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Studies on the transglucosylation reactions of cassava and Thai rosewood β-glucosidases using 2-deoxy-2-fluoro-glycosyl-enzyme intermediates

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Abstract

β-Glucosidases from cassava and Thai rosewood can synthesize a variety of alkyl glucosides using various alcohols as glucosyl acceptors for transglucosylation. Both enzymes were inactivated by 2-deoxy-2-fluoro-sugar analogues to form the covalent glycosyl-enzyme intermediates, indicating that the reaction mechanism was of the double-replacement type. The trapped enzyme intermediates were used for investigating transglucosylation specificity, by measuring the rate of reactivation by various alcohols. The glucosyl-enzyme intermediate from the cassava enzyme showed a 20- to 120-fold higher rate of glucose transfer to alcohols than the glucosyl-enzyme intermediate from the Thai rosewood enzyme. Kinetic analysis indicated that the aglycone binding site of the cassava enzyme was hydrophobic, since the enzyme bound better to more hydrophobic alcohols and showed poor transfer of glucose to hydrophilic sugars. With butanol, transglucosylation was faster with the primary alcohols than with the secondary or tertiary alcohol. Studies with ethanol and chloro-substituted ethanols indicated that the rate of transglucosylation was significantly faster with alcohols with lower pK_a values, where the reactive alkoxide was more readily generated, indicating that the formation of the alkoxide species was a major step governing the formation of the transition state in the cassava enzyme.

Keywords: Cassava linamarase; Transglucosylation specificity; Alkyl-β-D-glucoside synthesis; 2-Deoxy-2-fluoro-α-D-glucopyranosyl-enzyme intermediate

β-Glucosidases (EC 3.2.1.21) hydrolyze glycosidic bonds between non-reducing terminal glucose residues and alcohols of various structures. This reaction occurs by cleavage of the substrate to form a glucosyl-enzyme intermediate, which is typically hydrolyzed by water to yield glucose [1,2]. However, under suitable conditions, the glucose moiety may also be transferred to other acceptors, such as alcohols or sugars, to form glucosides or oligosaccharides by transglucosylation [3,4]. There appears to be no evidence that glycosyl hydrolases catalyze transglucosylation of alcohols in vivo. On the contrary, synthesis of alkyl glucosides in vivo, for example, the cyanogenic glucosides [5,6], tends to be catalyzed by other enzymes, such as glucosyl transferases, rather than to result from reverse hydrolysis or transglucosylation by family 1 glycosyl hydrolases. However, many alkyl β-glucosides have interesting potential applications, for example in solubilization of biological membranes [7], as drug carriers [8], and as synthetic flavor precursors [9]. Accordingly, enzymatic synthesis of anomerically pure alkyl glucosides by β-glucosidases is of much interest, since the procedure is stereo- and regio-specific, and may be easily carried out under mild conditions, without requiring protection and deprotection of the hydroxyl

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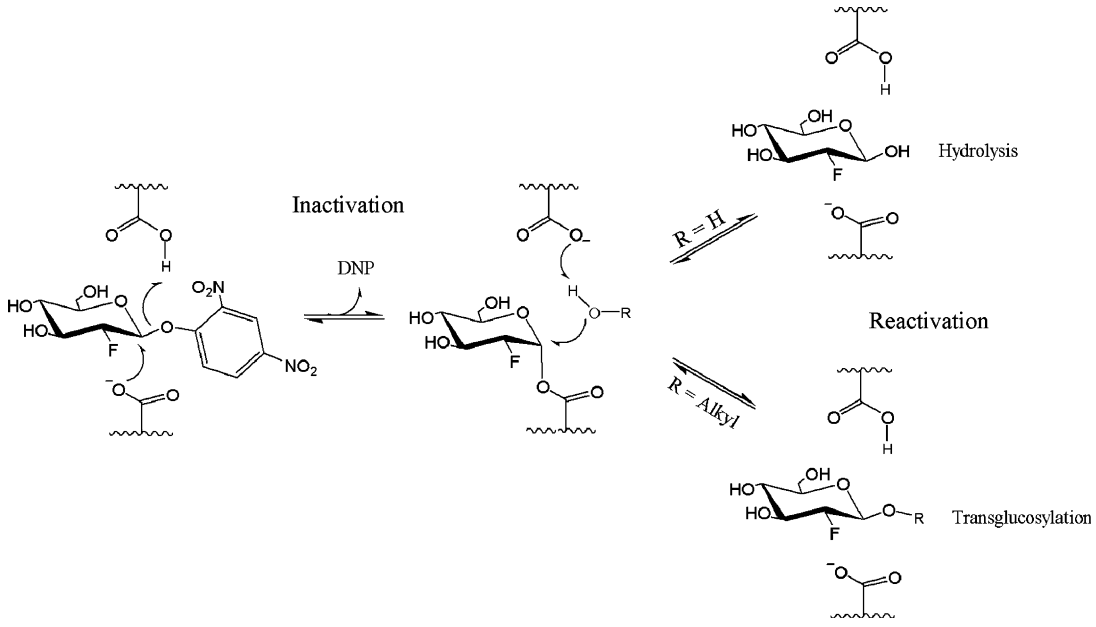


Scheme 1. Inactivation and reactivation mechanisms of β-glucosidase. The inactivator, 2FDNPG, is cleaved during the inactivation step (glycosylation of the enzyme) resulting in the accumulation of covalent glycosyl-enzyme intermediate. In the reactivation step, the activity of the enzyme is slowly recovered by hydrolysis, but may be regained more rapidly by transglucosylation with a suitable alcohol acceptor.

