglucosylamine, namely α-D-glucopyranosyl-β-D-glucopyranosylamine (**1**) and di-β-D-glucopyranosylamine (**2**) besides the *N*-acetylated derivative of (**2**) were tested as inhibitors of β-glucosidases.



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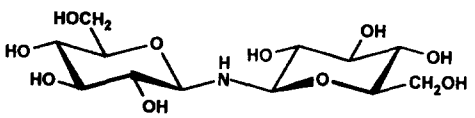

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☆ Glycosylamines, Part 4. For Part 3 see ref. [8]

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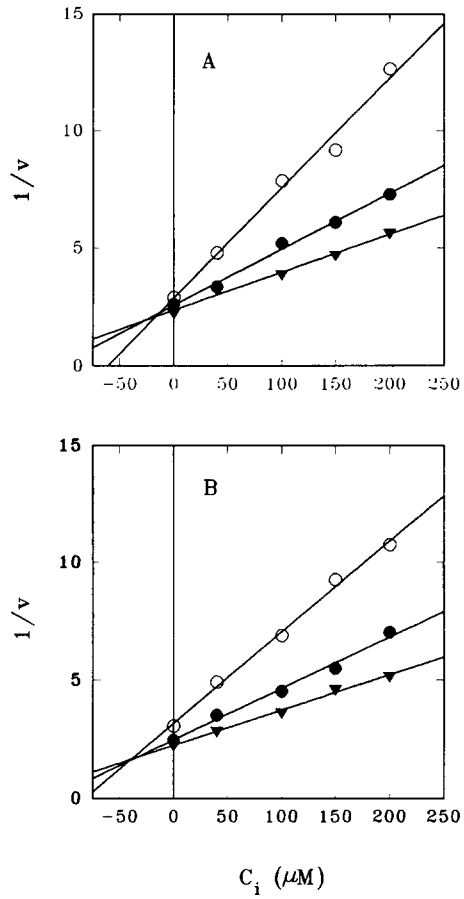

Fig. 1. Representative Dixon plots showing the inhibition of *Trichoderma* β-glucosidase with diglucosylamine **1** (part A), and **2** (part B). The concentration of 4-NPG as (○), 0.1; (●), 0.2; and (▼), 0.5 mg mL^{-1}.

Dixon plots were used for the determination of inhibition constants (K_i) with 4-nitrophenyl β-D-glucopyranoside (4-NPG) as the substrate. At pH 5, both diglucosylamines **1** and **2** competitively inhibited *Trichoderma* β-glucosidase (Fig. 1) with K_1

Table 1
Inhibitory constants $K_i \pm$S.E.M. of glycosylamines with β-glucosidase from *Trichoderma reesei* at pH 5 and 37°C

Inhibitor	K_i [a] (mM)
β-D-Glucosylamine	0.024 ± 0.004
Di-α,β-D-glucosylamine (**1**)	0.028 ± 0.010
Di-β-D-glucosylamine (**2**)	0.038 ± 0.007
N-Acetyl-di-β-D-glucosylamine	57.0 ± 12

[a] Values are means from three independent determinations.

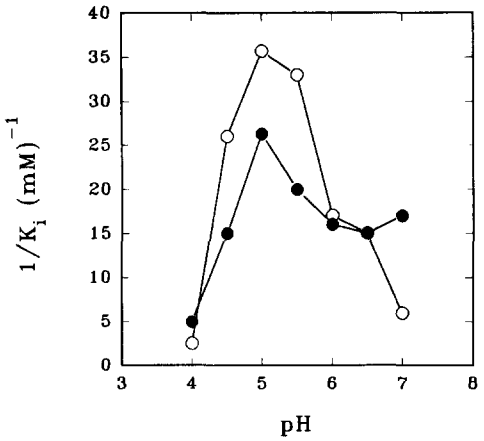

Fig. 2. Plot of $1/K_i$ versus pH for diglucosylamine **1** (○); and **2** (●) with β-glucosidase from *Trichoderma reesei*.

values of 28 and 38 μM respectively (Table 1). The similarity of K_i values indicates that only the β-glucosyl unit in the diglucosylamine molecule is recognized by the enzyme. This is in agreement with a comparable K_i value of 24 μM found for β-glucosylamine. *N*-Acetylation of **2** lead to a drastic increase in the K_i value. The reason for this loss of inhibitory capacity of *N*-acetylated **2** may be either a decreased basicity of the nitrogen atom or sterical hindrance by the *N*-acetyl group.

Of the two β-glucosidases tested, only the fungal enzyme was sensitive to diglucosylamines. This may be explained by the different specificity of the aglycon site of fungal and emulsin β-glucosidase; the natural substrates of the latter enzyme are aromatic glycosides.

