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Kinetic study of a thermostable β-glycosidase of *Thermus thermophilus*. Effects of temperature and glucose on hydrolysis and transglycosylation reactions

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A β-glycosidase of a thermophile, *Thermus thermophilus*, belonging to the glycoside hydrolase family 1, was cloned and overexpressed in *Escherichia coli*. The purified enzyme (Ttβgly) has a broad substrate specificity towards β-D-glucoside, β-D-galactoside and β-D-fucoside derivatives. The thermostability of Ttβgly was exploited to study its kinetic properties within the range 25–80 °C. Whatever the temperature, except around 60 °C, the enzyme displayed non-Michaelian kinetic behavior. Ttβgly was inhibited by high concentrations of substrate below 60 °C and was activated by high concentrations of substrate above 60 °C. The apparent kinetic parameters (k_{cat} and K_m) were calculated at different temperatures. Both k_{cat} and K_m increased with an increase in temperature, but up to 75 °C the values of k_{cat} increased much more rapidly than the values of K_m. The observed kinetics might be due to a combination of factors including inhibition by excess substrate and stimulation due to transglycosylation reactions. Our results show that the substrate could act not only as a glycosyl donor but also as a glycosyl acceptor. In addition, when the glucose was added to reaction mixtures, inhibition or activation was observed depending on both substrate concentration and temperature. A reaction model is proposed to explain the kinetic behavior of Ttβgly. The scheme integrates the inhibition observed at high concentrations of substrate and the activation due to transglycosylation reactions implicating the existence of a transfer subsite.

Keywords: **β-glycosidase, temperature dependence, kinetics, glucose, transglycosylation, (*Thermus thermophilus*)**

Introduction

Thermozymes are enzymes that thrive in microorganisms growing at temperatures above 60 °C. They have *in vitro* an optimal activity at very high temperatures (between 60 and 120 °C) and are generally more resistant to heat and most common protein denaturants than their counterparts from mesophilic sources [1–3]. The difference in thermostability between homologous enzymes from hyperthermophilic, thermophilic and mesophilic sources has been largely exploited for understanding the structural bases of protein stability [1,4–9]. Very recently, a comparative study of catalytic mechanisms of two β-glucosidases from the hyperthermophilic archaeon, *Pyrococcus furiosus*, and the mesophilic bacterium, *Agrobacterium faecalis*, showed that both enzymes utilize a similar two-step mechanism and that they stabilize similar transition states, suggesting that the active site architecture has evolved far less than the overall protein structure [10].

Due to their potential industrial applications, great effort is devoted to identifying new thermostable glycosidases and to improving the properties of existing ones. To be of effective use, the enzymes must generally possess a good thermostability and an activity at temperatures compatible with the stability of the substrates and products. Therefore, the enzymes from thermophiles (growth at temperatures between 60 and 80 °C) may be preferred to the enzymes from hyperthermophiles (growth at temperatures above 80 °C) since the former are not only thermoresistant but also display an optimal activity at lower temperatures, which can be of great interest for many applications and particularly for carbohydrate hydrolysis and synthesis [11–14]. The fact that glycosidases are often inhibited by the reaction products is also a hindrance to their biotechnological use. For all these reasons, the choice of a

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particular enzyme for developing applications can be guided by its kinetic behavior.

The present report deals with a β-glycosidase from *Thermus thermophilus* (Ttβgly) that we have recently cloned, sequenced and overexpressed in *Escherichia coli* [15]. The recombinant enzyme is monomeric with a molecular mass of 49-kDa. Its amino acid sequence shows strong identity with those of β-glycosidases belonging to the glycoside hydrolase family 1 [15–17]. Ttβgly displays a broad substrate specificity as is the case with most enzymes of family 1. Ttβgly functions optimally at 88 °C, at a pH of 6.5–7.0 and is stable during several days at 70 °C. In this study we take advantage of the thermostability of Ttβ-gly to examine its kinetic characteristics in an extended temperature range. Substrate inhibition or activation kinetics are observed depending on the temperature conditions. We also describe the effects of glucose on the hydrolysis and transglycosylation reactions catalyzed by the enzyme. The results show the practical potential of Ttβ-gly and should be useful for successful applications.