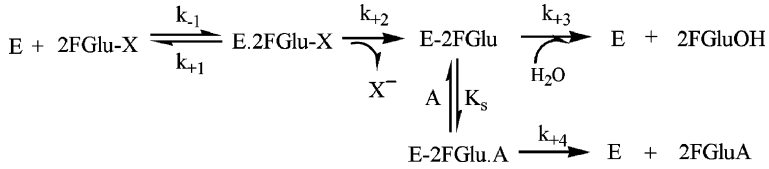
groups [10,11]. Our previous work has shown that β-glucosidases from Thai rosewood (*Dalbergia cochinchinensis* Pierre) and cassava (*Manihot esculenta* Crantz) can synthesize a variety of alkyl-β-D-glucosides via transglucosylation of alcohols, with different enzymes showing different specificities toward the glucosyl acceptor [12,13]. Remarkably, cassava β-glucosidase (linamarase) is the only β-glucosidase described, which can produce alkyl-β-D-glucosides from tertiary alcohols in high yields [13].

Studies of various glycosidases suggest that both glycone and aglycone binding sites of these enzymes are important in determining specificity and rate of catalysis [14–16]. The specificity of glycosidases for the glycone moiety (glycosyl donor) during cleavage of glycosides may be conveniently studied by using nitrophenyl glycosides that generate yellow colored products when cleaved [17–19]. However, the specificity for the aglycone moiety (glycosyl acceptor) in transglycosylation reactions has not been well studied because the reaction does not generally produce any absorbance change. Previously, the equilibrium yield of alkyl glucoside product formed from transglucosylation was used as an indicator of the specificity of the enzyme towards the aglycone moiety [20,21]. However, such values are not direct measures for comparing the reactivity of β-glucosidases toward different alcohol substrates, because the newly formed products may also be further degraded by secondary hydrolysis with time.

Withers et al. [22–25] have developed mechanism-based glycosidase inactivators, 2-deoxy-2-fluoroglycosides, which have been shown to inactivate the enzyme via the accumulation of covalently linked glycosyl-enzyme intermediates, that are hydrolyzed at very low rates. However, the inactivated enzyme, which is catalytically competent, can be rescued in the presence of suitable glycosyl acceptors leading to the reactivation of the enzyme (Scheme 1). These mechanism-based inactivators have been used as affinity-labels to unequivocally identify the active site nucleophiles in a wide variety of glycosidases [26]. The trapped glycosyl-enzyme intermediate has been also applied for rapid screening of the glucosyl acceptor specificity of a variety of glycosidases, such as *Agrobacterium* β-glucosidase, *Bacillus circulans* β-galactosidase, *Cellulomonas fimi* β-mannosidase and xylanase/glucanase, *Xanthomonas manihotis* β-galactosidase, and *Streptomyces lividans* cellulase, toward various saccharides and glycosides by measuring the rate of reactivation of the glycosyl-enzyme intermediates [27]. Glucosyl acceptors that can produce a higher regain of enzyme activity are those which have better specificity and reactivity for the transglucosylation reaction.

In this study, we have investigated the transglucosylation activities of Thai rosewood β-glucosidase (dalcochinase) and cassava β-glucosidase (linamarase) by initially reacting the enzymes with 2′,4′-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside (2FDNPG)[1] to

[1] *Abbreviations used:* 2FDNPG, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside; 4-MuGlu, 4-methylumbelliferyl-β-D-glucopyranoside; 4-Mu, 4-methylumbelliferone; E, free enzyme; 2FGlu-X, 2-deoxy-2-fluoroglucosides with alkyl group substituents; E-2FGlu, 2-deoxy-2-fluoro-glucosyl-enzyme intermediates; 2FGluOH, 2-deoxy-2-fluoro-glucopyranoside; 2FGluA, 2-deoxy-2-fluoro-glucoside with glucosyl acceptor (a product from the reaction of E-2FGlu with alcohols).



Scheme 2. Kinetic scheme for inactivation and reactivation of β-glucosidase. Reaction of the active enzyme with the inactivator 2FDNPG (here shown as 2FGlu-X) yields the inactive E-2FGlu intermediate. The dissociation constant for the inactivator (K_i) can be described by the term k_{-1}/k_{+1}. Values for the inactivation rate constant (k_i) can be represented by k_{+2} which is the first-order rate constant for formation of the glucosyl-enzyme. This intermediate can be further decomposed by hydrolysis with water to yield 2FGluOH with rate constant k_{+3}. In the presence of ligand, such as alcohol A, transglucosylation yielding 2FGluA can occur with a rate constant k_{+4} and a ligand dissociation constant represented by K_s.

trap the enzyme in the form of the covalent 2-deoxy-2-fluoro-glucosyl-enzyme intermediate (E-2FGlu) (Scheme 2). The active enzyme (E) can be regenerated by hydrolysis with water to yield 2-deoxy-2-fluoro-glucose (2FGluOH) or by transglucosylation with an alcohol (A) to yield the alkyl-2-deoxy-2-fluoro-glucoside (2FGluA). The kinetic parameters of the transglucosylation reaction have been determined and compared for different alcohols. This report is the first investigation of the transglucosylation specificity of β-glucosidases toward non-carbohydrate acceptors by measuring the kinetic constants of the transglucosylation reaction.

Materials and methods

Reagents

All glucosyl acceptors used in the reaction mixture were of analytical grade. Enzyme inactivator, 2′,4′-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside (2FDNPG), and 4-methylumbelliferyl glucosides substrate (4-MuGlu) were purchased from Sigma–Aldrich Co. Cassava linamarase and Thai rosewood dalcochinase were purified in our laboratory as reported elsewhere [18,19].

Measurement of enzyme activity by continuous fluorimetric assay

All enzymatic assays were performed at 30 °C in 0.1 M sodium acetate buffer, pH 5.5, in a final volume of 1 ml. A continuous fluorimetric assay based on the hydrolysis of 4-methylumbelliferyl glucosides (4-MuGlu) at saturable concentrations was used to monitor enzyme activity by measuring the rate of 4-methylumbelliferone (4-Mu) release [28] (excitation wavelength = 350 nm, emission wavelength = 450 nm, and slit width = 3 nm), using a spectrofluorometer (SHIMADZU, RF-5301PC) equipped with a circulating-water bath. Controls, in which the enzyme solution was replaced by acetate buffer, showed that the rate of non-enzymatic hydrolysis of 4-MuGlu was negligible. Fluorescence readings were taken at 1 s intervals for 60 s and data were converted to ASCII format, followed by analysis using linear regression with the Prism 3 program (Graphpad software). Enzyme activity was calculated from the slope of fluorescence increase.

