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Cellular zwitterionic metabolite analogs simultaneously enhance reaction rate, thermostability, salt tolerance, and substrate specificity of α-glucosidase

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ABSTRACT

We investigated the structural effects of metabolite analogs derived from a naturally-occurring zwitterionic metabolite, glycine betaine, on the activity of several hydrolases. The initial velocities of the hydrolases were enhanced by the addition of the solutes into the buffer solution. Based on a detailed study using α-glucosidases, the acceleration efficiency of the enzymatic activity was strongly induced by solutes possessing bulky and aliphatic ammonium cations, indicating that enhancement of activity by the solutes depended on their chemical structures. Kinetic analysis revealed that the acceleration of the hydrolysis reaction was related to both the decrement of K_m and increment of V_{max} values. Furthermore, the addition of the metabolite analogs enhanced not only the rate constant but also the thermostability, salt tolerance, and substrate specificity of α-glucosidase simultaneously through the reduction of conformational perturbation of the enzyme.

1. Introduction

Enhancement of enzymatic activity is of great significance for diagnostic, industrial, and biological applications.[1] In order to obtain active enzymes, several strategies are used, such as screening for new enzymes from natural sources, site-specific mutation of enzymes utilizing genetic engineering, the addition of organic molecules in the reaction buffer.[1] Above all, the addition of organic molecules is particularly useful because it is easy and does not require time-consuming enzymatic modifications. Usually, polyols,[2–4] amino acids,[5–7] small metabolites[7,8] such as glycine betaine and L-ectoine, proteins,[9] polymers[9] such as polyethylene glycol (PEG), and surfactants[10] are used as additives. In spite of their promise, however, there are also problems in their usage; for example, combining several additives results in a large decrement in enzymatic activity, although each additive alone strongly activates the enzyme.[11] Accordingly, improvements in the buffer conditions are required.[3,12]

Among the additives used, naturally-occurring zwitterionic metabolites, such as glycine betaine and L-ectoine, show different behaviors from the others, owing to their inert properties.[7,8] These zwitterionic metabolites are produced in cells from various biomolecular scaffolds, such as saccharides, amino acids, and lipids, to regulate the osmotic gradient in the cytoplasm, enabling cells to survive in extremely harsh environments.[13] Because of the diversity of their chemical structures, it is difficult to predict which metabolite structure will be optimal for use as an additive for various biomolecular reactions.

We recently synthesized metabolite analogs derived from a naturally-occurring zwitterionic metabolite,[14] glycine betaine (*N,N,N*-trimethylglycinate). To investigate the specific structural effects of zwitterionic metabolites on biomolecules, we changed the linker length between the ammonium cation and carboxylate anion (solutes **2**, **3**), the bulkiness of the ammonium group (solutes **4**–**8**), or the number of intramolecular zwitterions (solute **9**) (Fig. 1). These metabolite analogs destabilize DNA duplexes differently depending on their chemical structure. In particular, solutes possessing bulky and aliphatic ammonium cations are more destabilizing than glycine betaine. Interestingly, while these analogs strongly destabilize DNA duplexes, they do not disrupt the duplex structure at all.[14] These results show that even inert analogs possess properties similar to naturally-occurring metabolites.

In this study, we extended our zwitterionic metabolite analogs to investigate their effects on various enzymatic reactions. To date, there have been many studies on the effects of natural metabolites on the activity of various enzymes.[2–9] Most metabolites, rather than enhancing the activity of enzymes, may instead counteract the decreased activity induced by an increase in urea or salt concentrations, or denaturation caused by heating or freezing.[15–17] Several groups have reported the facilitation of enzymatic activity by the addition of naturally-occurring metabolites into the reaction

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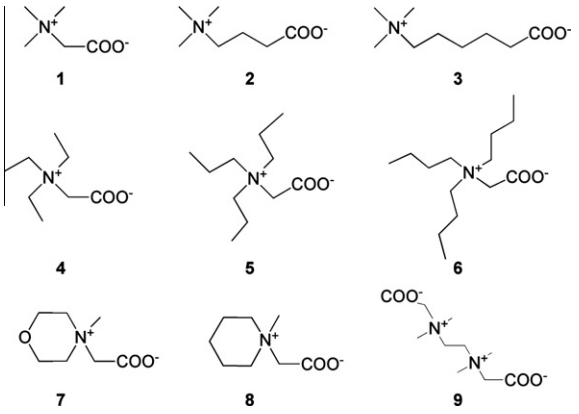

Figure 1. Chemical structure of synthetic zwitterionic solutes.

buffer. For example, Matholouthi et al. reported that carbohydrates such as sucrose, trehalose, glucose, and sorbitol, which decreased water activity in buffer solutions, intensified the enzymatic activity of a protease (thermolysin) and two glycohydrolases (pullulanase and inulinase).[4] Rao and co-workers reported that the activity of a glycohydrolase (xylanase) is enhanced by the addition of glycine.[6] However, enhanced activity was induced by only glycine and not by other amino acids or derivatives. Considering these reports, naturally-occurring metabolites, such as polyols and amino acids, should have the potential to facilitate enzymatic reactions. However, there have been few structural studies. Thus, we investigated the effects of our metabolite analogs on the activity of enzymes. We selected several hydrolases to study the effects of our metabolite analogs since these hydrolases have already been studied using naturally-occurring metabolites.[4,6,9]