Materials and methods

Microorganisms and plasmids

Escherichia coli and *Thermus thermophilus* HB 27 [18] strains were cultivated in LB medium at 37 and 65 °C respectively. Selection of ampicillin-resistant (100 µg/ml) *E. coli* was performed on LB agar plates. Expression of the tt-β-gly gene was carried out in *E. coli* BL21 (DE3) strain (Novagen) using the vector pET21a (Novagen). Overexpression and purification of the Ttβgly enzyme were reported previously [15].

Chemicals

Synthetic substrates (*p*-nitrophenyl-β-D-glycosides) and monosaccharides (galactose, glucose and fucose) were purchased from Sigma-Aldrich. All other chemicals and reagents were of analytical grade.

Kinetic studies

All kinetic studies were performed with a Kontron Uvikon 860 spectrophotometer, equipped with a cell holder system connected to a circulating water bath which maintained the cuvettes at a constant temperature. Quartz cuvettes of 1-cm path length were employed for all experiments. The rates of enzymatic hydrolysis for all the *p*-nitrophenyl derivatives were determined using a continuous assay. An appropriate concentration of substrate in 1 ml of 100 mM phosphate buffer (pH 7.0) was prewarmed to the desired temperature. Reaction was initiated by the addition of enzyme (5 µl, about 1.3 µg) and the substrate hydrolysis reaction was monitored by measuring the increase in absorbance at 420 nm due to the liberation of *p*-nitrophenoxide anion. The reference cuvette contained all reactants except the enzyme. The calibration curves (*p*NP) were performed under the same conditions of pH and temperature since the proportion of the nitrophenoxide anion present in the total amount of nitrophenol depends not only on pH but also on temperature [19].

The kinetic parameters, K_m and k_{cat}, were determined from Lineweaver-Burk and Eadie representations using at least 10 different substrate concentrations ranging, where possible, from approximately 0.05 mM to 20 mM. Each experimental point was determined at least in triplicate and in all cases the initial rate was used for plotting.

Protein concentration was determined by the bicinchoninic acid method [20] using bovine serum albumin as the standard.

The unit of activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol of *p*-nitrophenyl-β-D-glucoside, used as substrate (1 mM), per minute at 65 °C under the conditions described above.

Capillary electrophoresis

Aliquots of the enzymatic reaction (25 µl) were withdrawn at different times and mixed with 5 µl of 10 mM galactose and 30 µl of derivatizing solution of sugars containing 0.3 g of 4-amino-benzonitrile in a methanol–acetic acid mixture (9.5 : 0.5, v/v) and 0.1 g of sodium cyano-borohydride [21].

These solutions were warmed for 15 min at 90 °C. The analysis of transglycosylation and hydrolysis products generated from *p*-nitrophenyl-β-D-glucoside and *p*-nitrophenyl-β-D-fucoside was performed by capillary electrophoresis using a Beckman P/ACE system 5000 with an uncoated fused-silica capillary (47 cm total length, 40 cm effective length, 75 µm ID). Samples were introduced into the capillary by pressure (0.5 psi) at the cathode end. Separations were performed at 10 kV in the presence of 50 mM sodium tetraborate, pH 10.4 at 25 °C. Substrate and products were detected and quantified by UV-absorbance at 284 nm. Galactose was used as an internal standard. Between runs, the capillary was rinsed for 10 min with 100 mM NaOH followed by water for 5 min and then with 50 mM sodium tetraborate (pH 10.4) for 5 min.

Results

Substrate specificity

The enzyme appears to be highly specific for the β-linked sugars and it catalyzes essentially the hydrolysis of β-D-galactosides, β-D-glucosides and β-D-fucosides as shown previously [15]. The analysis by capillary electrophoresis of enzymatic reactions towards *p*-nitrophenyl-β-D-cellobioside and cellotetraose indicates that the enzyme is an exo-glycosidase since, in addition to the formation of glucose units, only *p*-nitrophenyl-β-D-glucoside and cellotriose were produced at first (data not shown).