The inhibitory properties of glycosylamines on β-glucosidases are explained by their ability to form salt bridges between the protonated glycosylated nitrogen of glycosylamine and a carboxylate ion at the active site of the enzyme whereby the protonation of the inhibitor may occur either from the solvent or by accepting the proton from an acidic group located at the active site of the enzyme [2,4].

In order to decide whether ionized or neutral forms of **1** and **2** were responsible for the inhibition, we have investigated the effect of pH on the inhibition constants K_i. As can be seen from Fig. 2 the maximum inhibitory capacity of both **1** and **2** was at pH 5, which coincided with the pH optimum for the enzyme activity [5]. By titration, we have estimated the apparent pK values for protonated **1** and **2** to be 3.14 ± 0.12 and 3.20 ± 0.16 respectively. Accordingly, we assume that the active forms of the inhibitors were unprotonated, i.e. neutral at pH 5.

Preliminary experiments indicated that β-glucosidases from *Aspergillus niger* and *Aspergillus phoenicis* were also effectively inhibited by compounds **1** and **2** (results not shown).

Diglucosylamines are labile in aqueous solution. The change of specific optical rotation at the conditions used for β-glucosidase assays (pH 5, 37°C) followed the

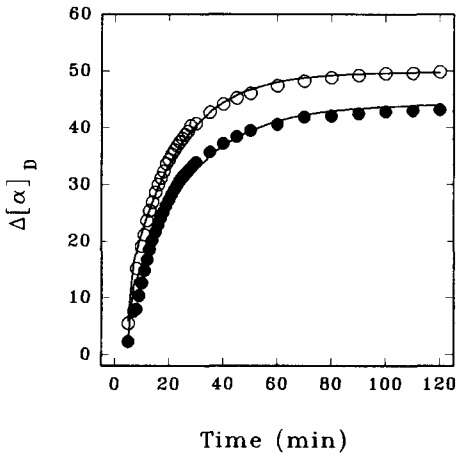

Fig. 3. Time course of the change of specific optical rotation of diglucosylamine **1** (○); and **2** (●) at pH 5.5 and 37°C.

first-order kinetics, with rate constants of hydrolysis (k_h) of 0.04452 and 0.05308 min^{-1} respectively (Fig. 3).

1. Experimental

Enzymes and the assays.—As the source of β-glucosidase, a crude enzyme preparation from *Trichoderma reesei* QM 9414 cultivated with cellulose as the substrate [6] was used. The dialyzed and lyophilized enzyme (2 g) was dissolved in water and the liquid (200 mL) was poured through a column of microcrystalline cellulose (25 mL). The filtrate was concentrated by ultrafiltration, dialyzed against distilled water and lyophilized. Isoelectric focusing, followed by detection of the separation gel with 4-methylumbelliferyl β-D-glucoside as an activity stain, proved the presence of essentially one β-glucosidase isoenzyme with pI 8.7. Sweet almond β-glucosidase was purchased from Serva (Heidelberg).

The assays with inhibitors were performed in 0.05 M citrate–phosphate buffers of varying pH, at two different concentrations (0.2 and 0.5 mg/mL) of 4-nitrophenyl-β-D-glucoside as the substrate in a total vol of 0.5 mL at 37°C for 10 min. To minimize the decomposition of the inhibitors, their solutions in the appropriate buffers were prepared always immediately before use. The reaction was stopped by the addition of 2 mL 4% Na_2CO_3 (w/v) and the absorbance was measured at 410 nm. The measurements were done in triplicate for each concentration of the substrate. The K_i values were obtained graphically from Dixon plots employing linear regression.

β-Glucosylamine and diglucosylamines used in this study were prepared as described previously [7,8]. Crystalline *N*-acetyl-di-β-D-glucosylamine was synthesized by the procedure of Brigl and Keppler [9].

pK_a values of diglucosylamines were determined according to Legler [10] by dissolving an accurately weighed amount of the sugar (50 μmol) in water (10 mL), adding one-half of the calculated amount of 0.1 M HCl and immediately measuring the pH with a digital pH-meter (Radiometer, Type PHM64). Measurements were performed in triplicate.

The decomposition of diglucosylamines at pH 5 and 37°C was followed by measuring the change of optical rotation of the sugar solution in 0.05 M citrate–phosphate buffer (ca. 0.5) in a Perkin–Elmer, Model 241 polarimeter. The kinetic constants were computed by fitting the data to equation $\Delta[\alpha]_D = [\alpha]_{DE}\ [1 - \exp\ (-k_h t)]$, where $\Delta[\alpha]_D$ is the difference between the specific optical rotation at the given time and the value at the time zero, $[\alpha]_{DE}$ is the specific optical rotation at the equilibrium, k_h is the rate constant of diglucosylamine hydrolysis and t is the time in min.

Acknowledgements

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