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Influence of water-miscible solvents on hydrolytic activity of crude almond β-glucosidase

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Abstract

The influence of polar solvents on the hydrolysis of cellobiose (4-*O*-(β-D-glucopyranosyl)-β-D-glucopyranose) and *p*-NPG (*p*-nitrophenyl β-D-glucopyranoside) by crude almond β-glucosidase has been measured. THF and acetonitrile diminish the initial reaction rate by a factor of 2 at a concentration of 10%, while DMF, DMSO and 2-methyl-2-butanol have little effect at this concentration, but induce a decrease at higher concentrations.

The specificity constant V_{max}/K_M of the crude enzyme in aqueous solution was 200-fold higher for *p*-NPG than for cellobiose, indicating that between the two β-glucosidase activities present in almond, prunasin hydrolase may be more important than amygdalin hydrolase. However, the specificity constant for *p*-NPG was more affected by the presence of DMF, since it dropped six-fold for a 20% concentration of solvent while in the case of cellobiose, the drop was only 1.6-fold for the same solvent concentration.

Keywords: β-Glucosidase; Hydrolysis; Organic solvent

1. Introduction

The use of glycosidases to catalyze the synthesis of various glycosides by condensation in low water environments has attracted considerable interest in recent years. These reactions can lead directly to anomerically pure products, without side reactions using non-activated substrates [1,2]. However, glycosidases can be very sensitive to the low water activities often used to foster condensation [3,4]. To circumvent this, changes can be made to the reaction media. For instance, higher reaction rates and yields can be obtained using almond β-glucosidase in the presence of water-miscible organic solvents. Adding 20% DMF to 1-octanol at a water activity of 0.53 can increase octyl β-D-glucopyranoside final concentration from 40 to 100 mM, and increase the rate of reaction eight-fold [4].

To help understand the behavior of glycosidases in the presence of polar solvents, the influence of co-solvents on the hydrolysis of two glucosides was investigated. Kinetics of cellobiose and *p*-nitrophenyl glucoside hydrolysis catalyzed by almond β-glucosidase in aqueous-organic media were studied.

2. Materials and methods

β-Glucosidase from almonds (*Prunus dulcis*) available as a crude lyophilized powder (2.85 units per mg of solid, 100% protein according to the supplier), cellobiose, gentiobiose, *p*-nitrophenyl β-D-glucopyranoside (*p*-NPG), *p*-nitrophenol, and 2-methyl-2-butanol were purchased from Sigma–Aldrich (Oakville, Ont., Canada). DMSO, sulfuric acid and succinic acid were purchased from Anachemia (Montreal, Que., Canada). DMF and HPLC-grade acetonitrile were obtained from Fisher Scientific (Montreal, Que., Canada). All water used for reactions and HPLC analyses was HPLC-grade and purchased from J.T. Baker (Phillipsburg, NJ, USA). Glucose was obtained from BDH (Montreal, Que., Canada). Acetic acid was purchased from EM Science (Gibbstown, NJ, USA).

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2.1. Cellobiose hydrolysis reactions at different solvent concentrations

All hydrolytic reactions were performed at ambient temperature using 76.6 mM cellobiose in a final volume of 10 ml with 0.25 mg solubilized almond β-glucosidase. 0.01 M succinate buffer pH 5.0 was used. The volume of co-solvent was adjusted to achieve the desired ratio of buffer/co-solvent.

2.2. K_M and V_{max} study of cellobiose hydrolysis reaction

To various aliquots of 250.0 mM cellobiose in buffer (0.01 M succinate buffer pH 5) was added sufficient volume of buffer and DMF to make the volume up to 9 ml. The reactions were initiated by the addition of 1 ml of β-glucosidase solution (0.351 mg/ml in buffer). The final DMF concentration was 0, 5, 10, or 20%. The kinetic parameters were determined by non-linear regression. The error bars on the graphs represent the standard error of the estimates [5].

2.3. K_M and V_{max} study of p-nitrophenyl β-D-glucopyranoside

To various aliquots of 100.0 mM *p*-NPG solution in buffer (0.01 M succinate buffer pH 5) was added sufficient volume of buffer and DMF to make the volume up to 9.9 ml. The reactions were initiated by the addition of 0.1 ml of β-glucosidase solution (0.351 mg/ml in buffer). The final DMF concentration was 0, 5, 10, or 20%.