The apparent kinetic parameters of Ttβgly were determined at 60 °C using, for purpose of comparison, substrates carrying the same aglycone (*p*-nitrophenyl). The results, reported in Table 1, display that the β-D-glucoside and β-D-fucoside

Table 1. Kinetic parameters of *T. thermophilus* β-glycosidase towards *p*-nitrophenyl-β-D-glycosides.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
p-nitrophenyl-β-D-galactoside	5.6	82.9	14.8
p-nitrophenyl-β-D-glucoside	0.10	23.4	227
p-nitrophenyl-β-D-fucoside	0.12	29.7	247

The experiments were carried out at 60 °C and pH 7.0. The substrate concentration range used was 0.05–10 mM for *p*-nitrophenyl-β-D-glucoside or β-D-fucoside and 1–20 mM for *p*-nitrophenyl-β-D-galactoside. In all cases the enzyme concentration was 1.3 μg.

derivatives are, by far, better substrates than the β-D-galactoside derivative in terms of catalytic efficiency, which is given by the k_{cat}/K_m ratio. In the continuation of this work, the kinetic behavior of Ttβgly was mainly studied with *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc) and *p*-nitrophenyl-β-D-fucopyranoside (*p*NPFuc) as substrates. Both could be used in a large range of substrate concentrations on both sides of the K_m values and the kinetics could be followed at initial velocity during a relatively long reaction time.

Effect of temperature on the kinetic behavior of Ttβgly

The effects of temperature on the kinetics of Ttβgly were investigated between 25 and 80 °C. When the kinetics were performed below 60 °C, Ttβgly was inhibited by high substrate concentrations (Figure 1A). At temperatures higher than 60 °C, the inhibition phenomenon was no longer observed but, on the contrary, an activation was obtained at high substrate concentrations (Figure 1B). Finally, around 60 °C, Ttβgly displayed Michaelian behavior.

The apparent kinetic parameters for the hydrolysis of *p*NPGlc catalyzed by Ttβgly were determined at different temperatures by the double-reciprocal method of Lineweaver-Burk (1/v versus 1/s) and also by the Eadie-Hofstee plot (v versus v/s). In both cases, except for around 60 °C, the graphs were non-linear. Eadie-Hofstee plots showed a biphasic character (graphs not shown), while Lineweaver-Burk plots displayed a concave downward or upward trend depending on the reaction temperature (Figure 2). Data are reported in Table 2 in which K_m^L and K_m^H are the Michaelis constants and k_{cat}^L and k_{cat}^H are the catalytic rate constants, derived by extrapolation from the linear segments of the plots obtained for the low and high substrate concentrations respectively. Both the K_m^L and k_{cat}^L values, which represent the primary activity of the enzyme, increase with an increase in temperature, but the K_m^L values vary much less rapidly than those of k_{cat}^L, so that the catalytic efficiency (k_{cat}^L/K_m^L ratio) is increased 7-fold between 25 and 75 °C. The effect in each case may be due to a combination of factors including inhibition and stimulation due to transglycosylation reactions.

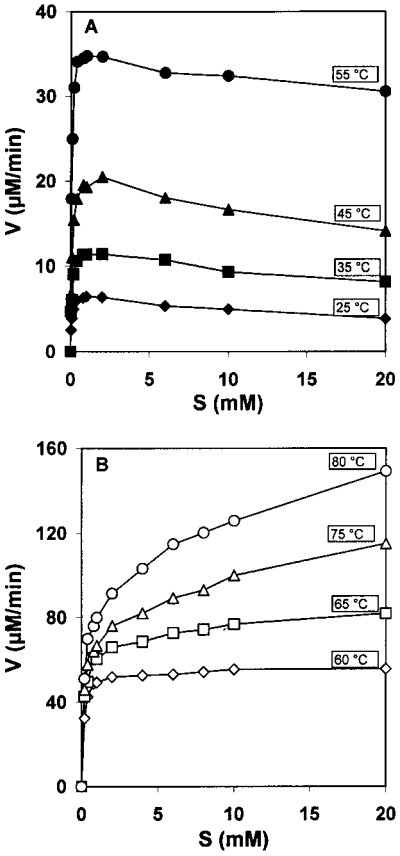


Figure 1. Effect of temperature on the kinetic behavior of *T. thermophilus* β-glycosidase. The experiments were carried out at the temperatures indicated in 100 mM sodium phosphate buffer (pH 7.0) with *p*-nitrophenyl-β-D-glucoside as substrate. Temperature range: A) 25–55 °C; B) 60–80 °C.