Inactivation kinetics

The inactivation kinetics of each β-glucosidase was measured by incubating the enzyme with several concentrations of 2FDNPG at 30 °C in 0.1 M sodium acetate buffer, pH 5.5, and assaying the residual activity of these enzymes at different time intervals. The concentrations of 2FDNPG used were in the 0.5–2.5 μM range, while enzyme concentration in the inactivation reaction was typically about 100–200 μg/ml, to maintain a pseudo-first-order condition. The inactivation reaction appeared to proceed as a first-order decay since the plot of ln V/V_0 against time was linear (where V and V_0 represent the initial velocity of the reaction in the presence and the absence of inhibitor, respectively). The observed rate constants (k_{obs}) at each inactivator concentration were determined from the slope of the plot of ln V/V_0 versus time. Rate constants for the inactivation step (k_i) and the dissociation constant for the inactivator (K_i) were calculated from the double reciprocal plot of Eq. (1) [22].

$$k_{obs} = k_i[I]/(K_i + [I]). \quad (1)$$

Preparation of trapped glucosyl-enzyme intermediate

The trapped 2-deoxy-2-fluoro-glucosyl-enzyme intermediates (E-2FGlu) were freshly prepared by incubating the enzymes with 2FDNPG (50–100 μM) until <5% of the original activity remained. Then, excess 2FDNPG was removed by concentrating the mixture to a volume of 50–100 μl using an ultrafiltration unit with molecular weight cut-off of 30 kDa (Millipore), and fresh buffer was added to make up the final volume to 1–2 ml. This process was repeated 2–3 times to ensure that excess inactivator was completely removed. The trapped intermediates were kept at 4 °C prior to use for enzymatic studies.

Analysis of transglucosylation reaction

Aliquots of the E-2FGlu were incubated at 30 °C in the presence of 0.1 M acetate buffer alone (control) or

in the presence of 0.01–2.5 M alcohols. These concentrations of alcohols were chosen to maintain pseudo-first-order conditions for the transglucosylation and to keep all incubation mixtures fully miscible as single-phase solutions. When the normal active enzyme was incubated with alcohols at the concentrations specified, there was no significant loss of activity due to protein denaturation. In the transglucosylation reaction, alcohol reacts with the E-2FGlu to yield the active enzyme (E) and the 2FGluA product, while water can also hydrolyze the trapped glucosyl-enzyme to produce E and 2FGluOH in the hydrolysis pathway (Scheme 2). However, the control experiment, where only acetate buffer was incubated with the E-2FGlu, produced only negligible amounts of active enzyme (see Results). Therefore, the amount of the active enzyme ([E]) regained over time was derived mainly from the transglucosylation pathway and may be used to represent the progress of the transglucosylation reaction. Since, the concentration of E was directly related to the initial velocity of the assay reaction (V), values of V were used to follow the progress of the transglucosylation occurring after addition of alcohol.

Results

Inactivation kinetics

Incubation of cassava and Thai rosewood enzymes with excess 2FDNPG resulted in time-dependent inactivation of the enzyme in a pseudo-first-order manner (Fig. 1A). The value of k_{obs} increased with higher concentration of 2FDNPG (Fig. 1A) but approached a limiting value (data not shown), indicating that the inactivation reaction involved at least two-steps (Scheme 2). Rate constants for the inactivation could be calculated from Eq. (1) [22]. Data were analyzed by plotting the $1/k_{obs}$ versus 1/[2FDNPG] (Fig. 1B) to yield the value of K_i and k_i according to the double reciprocal of Eq. (1). The k_i and K_i for the reaction of each enzyme with 2FDNPG were as follows: cassava β-glucosidase, $k_i = 0.28 \pm 0.02\ min^{-1}$, $K_i = 6.78 \pm 0.07\ \mu M$; Thai rosewood β-glucosidase, $k_i = 0.82 \pm 0.35\ min^{-1}$, $K_i = 15.9 \pm 0.3\ \mu M$.

Protection from inactivation by δ-gluconolactone

We investigated whether gluconolactone, a competitive inhibitor of β-glucosidase hydrolysis of *p*-nitrophenyl-β-D-glucoside and 4-methylumbelliferyl glucoside [18,19], could protect the enzymes from inactivation by 2FDNPG. The experiment was performed by adding 50 μM δ-gluconolactone into the inactivation reaction using 2.5 μM 2FDNPG and comparing the rate of inactivation to that of the control reaction without δ-gluconolactone. The results showed that under these conditions,