2.4. Equipment

All HPLC analyses were performed using a Waters Millennium^{32} liquid chromatography system purchased from Waters Scientific (Mississauga, Ont., Canada) and equipped with a Waters 410 refractive index detector (RI), a Waters 600E or a Model 590 solvent delivery system, and a Waters 717 or 712 autosampler.

2.5. HPLC analyses for glucose detection

The appearance of glucose in the cellobiose hydrolysis reactions was monitored by HPLC using the polymeric column ICSep ICE-ION-300, 8 μm (0.78 cm × 30 cm) (Transgenomic, Omaha, NE, USA, supplied by Transition Technologies Inc., Toronto, Ont., Canada), connected to an RI detector. The mobile phase was 0.0085 M sulfuric acid, the flow was maintained constant at 0.5 ml/min and the column temperature was 60 °C. To obtain initial reaction rates, at preset time intervals, aliquots of hydrolysis reaction mixture were injected on the HPLC.

2.6. HPLC analyses for p-nitrophenol detection

The appearance of *p*-nitrophenol in the *p*-NPG hydrolysis reactions was monitored by HPLC because of the low concentrations of *p*-NPG used. A CSC-Inertsil, 150A/ODS2, 5 μm (25 cm × 0.45 cm) HPLC column (supplied by CSC, Montreal, Que., Canada), was used for separation and an RI detector for detection. The mobile phase was 65/35 1 mM acetic acid/acetonitrile (v/v), the flow was maintained constant at 1.4 ml/min and the column temperature was 25 °C. To obtain initial reaction rates, at preset time intervals, aliquots of hydrolysis reaction mixture were injected on the HPLC.

3. Results and discussion

The hydrolysis of cellobiose in succinate buffer at pH 5.0 was performed at room temperature with an initial rate of 0.435 μmol min^{-1} mg protein^{-1} of cellobiose hydrolyzed. The addition of polar miscible co-solvents affects the reaction differently as can be seen in Fig. 1 (solid lines). Hydrolysis is greatly impaired by THF and acetonitrile, the enzyme losing half of its catalytic activity in the presence of only 10% of these solvents. The effect of DMF, DMSO and 2-methyl-2-butanol is less pronounced. Up to 10% of these co-solvents does not affect the initial rate of the reaction. Concentrations higher than 10% of 2-methyl-2-butanol lead to phase separation. DMSO and DMF induce a loss of activity at 20 and 30%, respectively. However, results previously obtained with the same enzyme in synthesis lead to completely different observations [4]. The synthetic rates are almost two orders of magnitude lower than the hydrolysis rates, but no loss of catalytic activity is observed with the addition of DMF, acetonitrile or 2-methyl-2-butanol up to 30%. The immobilization of the almond β-glucosidase on XAD-4, the high glucose concentration and the low water activity of the system can contribute to the protection

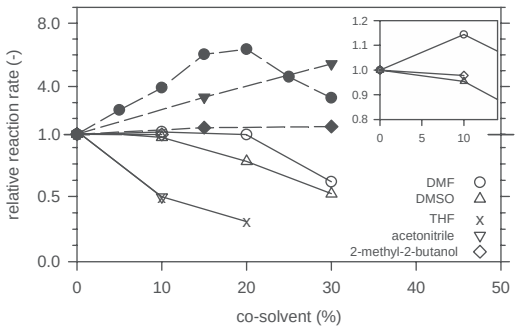

Fig. 1. Effect of the presence of organic miscible co-solvents on the initial enzyme activity in hydrolysis (solid lines) and synthesis (dashed lines). The reference hydrolytic activity of 1 corresponds to the hydrolysis of cellobiose without any addition of co-solvent at ambient temperature at an initial rate of 0.435 μmol min^{-1} mg protein^{-1} (76.6 mM cellobiose; 0.01 M succinate buffer pH 5.0; 0.25 mg solubilized almond β-glucosidase). The reference synthetic activity of 1 corresponds to the synthesis of octyl glucoside in *n*-octanol without any addition of co-solvent at 50 °C at an initial rate of 0.0083 μmol h^{-1} mg protein^{-1} (300 mg glucose; 2 ml *n*-octanol; almond β-glucosidase immobilized on XAD-4; a_W preset at 0.75) [4].

of the enzyme in non-aqueous media. On the contrary, in the hydrolysis experiments, the enzyme (as well as all the other components) was in solution, thus directly exposed to co-solvent, which represents more drastic conditions for the enzyme, even with lower percentages of co-solvents in the media. In addition, the water activity of the media remains high, favoring denaturing processes. This is different from what was observed by Huneke et al. for the hydrolysis of various nitrophenyl glycosides by the β-glycosidase from the thermophilic archaeon *Sulfolobus solfataricus* [6]. In this case, the rate of hydrolysis was increased up to 1.25-fold in the presence of butan-2-one, methyl acetate, and acetonitrile.