Transglycosylation reactions

Activation at high substrate concentrations has already been observed with various β-glycosidases as is the case for the hydrolysis of *p*-nitrophenyl-β-D-glucoside and cellobiose by β-glucosidase (Bgl1) from *Streptomyces* sp [22], of nitrophenyl derivatives of β-D-galactoside, β-D-glucoside and β-D-fucoside by aryl-β-hexosidase from bovine liver [23] or by β-fucosidases from *Achatina balteata* [24], of nitrophenyl-β-D-xylosides and 2,4-dinitrophenyl-3-deoxy-3-fluoro-β-D-glucoside by β-D-glucosidase from *Agrobacterium faecalis* [25,26] and of *p*-nitrophenyl-β-D-xyloside and *p*-nitrophenyl-α-L-arabinoside by β-glucosidase from *Pyrococcus furiosus* [10]. In the case of aryl-β-hexosidase from bovine liver [23] and β-glucosidase from *Agrobacterium faecalis* [25,26] and *Pyrococcus furiosus* [10], the rate enhancement over that expected at higher substrate concentrations was ascribed to transglycosylation reactions. To test this hypothesis, the liberation of both *p*-nitrophenol and sugar was monitored in the Ttβgly-catalyzed kinetics of the hydrolysis of *p*NPFuc. A simple hydrolysis was observed at low substrate concentrations since the ratio of fucose to *p*-nitrophenol was equal to 1.0 while at high substrate concentrations it was lower than 1.0

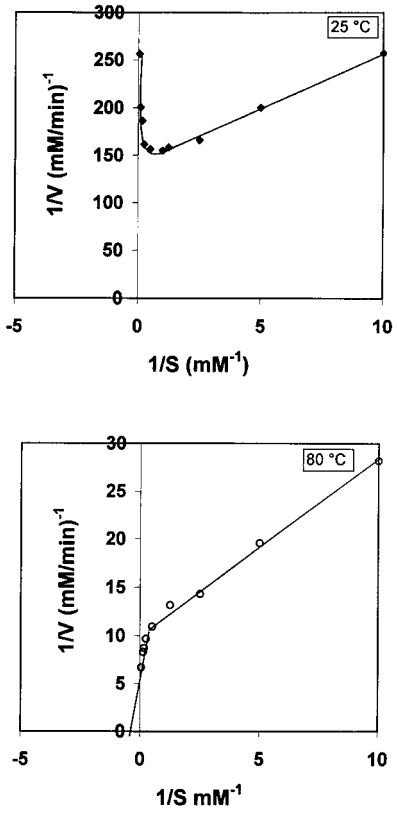


Figure 2. Lineweaver-Burk plots for the hydrolysis of *p*-nitrophenyl-β-D-glucoside by *T. thermophilus* β-glycosidase at 25 °C and 80 °C.

Table 2. Kinetic parameters of *T. thermophilus* β-glycosidase determined at different temperatures.

T °C	K_m^L (μM)	k_{cat}^L (s^{-1})	k_{cat}^L/K_m^L ($mM^{-1}\,s^{-1}$)	K_m^H (*mM*)	k_{cat}^H (s^{-1})	k_{cat}^H/Km^H ($mM^{-1}\,s^{-1}$)
25	79	3.6	46			
35	84	6.5	77			
45	87	11.3	130			
55	95	19.2	202			
60	103	23.4	227			
65	109	27.9	256	0.7	30.5	44
70	117	35.3	301	1.1	43.9	40
75	127	39.0	307	2.6	67.2	26
81	173	50.0	289	2.4	83.4	35

The experiments were carried out at pH 7.0 with *p*-nitrophenyl-β-D-glucoside as the substrate.