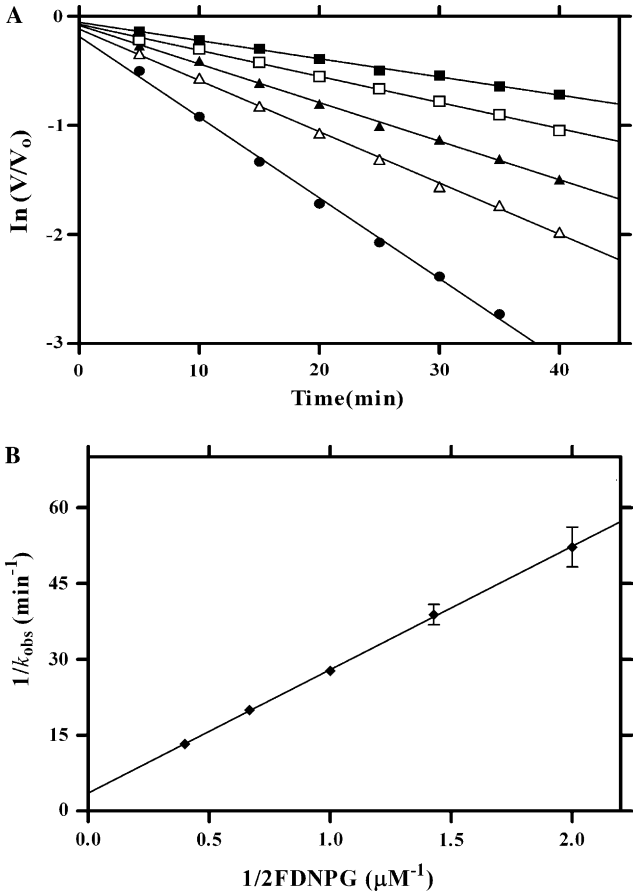


Fig. 1. Inactivation of cassava linamarase by 2FDNPG. The enzyme (100–200 μg/ml) was incubated with several concentrations of 2FDNPG (0.5–2.5 μM) at 30 °C in 0.1 M sodium acetate buffer, pH 5.5, and residual activity of the enzyme was assayed at different times. (A) Semi-logarithmic plot of residual activity versus time over the range of inhibitor concentrations: ■, 0.5 μM; □, 0.7μM; ▲, 1.0 μM; △, 1.5 μM; ●, 2.5 μM. (B) Double-reciprocal plot of the first-order rate constants (k_{obs}) from (A) versus 2FDNPG concentration.

the rate of enzyme inactivation by 2FDNPG was decreased by ~50% in presence of δ-gluconolactone (data not shown). This indicated that the two inhibitors compete for the same active site. The k_{obs} of inactivation was decreased from $0.074 \pm 0.002\ min^{-1}$ to $0.039 \pm 0.001\ min^{-1}$ for cassava β-glucosidase and from $0.116 \pm 0.001\ min^{-1}$ to $0.063 \pm 0.001\ min^{-1}$ for Thai rosewood β-glucosidase.

Validity of using 2-deoxy-2-fluoro-glucosyl-enzyme intermediates for studying transglucosylation

To demonstrate that the trapped intermediates of cassava and Thai rosewood β-glucosidases were not irreversibly inactivated and could regain their normal catalytic function, the trapped E-2FGlu was incubated with 0.5 M ethanol, as described in Material and methods. Progress of the reaction was measured from the activity regained (V) [27] at various times after the start

of the incubation. To correct for possible variations in the amount of enzyme used in each experiment, results were expressed relative to the estimated full activity of the trapped enzyme (V_0), which was calculated from protein absorbance at 280 nm after removing excess inhibitor, multiplied by the initial activity of the enzyme prior to inhibition. The results showed that the activity could be recovered over time after incubation with ethanol (Fig. 2A), and that the plot of $\ln[(V_0 - V)/V_0]$ versus time was linear (Fig. 2B).

Studies were also performed on the stability of the E-2FGlu intermediate to hydrolysis by water (upper part of Scheme 2). Results showed that the hydrolysis reaction proceeded slowly and also appeared to be first order (Fig. 2B). Rate constants for reactivation ($k_{re,obs}$) of cassava linamarase and Thai rosewood dalcochinase by the hydrolysis reaction were calculated to be 0.000596 min^{-1} ($t_{1/2} = 19$ h) and 0.000236 min^{-1} ($t_{1/2} = 48$ h) at 30 °C, respectively, indicating that the intermediate trapped

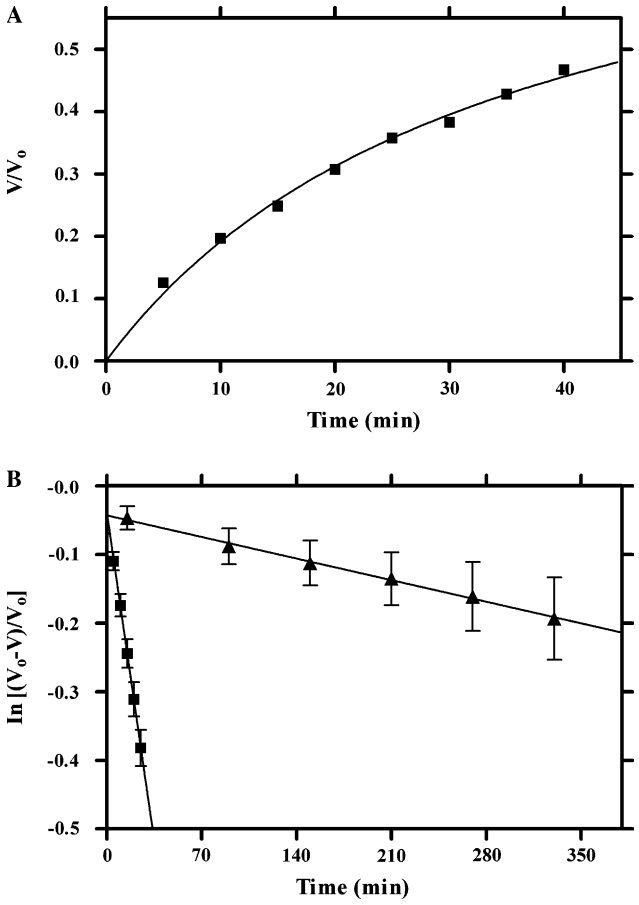


Fig. 2. Reactivation of inactivated cassava linamarase by 0.5 M ethanol at various times. Aliquots of the E-2FGlu were incubated at 30 °C in the presence of 0.1 M acetate buffer alone (control) or in the presence of 0.5 M ethanol and assayed for the return of enzyme activity at various times. (A) Direct plot of relative activity regained versus time. (B) Semi-logarithmic plot of activity versus time: (▲) buffer alone; (■) 0.5 M ethanol. The value of $k_{re,obs}$ was determined from the linear slope of the regression line.

was quite stable towards hydrolysis ($k_3 \ll k_2$,Scheme 2) and valid for use in our study. Nevertheless, the trapped glucosyl-enzyme intermediate was freshly prepared, kept at 4 °C, and normally used within a day to avoid interference from hydrolysis. Other glycosidases also showed a similar range of half-life for hydrolysis, such as *Escherichia coli* β-galactosidase ($t_{1/2} = 11.5$ h) [29], *Candida albicans* exo-β-1,3-glucanase ($t_{1/2} = 9.4$ h) [30], and *Cellulomonas fimi* β-mannosidase ($t_{1/2} = 5.7$ h) [31].