In the synthetic reaction, the presence of 2-methyl-2-butanol produces minor changes while the addition of DMF or acetonitrile increases greatly the production rate of octyl glucoside. As shown in Fig. 1 (dashed lines), the presence of acetonitrile up to 30% increases the condensation rate more than five times while 20% DMF increases it by a factor of 6. It has been proposed that the addition of DMF or acetonitrile, both having high dielectric constants (38.2 and 36.6, respectively) raises the dielectric constant of the medium ($\epsilon_{\text{octanol}} = 8.1$) and thus increases the flexibility of the protein, resulting in a higher rate. This is supported by the fact that the addition of 2-methyl-2-butanol ($\epsilon = 5.8$) induces no change in the rate [4]. This explanation, however, is not applicable or at least is not sufficient for the hydrolytic reaction catalyzed by the solubilized enzyme. Considering that the dielectric constant of a mixture of solvents varies linearly with the volume fraction of the components [7,8], we calculated the values of ϵ, for the 10% mixtures of the solvents using the data for the pure solvents at 20 °C [9]. The results are as follows: 2-methyl-2-butanol, 72.8, THF, 72.9, acetonitrile, 75.8, DMF, 76.0, and DMSO, 76.9. As can be seen, the values of the dielectric constant do not change drastically from that of pure water ($\epsilon = 80.2$) for 10% (v/v) of solvent, and the variation does not correlate with the change in enzymatic activity.

We have compared the behavior of the almond β-glucosidase in the hydrolysis of a disaccharide (cellobiose) and *p*-nitrophenyl glucoside (*p*-NPG), a chromogenic substrate commonly used for screening purposes. The steady-state kinetic constants $V_{\max}$ and K_M were determined in the presence of various percentages of DMF with these two substrates. As can be seen in Fig. 2, the rate profiles are completely different. The hydrolysis rate of cellobiose is not affected by the presence of DMF up to 20% and all the curves superimpose. $V_{\max}$ remains constant around $0.5\,\mu\text{mol}\,\text{min}^{-1}\,\text{mg}^{-1}$, and the little changes observed for K_M are very low, considering the errors in the K_M determination. On the other hand, the addition of DMF changes the hydrolysis rate profile of *p*-NPG, resulting mainly in a decrease in $V_{\max}$ from 4 to $1.7\,\mu\text{mol}\,\text{min}^{-1}\,\text{mg}^{-1}$, combined with a slight increase in K_M.

Fig. 3 shows that the specificity constant $V_{\max}/K_M$ remains quite stable for cellobiose while decreasing by almost one order of magnitude for *p*-NPG. This latter observation can be partly explained by the increased solubility of *p*-NPG in the presence of DMF (from 100 to more than 700 mM), the ground state stabilization of the substrate inducing an increase of K_M [10]. However, the fact that DMF