involving transglycosylation reactions. The products of transglycosylation were then identified by capillary electrophoresis and mass spectrometry (Figure 3), and corresponded to the transfer of the fucosyl group from the Ttβgly to another molecule of *p*NPFuc, leading to the formation of *p*NPFucFuc. The formation of ($\beta 1 \rightarrow 3$) linkage was largely favored as previously shown by studies performed under conditions of

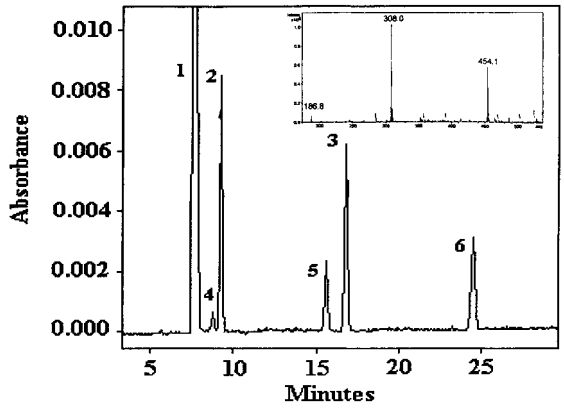


Figure 3. Capillary electrophoresis profile of transglycosylation and hydrolysis products generated from *p*-nitrophenyl-β-D-fucoside (10 mM) by *T. thermophilus* β-glycosidase. The analyzed sample was derived from an enzymatic reaction performed at 75 °C after 30 min-incubation of the substrate in the presence of the enzyme. Peak 1: derivatization reagent (4-aminobenzonitrile) of sugars; Peak 2: *p*-nitrophenyl-β-D-fucoside (*p*NPFuc); Peak 3: internal standard (galactose); Peak 4: *p*NPFuc-Fuc; Peak 5: fucose; Peak 6: *p*-nitrophenol. Inset: ES/MS spectrum: m/z = 308.0 $[M + Na]^+$, substrate (pNPFuc); m/z = 454.1 $[M + Na]^+$, transglycosylation product (pNPFuc-Fuc) and m/z = 186.8 $[M + Na]^+$, hydrolysis product (fucose).

Table 3. Transglycosylation reaction by *T. thermophilus* β-glycosidase.

T °C	*pNP* μ*mol/min*	*Fucose* μ*mol/min*	*pNPFuc-Fuc* μ*mol/min*	*Transglycosylation (%)*
37	0.104	0.087	0.017	16
55	0.108	0.079	0.029	27
75	0.106	0.061	0.045	42

The experiments were carried out in 2 ml of 100 mM sodium phosphate buffer, pH 7.0 with 10 mM *p*-nitrophenyl-β-D-fucoside. Reaction was initiated by addition of the enzyme: 4.2 μg at 37 °C, 1.7 μg at 55 °C and 0.6 μg at 75 °C.

non-initial velocity [27]. The presence of *p*NPFucFuc in the reaction mixture indicates that the substrate acts both as a glycosyl donor and as a glycosyl acceptor. Moreover, the results reported in Table 3 show that the amount of *p*-nitrophenyl-disaccharide obtained at the initial velocity using 10 mM *p*NPFuc increased with temperature.

Effect of glucose on the kinetic behavior of Ttβgly

The fact that the substrate participated not only as a glycosyl donor but also as a glycosyl acceptor prompted us to test for alternate acceptor compounds such as glucose. Addition of the latter to reaction mixtures altered the rate of *p*-nitrophenol formation from *p*-nitrophenyl-glycosides. For a given concentration of glucose, the effect was dependent upon both the concentration of substrate and the temperature of the assay. A decrease in the rate of release of *p*-nitrophenol was observed