Transglucosylation of cassava and Thai rosewood β-glucosidase

The transglucosylation activities of Thai rosewood and cassava β-glucosidases were compared by incubating the trapped E-2FGlu with various alcohols at a fixed concentration of 0.5 M. All reactions appeared to be first order since the plots of $\ln[(V_0 - V)/V_0]$ versus time were linear (data not shown). The observed rate constants for reactivation ($k_{re,obs}$) due to transglucosylation were calculated from the slope of the plots of $\ln[(V_0 - V)/V_0]$ versus time. Table 1 indicated that for cassava enzyme, tranglucosylation of all alcohols at a concentration of 0.5 M proceeded at least 10 times faster than the hydrolysis reaction. However, for the Thai rosewood enzyme, transglucosylation was not dramatically faster than hydrolysis. Under the conditions used, the transglucosylation of cassava enzyme with *iso*-butanol had the highest $k_{re,obs}$ value among all alcohols studied, with a rate constant of $958 \pm 152 \times 10^{-4}$ min^{-1}, approximately 160-fold faster than simple hydrolysis. However, with the Thai rosewood enzyme, the highest transglucosylation rate, found with *n*-butanol, was only 6- to 7-fold faster than the hydrolysis reaction (Table 1). This indicates that cassava linamarase has a remarkable preference for transglucosylation by alcohols, compared to hydrolysis, which agrees with our previous observations that cassava β-glucosidase was the only enzyme that could transglucosylate tertiary alcohols [13]. The $k_{re,obs}$ for transglucosylation of *tert*-butanol by cassava enzyme was approximately 8-fold faster than simple hydrolysis, while Thai rosewood enzyme did not show any substantial difference in the $k_{re,\ obs}$ (Table 1).

Transglucosylation by glucose and fucose was also studied (Table 1), but with both enzymes, these sugars acted as poor glucosyl acceptors compared to the alcohols, since $k_{re,obs}$ of their reactions were similar to those of hydrolysis. Studies of the inhibitory effect of D-glucose on the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) by cassava enzyme showed that D-glucose behaved as a competitive inhibitor (data not shown). This indicates that D-glucose only competes with *p*-NPG for the free form of enzyme, so that it is likely that the glucose moieties of both compounds bind to the glycone site. This result also indicates that there is insignificant binding of D-glucose to the glucosyl-enzyme

Table 1
The observed reactivation rate constants ($k_{re,obs}$) for a series of 0.5 M alcohol and monosaccharide acceptors, comparing between cassava linamarase and Thai rosewood dalcochinase

Acceptor	$k_{re,obs}$ (min^{-1})	
	Cassava enzyme	Thai rosewood enzyme
Control (no acceptor)	$(6 \pm 1) \times 10^{-4}$	$(2.3 \pm 0.3) \times 10^{-4}$
Methanol	$(62 \pm 13) \times 10^{-4}$	$(2.7 \pm 0.4) \times 10^{-4}$
Ethanol	$(137 \pm 23) \times 10^{-4}$	$(3.6 \pm 0.2) \times 10^{-4}$
n-Propanol	$(600 \pm 98) \times 10^{-4}$	$(6.8 \pm 0.8) \times 10^{-4}$
n-Butanol	$(444 \pm 83) \times 10^{-4}$	$(15.0 \pm 0.5) \times 10^{-4}$
2-Butanol	$(244 \pm 46) \times 10^{-4}$	$(3.7 \pm 0.4) \times 10^{-4}$
tert-Butanol	$(47 \pm 6) \times 10^{-4}$	$(3.1 \pm 0.4) \times 10^{-4}$
iso-Butanol	$(958 \pm 152) \times 10^{-4}$	$(8.7 \pm 0.5) \times 10^{-4}$
Glucose	$(7 \pm 2) \times 10^{-4}$	$(2.8 \pm 0.3) \times 10^{-4}$
Fucose	$(9 \pm 2) \times 10^{-4}$	$(3.6 \pm 0.2) \times 10^{-4}$

form (a similar intermediate to the trapped E-2FGlu), where D-glucose would be required to bind at the aglycone site. This again agrees well with data in Table 1 that the aglycone binding site of cassava linamarase prefers to bind to alcohols more than to sugars.

Rate constants for transglucosylation of cassava β-glucosidase

Since, the cassava enzyme was so efficient in catalyzing the transglycosylation reaction using various alcohols, we were interested to determine the rate constants associated with each step in transglucosylation. When the trapped E-2FGlu was incubated at 30 °C in presence of various concentrations of different alcohols, the active enzyme (E, Scheme 2) was regenerated as the transglucosylation proceeded. The initial velocity of the enzyme regained (V) was used to represent the amount of E formed from the transglucosylation pathway, since the control experiment showed that the hydrolysis reaction was relatively slow (Table 1 and previous section). The observed pseudo-first-order return of activity ($k_{re,obs}$) at each concentration of alcohol acceptor was determined from the slope of the plots of ($\ln [(V_0 - V)/V_0]$) as described in the previous section.