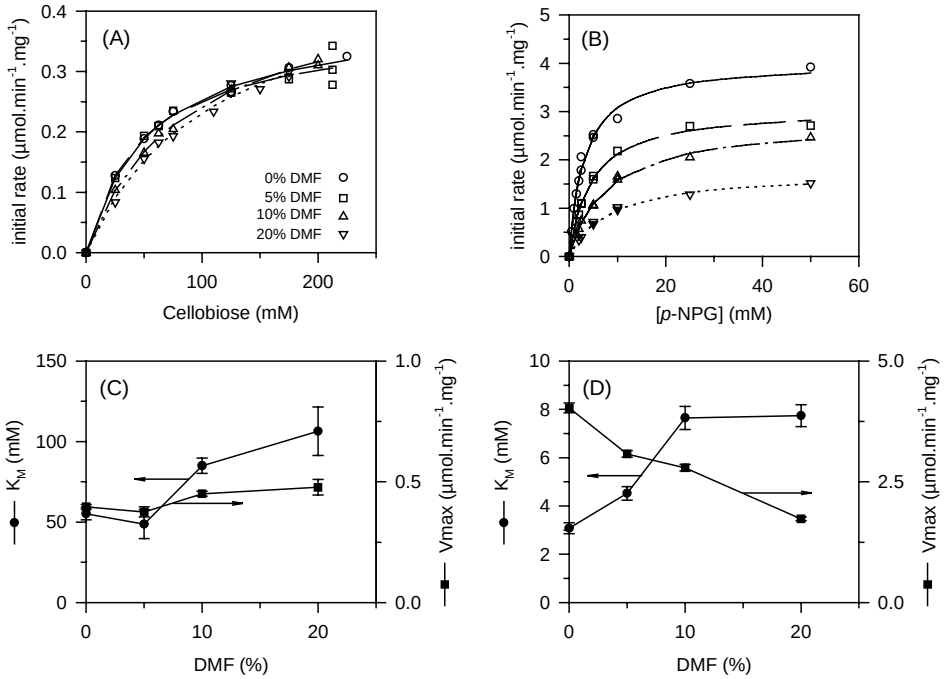

Fig. 2. Effect of DMF on the initial rate of hydrolysis (A, B) and on the kinetic parameters $V_{\max}$ and K_M (C, D) using cellobiose (A, C) or *p*-NPG (B, D) as the substrate.

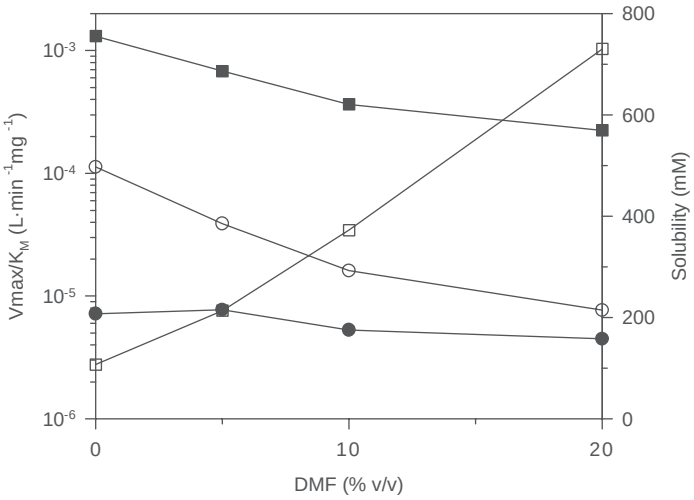

Fig. 3. Variation of V_{max}/K_M (closed symbols) and of the substrate solubility (open symbols) in the presence of DMF. ((■) *p*-NPG, (●) cellobiose, solubility measurements performed at ambient temperature).

decreases V_{max} indicates that the solvent has other effects on the enzyme-catalyzed reaction. This is confirmed by the observation that decreasing solubility of cellobiose (500 mM at 0% and 215 at 30% DMF) does not lead to an increase in V_{max}/K_M that would be expected if ground state stabilization of the substrate was the sole factor.

We also measured $K_M = 250$ mM and $V_{max} = 0.713$ μmol min^{-1} mg^{-1} ($V_{max}/K_M = 2.85\times10^{-6}$ l min^{-1} mg^{-1}) for the hydrolysis of gentiobiose (data not shown). The fact that the crude enzyme obtained from almond has a much higher specificity constant for *p*-NPG (1.31×10^{-3} l min^{-1} mg^{-1}) than for cellobiose (7.18×10^{-6} l min^{-1} mg^{-1}) and gentiobiose indicates that the main activity present in this extract is most probably prunasine hydrolase rather than amygdaline hydrolase. The glycosidic bond in prunasine (D-mandelonitrile-β-D-glucoside) with its aryl aglycon is more similar to the one in *p*-NPG than the 1–6 glycosidic bond between two glucose units cleaved by amygdaline hydrolase in the gentiobiose moiety of amygdaline. It is interesting to note that this extract is slightly more active towards the β 1–4 glycosidic bond of cellobiose than the β 1–6 bond of gentiobiose, although the natural substrate of one of the activities present (amygdalin hydrolase) is hydrolyzed at a β 1–6 bond.

It is known that crude extracts of almond β-glucosidase contain many β-glucosidase activities [11,12], however these enzymes have not been characterized, apart from the fact that at least one of them belongs to family 1 of glycosidases [13]. In another member of the *Prunus* genus, *Prunus serotina*, it has been shown that the seeds contain many β-glucosidases: up to four amygdaline hydrolases and three prunasine hydrolases [14], to which three other prunasine hydrolases present in seedling shoots should be added [15]. However, the seven β-glycosidases from *P. serotina* seeds are not active towards cellobiose and gentiobiose, showing some difference with the almond extract for which we found activity [16,17]. A better understanding of the activity of the various almond β-glucosidases towards their substrates and the linkage with their synthetic activity will require the purification of these enzymes and their kinetic characterization.

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