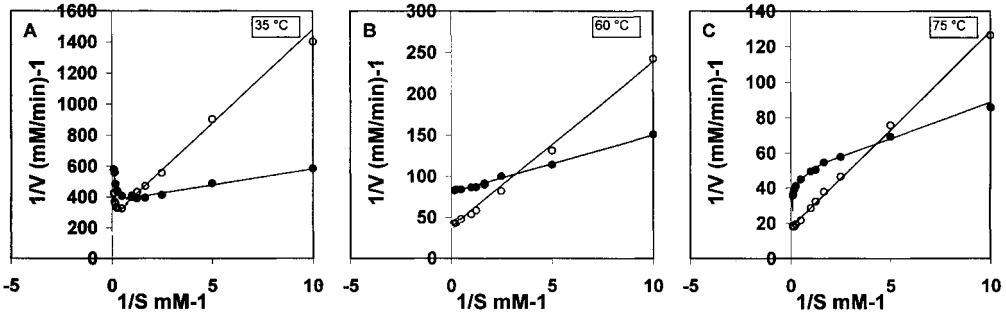


Figure 4. Lineweaver-Burk plots for the hydrolysis of *p*-nitrophenyl-β-D-glucoside by *T. thermophilus* β-glycosidase in the presence of glucose. The experiments were performed at 35 °C (A), 60 °C (B) and 75 °C (C) with glucose (●) 0 mM, (○) 100 mM.

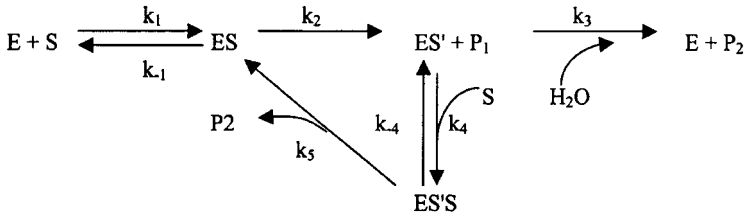
below a critical concentration of substrate. Above this substrate concentration, addition of glucose resulted in an increased rate of *p*-nitrophenol release. The critical concentration of substrate decreases as the temperature of the reaction rises. Thus, an activation by 100 mM glucose occurred from a *p*NPGlc concentration of 1.2 mM at 35 °C, 0.3 mM at 60 °C and 0.2 mM at 75 °C (Figure 4). In addition, the transfer of the fucosyl residue from the fucosyl-enzyme intermediate to the sugar moiety of *p*NPFuc could be inhibited by the addition of 100 mM glucose to the incubation medium.

Discussion

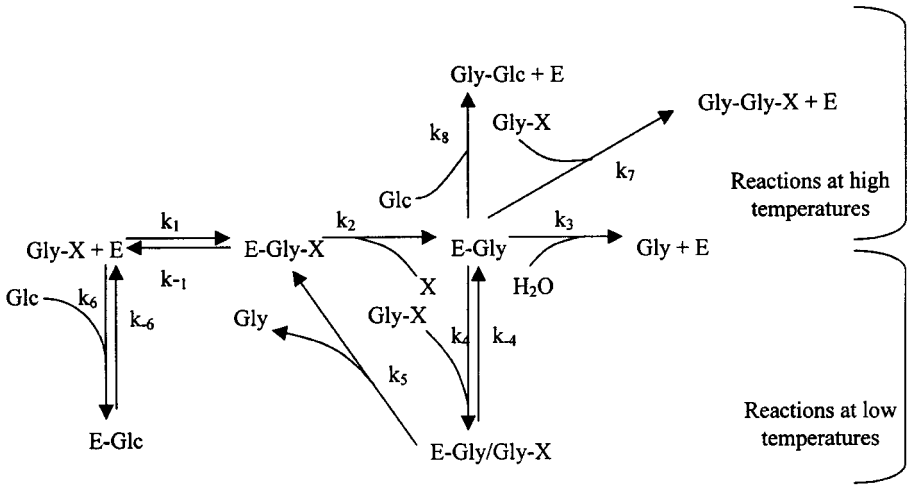
Much research has been devoted to the thermostability of enzymes from thermophilic bacteria due to their potential industrial applications. The studies have provided useful information about the structural factors affecting their thermostability [2–9]. Much less attention has been focused on the dependence of the kinetic behavior of thermoenzymes on temperature. Only a few studies have taken advantage of the thermostability and thermophilicity of thermophile β-glycosidases to study their kinetic properties at different temperatures [28–30]. Such a study was performed with a thermostable enzyme from *Sulfolobus solfataricus* [30]. The kinetic data showed no atypical behavior of the enzyme in the studied temperature range (30–80 °C). However, kinetic behavior similar to that of Ttβgly was reported for a β-galactosidase from the extreme thermoacidophile archae *Caldariella acidophila* [29]. The enzyme was inhibited by excess substrate at low temperatures and was activated by excess substrate at high temperatures. The model which was proposed by the authors to explain the kinetic behavior of the enzyme assumes the binding of an additional substrate molecule to the glycosyl-enzyme intermediate (Scheme 1).