Kinetics of reactivation based on Scheme 2 was analyzed according to Eq. (2) [23]. To simplify the calculation, the term k_3 was neglected, since spontaneous hydrolysis occurred much more slowly than transglucosylation ($k_3 \ll k_4$) Under these circumstances, k_4 (transglucosylation rate constant) and K_s (dissociation constant of ligand) can be obtained by fitting the observed rate constants for reactivation to Eq. (3) (where [A] represents alcohol concentration).

$$\text{rate} = -\frac{\mathrm{d}[\text{E-2FGlu}]}{\mathrm{d}t} = \left(k_3 + \frac{k_4[\text{A}]}{K_s + [\text{A}]}\right)[\text{E-2FGlu}]. \quad (2)$$

$$k_{re,obs} = k_4[\text{A}]/(K_s + [\text{A}]). \quad (3)$$

As shown for the transglucosylation reaction with *n*-butanol, the reaction appeared to be pseudo-first-order as the plot of ($\ln [(V_0 - V)/V_0]$) versus time was linear (Fig. 3A). When $k_{re,obs}$ was plotted as a function of *n*-butanol concentration, the rate constant was shown to depend on the concentration of *n*-butanol in a saturable fashion (Fig. 3B). This indicated that transglucosylation required at least two-steps (lower part, Scheme 2). With cassava enzyme, the rate of the hydrolysis was relatively small ($\sim 6 \times 10^{-4}\ min^{-1}$) when compared to the range of $k_{re,obs}$ found with all alcohols (~ 60–$960 \times 10^{-4}\ min^{-1}$), so that values of k_4 (transglucosylation rate constant) and K_s (dissociation constant of alcohol) can be analyzed according to Eq. (3). Data from the *n*-butanol reaction were analyzed according to the direct plot (Fig. 3B). Results yielded values of 184 mM for K_s and $58 \pm 1 \times 10^{-3}\ min^{-1}$ for k_4, respectively (Table 2). A previous study by Street et al. [23] has shown that the trapped glucosyl-enzyme intermediate of *Agrobacterium* β-glucosidase can be reactivated by the β-glucosyl benzene acceptor. The reactivation rate followed pseudo-first-order kinetics and was dependent on the concentration of β-glucosyl benzene in a saturable fashion.

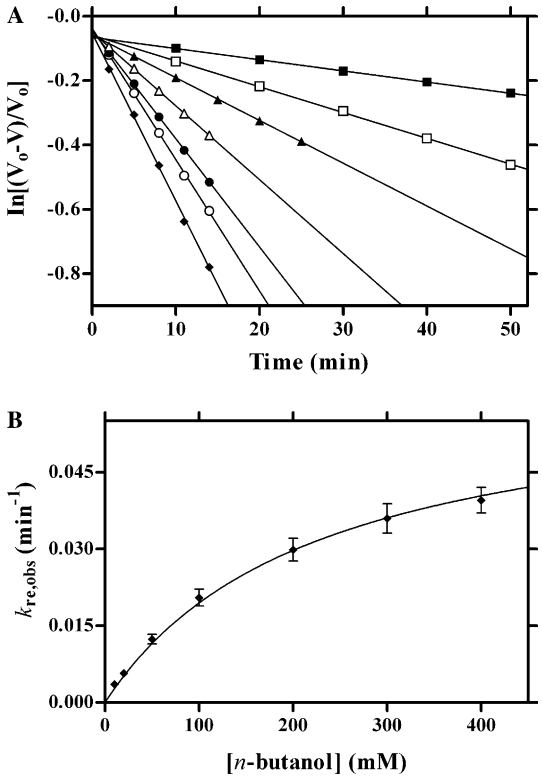


Fig. 3. Reactivation of the inactivated cassava linamarase by *n*-butanol at various concentrations. Aliquots of the E-2FGlu were incubated at 30 °C in the presence of various *n*-butanol concentrations and assayed for the return of activity of this enzyme at different times. (A) Semi-logarithmic plot of activity versus time at indicated concentrations of *n*-butanol: (■) 10 mM; (□) 20 mM; (▲) 50 mM; (△) 100 mM; (●) 200 mM; (○) 300 mM; (♦) 400 mM. (B) Direct plot of first-order rate constants from (A) versus alcohol concentration.

Table 2
Kinetic parameters of transglucosylation by cassava linamarase using various alcohols

Acceptor	k_4 (transglucosylation) (min^{-1})	K_s (ligand dissociation constant) (mM)	k_4/K_s (transglucosylation efficiency) ($mM^{-1}\,min^{-1}$)
Methanol	$(18 \pm 1) \times 10^{-3}$	1228 ± 70	$(1.5 \pm 0.5) \times 10^{-5}$
Ethanol	$(21 \pm 1) \times 10^{-3}$	255 ± 3	$(8 \pm 1) \times 10^{-5}$
n-Propanol	$(61 \pm 3) \times 10^{-3}$	157 ± 16	$(39 \pm 5) \times 10^{-5}$
n-Butanol	$(58 \pm 1) \times 10^{-3}$	184 ± 7	$(32 \pm 5) \times 10^{-5}$
n-Pentanol	$(34 \pm 2) \times 10^{-3}$	112 ± 9	$(30 \pm 3) \times 10^{-5}$
iso-Propanol	$(35 \pm 1) \times 10^{-3}$	317 ± 22	$(11 \pm 2) \times 10^{-5}$
2-Butanol	$(32 \pm 1) \times 10^{-3}$	200 ± 10	$(16 \pm 2) \times 10^{-5}$
tert-Butanol	$(14 \pm 1) \times 10^{-3}$	968 ± 100	$(1.4 \pm 0.5) \times 10^{-5}$
iso-Butanol	$(132 \pm 11) \times 10^{-3}$	154 ± 28	$(86 \pm 8) \times 10^{-5}$

Table 2 summarizes the values of k_4 and K_s of various alcohol acceptors for the transglucosylation reaction with cassava enzyme. The results clearly show that *iso*-butanol reacted with E-2FGlu intermediate with the highest rate constant for the transglucosylation step ($132 \times 10^{-3}\,min^{-1}$), while transglucosylation of other alcohols occurred with rate constants of ~10–$60 \times 10^{-3}\,min^{-1}$. Table 2 also showed that most alcohols bound to the E-2FGlu intermediate with similar binding strength ($K_s \sim 150$–300 mM). Exceptions were methanol ($K_s = 1228$ mM) and *tert*-butanol ($K_s = 968$ mM), which appeared to show lower preference for binding to the active site of cassava enzyme.

We also explored whether the reactivity of the alcohol in the transglucosylation reaction is directly related to its basicity or nucleophilic strength by comparing the rate of reactivation ($k_{re,obs}$) by alcohols with different pK_a values. Ethanol and its derivatives, 2-chloroethanol, 2,2-dichloroethanol, and 2,2,2-trichloroethanol, at fixed concentrations of 10 mM were chosen as glucosyl acceptors in this study, since they are similar in structure but have significantly different pK_a values, due to the electron withdrawing chloro-group. The values of $k_{re,obs}$ are summarized in Table 3 showing that the transglucosylation of cassava enzyme with 2,2,2-trichloroethanol (p$K_a \sim 12.2$) has the highest $k_{re,obs}$, with a rate constant of $1333 \pm 32 \times 10^{-4}\,min^{-1}$, which is about 160-fold faster than that of ethanol (p$K_a \sim 15.5$). When the log of $k_{re,obs}$ was plotted against the pK_a value of the alcohol (Fig. 4), the relationship was linear with the β value of -0.65 ($R = 0.98$, Fig. 4). This study clearly implies that the removal of proton from the alcohol (possibly by the active site Glu) is a significant factor governing the formation of the transition state during the transglucosylation reaction, since the rate of the reaction was increased with alcohols of lower pK_a.

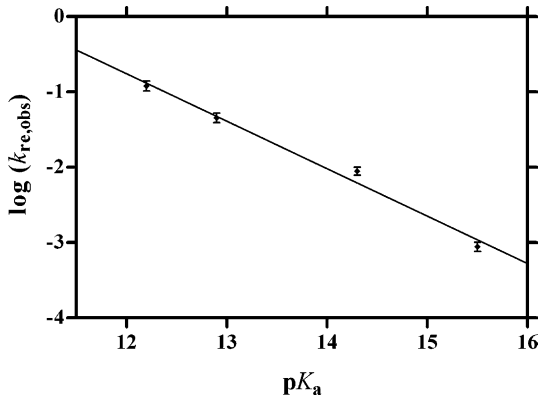


Fig. 4. Correlation between logarithms of the observed rates of reactivation ($k_{re,obs}$) of inactivated cassava β-glucosidase and the pK_a values of ethanol and its derivatives (a Bronsted plot). The linear fit gives a slope (−value) of −0.65 and $R = 0.98$.

Table 3
Substituent effects of ethanol and its derivatives on the observed reactivation rate constants ($k_{re,obs}$) of inactivated cassava β-glucosidase

Acceptor	pK_a of alcohol[a]	$k_{re,obs}$ (min^{-1})
Ethanol	15.5	$(8 \pm 0.2) \times 10^{-4}$
2-Chloroethanol	14.3	$(92 \pm 6) \times 10^{-4}$
2,2-Dichloroethanol	12.9	$(487 \pm 26) \times 10^{-4}$
2,2,2-Trichloroethanol	12.2	$(1333 \pm 32) \times 10^{-4}$

Aliquots of the E-2FGlu were incubated with 10 mM alcohol acceptor at 30 °C in 0.1 M sodium acetate buffer, pH 5.5, and assayed for the return of activity of this enzyme at different time intervals. The observed rate constants for reactivation ($k_{re,obs}$) due to transglucosylation were calculated from the slope of the plots of $\ln[(V_0 - V)/V_0]$ versus time.

[a] pK_a values were taken from [43].

Discussion

This study has shown that the use of the trapped 2-deoxy-2-fluoro-glycosyl-enzyme intermediate is a valid means to investigate the transglucosylation of alcohol acceptors, catalyzed by plant β-glucosidases. 2-deoxy-2-fluoro-sugar analogues have proven to be valuable mechanistic probes for studying the reaction mechanism of tranglucosylation by various glucosidases, using a variety of sugars as glycosyl acceptors [27]. These compounds have been shown to be active site-directed inhibitors reacting with the catalytic nucleophile [32,33]. In the reaction of Thai rosewood and cassava β-glucosidase, 2FDNPG effectively inactivated the enzyme and

competed with δ-gluconolactone, indicating that the compound directly reacted with the active site. The higher reactivity of 2FDNPG toward the Thai rosewood enzyme than toward the cassava enzyme (0.82 min^{-1} versus 0.28 min^{-1}) also implies that the residue acting as the nucleophile in the Thai rosewood enzyme is more reactive than that in cassava enzyme for the cleavage reaction (first part of Scheme 2). Previous sequence analysis has shown that the probable nucleophile is Glu-419 for Thai rosewood and Glu-413 for cassava enzyme, both of which are located in the highly conserved motif I/VTENG [34–36]. The ability of 2FDNPG to trap both enzymes as the glucosyl intermediates also implies that the reactions of these enzymes proceed via double displacement mechanisms (Scheme 1) [15], which agree well with our previous findings that the configuration of alkyl glucoside products of these two enzymes are retained [12,13].