This model, that was first proposed by Krupka and Laidler [31] to interpret the partial inhibition observed with acetylcholinesterase at high substrate concentration, can explain both the activation and the inhibition by excess substrate depending on whether the rate of dissociation of the ES'S ternary complex is higher or lower than the rate of deglycosylation of the ES' intermediate. However, such a scheme does not integrate the transglycosylation reactions observed with Ttβgly for which the substrate itself can serve as a glycosyl acceptor.

Ttβgly belongs to the glycoside hydrolase family 1 that brings together a large number of β-glycosidases characterized by a wide range of substrate specificity and quite a high degree of sequence homology [16,17]. In addition, these enzymes catalyze glycosidic linkage hydrolysis as well as transglycosylation reactions, namely the transfer of glycosyl residues from glycoside substrates to acceptors other than water [10,25,26,32]. Thus the unconventional kinetics observed with β-glycosidases has often been ascribed to a mechanism of transglycosylation involving the existence of a specific acceptor-binding subsite [23,33–35]. This explanation is reinforced by the recent study of the crystal structure of β-glycosidase A from *Bacillus polymyxa* (Bg1A) which is also a member of family 1 of the glycoside hydrolases [36]. The geometry of the active site cavity of Bg1A reflects both hydrolytic and transglycosylating activities with the existence of subsites able to accommodate substrates longer than disaccharides and to explain the transglycosylation reactions.



Scheme 1.



Scheme 2.

Likewise Ttβgly is capable of hydrolyzing short-chain oligosaccharides and the enzyme presents a high degree of amino acid sequence identity (42.6%) with *Bacillus polymyxa* (Bg1A) [15]. The identity was much more high (86%) at the level of the amino acid residues of the two active sites as demonstrated by structural studies from Bg1A (Protein Data Base : 1BGA) [36,37] and sequence alignments. These data, together with the fact that homologous enzymes from mesophilic and thermophilic sources have an active site architecture that has evolved far less than the overall protein structure [10], suggest that the topography of the active site of Ttβgly might be very similar to that of *Bacillus polymyxa* (Bg1A).

The data reported in this study support a model (Scheme 2) in which Ttβgly could be partially inhibited by excess substrate whatever the temperature, but with the increase of this parameter, a transfer subsite might become effective and catalyze transglycosylation reactions leading to the autocondensation of substrate (formation of *p*-nitrophenyl-disaccharides).

Scheme 2 is based on the clearly established mechanism of retaining glycosidases. The first step leads to formation of the Michaelis complex (E-Gly-X) while the second step involves the liberation of the aryl aglycone (X = *p*NP) and the simultaneous formation of the glycosyl-enzyme intermediate (E-Gly). At low temperatures and at high concentrations of substrate (Gly-X), the phenomenon of inhibition (E-Gly/Gly-X) was essentially observed while at higher temperatures, the transglycosylation reactions became predominant (formation of Gly-Gly-X).

Glucose, when it was included in the assay medium, could behave as an inhibitor at the active site of TtβGly (E-Glc) but also as a glycosyl acceptor at a transfer subsite leading to the formation of disaccharides (Gly-Glc). For a given concentration of glucose (e.g. 100 mM), the effect of carbohydrate on the kinetic behavior of TtβGly depended on both substrate concentration and temperature. At low concentrations of substrate, glucose competed with the substrate for its binding to the active site of TtβGly while from a critical substrate concentration, glucose behaved especially as a glycosyl acceptor at the transfer subsite.

Since the observed activation became greater as the temperature of the reaction mixture increased, we postulate that the accessibility at the transfer subsite is dependent on temperature. To support this interpretation, structural and molecular modelling studies on TtβGly are now in progress.

Acknowledgements

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