Results from this investigation also suggest that the aglycone binding sites of these two enzymes are hydrophobic. Results from Table 1 showed that monosaccharides, glucose and fructose, give poor transglucosylation of E-2FGlu compared to that obtained with alcohols, indicating that the architecture of the aglycone binding sites do not accommodate monosaccharide well. This preference suggests that the aglycone binding sites interact better with the hydrocarbon moiety of the alcohol than with the hydroxyl groups of sugars, or that they prefer the straight chain structure of an alcohol to the cyclic structure of sugars. Inhibition study indicated that D-glucose acted as a competitive inhibitor toward *p*-nitrophenyl-β-D-glucoside hydrolysis suggesting that the sugar compounds do not bind well to the glucosyl-enzyme intermediate (where the only available binding site is the aglycone part) and implying that the aglycone site of this enzyme is hydrophobic. The aglycone binding specificity of these plant glucosidases appear to be quite different from the specificity of microbial glucosidases [27]. Previous studies of Blanchard and Withers [27] have shown that six glycosidases from various sources of microorganism can be reactivated well with various saccharides and glycoside acceptors. When the binding constants of alcohols to cassava enzyme were considered (Table 2), *n*-pentanol, the alcohol with the highest degree of hydrophobicity in our study, bound to the enzyme with the smallest K_s value. Results of Table 2 also indicate that methanol binds to the enzyme with the highest K_s value, implying that lack of hydrocarbon side chains decreases hydrophobic interaction between enzyme and substrate, and decreases the required interaction between the substrate and the aglycone binding site. However, compounds with similar hydrophobicity (*n*-butanol, 2-butanol, *tert*-butanol, and *iso*-butanol) still have different K_s values, with the highest value being for *tert*-butanol (968 ± 100 mM) and the lowest being for *iso*-butanol (154 ± 28 mM), suggesting that correct geometry is also required for optimum binding of alcohols to the cassava enzyme. Thus, it seems that the aglycone site can accommodate alkyl moieties of up to five carbon atoms and that the methyl group at the 2-position of *iso*-butanol does not obstruct the binding. In addition, the K_s values in Table 2 also indicate that the interaction of these ligands with the enzyme is not superbly tight, since they are all in the millimolar range. Previous study by Street et al. [23] also shows that β-glucosyl benzene binds to the *Agrobacterium* β-glucosidase with similar strength (K_s value of 59 ± 3 mM).

Table 2 indicates that primary alcohols generally show higher reactivity in the transglucosylation step (k_4) than secondary and tertiary alcohols. This may be seen in the butanol series, where *n*-butanol and *iso*-butanol have higher values of k_4 than those of 2-butanol and *tert*-butanol. This observation agrees with our previous reports [12,13] and other studies on glycosidases [37–39], which show that the yields of glycosylation with tertiary alcohols are lower than those of secondary and primary alcohols. This indicates that the steric hindrance of alcohol nucleophile significantly affects the rate of the transglucosylation step as found in a typical SN_2 reaction [40]. The substituent at the carbon atom forming the glycosidic bond may somewhat shield the alkoxide ion from attacking the anomeric carbon atom of the glucosyl-enzyme intermediate. Possessing all three substituents as in the case of *tert*-butanol might block the accessibility of the alkoxide to the glucosyl-enzyme and result in the lowest k_4 as shown in Table 2. Although it is not clear at this stage why *iso*-butanol possesses the highest k_4 value, it may be speculated that the structure of *iso*-butanol provides a proper geometry for the general base residue to pull out a proton from the alcohol moiety, allowing the alkoxide intermediate to transglucosylate well with E-2FGlu. When the structures of *iso*-butanol and linamarin (isobutyronitrile-β-D-glucoside), a physiological substrate for the cassava enzyme, are compared, both compounds share a similar structure in the terminal dimethyl moiety. It may be envisaged that the general base–acid residues positioned to catalyze hydrolysis of linamarin could also efficiently carry out similar general acid–base catalysis in the transglucosylation reaction of *iso*-butanol. The terminal dimethyl moiety could serve as a docking point for positioning *O*-glycosyl-bond of linamarin or hydroxyl group of *iso*-butanol so that it is suitable for the general acid–base catalysis.

Transglucosylation was assumed to occur according to Scheme 1. According to this model, transglucosylation requires the removal of proton from the alcohol by the active site Glu to generate a more reactive nucleophile, the alkoxide, to attack the C1-position of the glycosyl moiety. Our results, shown in Table 3, indicate that this proton removal step might possibly be a major factor governing the rate of transglucosylation, since the rate of transglucosylation is higher with the lower pK_a

alcohols, which are more ready to generate alkoxide. A Bronsted plot (Fig. 4) yielded the β value of -0.65, implying that the transition state has developed a significant negative charge [41]. This contrasts with some other enzymatic reactions such as our recent studies which show that a flavoprotein hydroxylase carrying out electrophilic aromatic substitution mechanisms had a β value of only -0.07 from the Bronsted plot, indicating only a small degree of negative charge developed at the transition state in this case [42]. The results of Table 3 also established that the reaction of the cassava enzyme is likely to be as in Scheme 1, with proton removal to generate the reactive alkoxide being a major step governing transition state formation. Since it was shown in Table 1 that reactivation of cassava enzyme by all alcohol acceptors tested were considerably faster than reactivation of the Thai rosewood enzyme, it may be further speculated that the general base (Glu) of Thai rosewood enzyme is not as reactive as in cassava enzyme in removing the proton to generate the reactive alkoxide. It should be noted that similar studies using Thai rosewood enzyme were also carried out, but the rate of reactivation did not depend significantly on the pK_a value of the alcohols as found with the cassava enzyme (data not shown). This implies that unlike the cassava enzyme, the generation of the reactive nucleophile by the active site base is not a major factor governing transition state formation in Thai rosewood enzyme.

In conclusion, our study has illustrated the fundamental requirements of the transglucosylation reaction of these two plant β-glucosidases. The hydrophobic nature of the aglycone sites of both enzymes is possibly the main reason allowing the enzymes to carry out an efficient transglucosylation reaction with alcohols. Our study clearly shows that the reaction mechanisms of the enzymes are of the double-replacement type, with the step involving generation of the reactive alkoxide being a major step governing transition state formation. The kinetic parameters obtained will be useful for preparative synthesis of alkyl β-glucosides.

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