2. Results and discussion

2.1. Comparison of enzymatic hydrolysis in the absence and presence of synthetic zwitterionic solutes

According to our previous study,[14] solute **6** possessed the strongest destabilization property for DNA duplexes. Therefore, we first compared enzymatic reactions in the absence and presence of solute **6** (50 mM) using several hydrolases: two α-glucosidases of different origin, from *Bacillus stearothermophillus* and *Saccharomyces cerevisiae*, and a commercially available α-glucosidase from yeast, a β-glucosidase from almonds; and an alkaline phosphatase from calf intestines. Hydrolysis of the substrates (*p*-nitrophenyl-α-D-glucopyranoside for α-glucosidase, *p*-nitrophenyl-β-D-glucopyranoside for β-glucosidase, and *p*-nitrophenylphosphate for alkaline phosphatase) in the absence and presence of solute **6** at 37 °C was followed by monitoring the absorbance at 405 nm (A_{405}) for *p*-nitrophenol produced during enzymatic hydrolysis using a Multiskan microplate reader (Thermo Scientific Inc., Pennsylvania, USA).

Figure 2(a)–(e) shows plots of A_{405} versus incubation time for various hydrolases. In all cases, the slopes in the presence of solute **6** were larger than those in the absence of solute **6**, suggesting that the zwitterionic solute facilitated the hydrolysis reaction, even though the substrates and reaction mechanisms were different. Furthermore, the degree of facilitation differed even for α-glucosidases (Fig. 2(a)–(c)), implying that the acceleration effects of the zwitterionic solutes may be related to the amino-acid sequence or higher-order structure of the enzymes. To obtain further insights into the acceleration mechanism by zwitterionic solutes, we investigated their effects using α-glucosidases.

2.2. Influence of chemical structure of zwitterionic solutes

As additives, not only zwitterionic solutes **1–9**, but also glycerol, sorbitol, and *myo*-inositol were used as references, because these polyols have been reported to be useful additives for glucosidases.[4] The initial velocity of the hydrolysis reaction was calculated from the initial slope of the line generated by A_{405} versus incubation time plots using the molar extinction co-efficient of *p*-nitrophenol ($10900\ M^{-1}\ cm^{-1}$).

A comparison of the initial velocities of the hydrolysis reaction by α-glucosidase (from all three sources) in the presence of zwitterionic solutes **1**–**9**, glycerol, sorbitol, and *myo*-inositol (concentration of all solutes was adjusted to 50 mM) is presented in Figure 3, where the *y*-axis shows activity (activity = initial velocity in the presence of solute/initial velocity in the absence of solute). As shown in Figure 3, solute **6** possessed the highest activity for α-glucosidases among all the solutes, and solute **5** showed the second highest activity. Although the other solutes did not enhance activity, the hydrolysis reaction was facilitated to some extent by the addition of the synthetic zwitterionic solutes. In contrast, the presence of polyols decreased activity.

To better understand the structural effects of the synthetic solutes on the activity of α-glucosidase, we focused on α-glucosidase isolated from *B. stearothermophillus*, as it appeared to be more sensitive to the presence of the solutes. As shown in Figure 3, solutes **4**, **5**, and **6** enhanced activity by 1.13 ± 0.02, 1.54 ± 0.07, and 2.74 ± 0.02, respectively. These solutes have a common aliphatic ammonium group and become bulkier from **4** to **5** to **6**, indicating that solutes possessing bulkier ammonium groups tend to accelerate the enzymatic reaction more so than those possessing less bulky groups. Subsequently, to compare functional groups introduced outside the ammonium cation, we compared the activities of α-glucosidase in the presence of solutes **4**, **7**, and **8**, which all possess a similar bulky ammonium cation, but differ in their other functional groups. As a result, activity in the presence of solute **7** (1.05 ± 0.04) was smaller than the others, indicating that the polar oxygen atom located outside the ammonium cation reduced enzymatic activity. On the other hand, the activity was almost equal when the ammonium cation possessed either a linear or cyclic aliphatic chain, as observed for solutes **4** and **8** (1.13 ± 0.02 and 1.16 ± 0.06, respectively). The present results indicate that in order to facilitate the hydrolysis reaction by α-glucosidase, bulky and aliphatic ammonium cations in zwitterionic metabolites are necessary, and that there should be no polar moieties outside of the aliphatic chain.

2.3. Influence of solute concentration on the activity of α-glucosidase

Using solutes **1**, **4**–**6**, and glycerol, sorbitol, and *myo*-inositol, we next examined the dependence of enzymatic activity on the solute concentration. As shown in Figure 4, solute **1**, a naturally-occurring metabolite, did not alter the activity even at concentrations up to 1000 mM. In contrast, solute **4**, with an ethyl group instead of the methyl group (solute **1**) attached to the ammonium cation, showed a maximum activity of 1.62 at above 800 mM. Moreover, solute **5** showed a much higher maximum activity of 2.21 at 200 mM. The activity of α-glucosidase was most significantly affected in the presence of solute **6**; the maximum activity reached 2.74 at 30–50 mM. These results revealed that as the length of the alkyl chain increases, the effective concentration decreases and the maximum activity increases. In contrast, all polyols decreased the activity of α-glucosidase as the concentration increased, indicating that the acceleration effect is characteristic of these zwitterionic metabolite analogs. When the solute **6** concentration increased above 50 mM, the activity of α-glucosidase

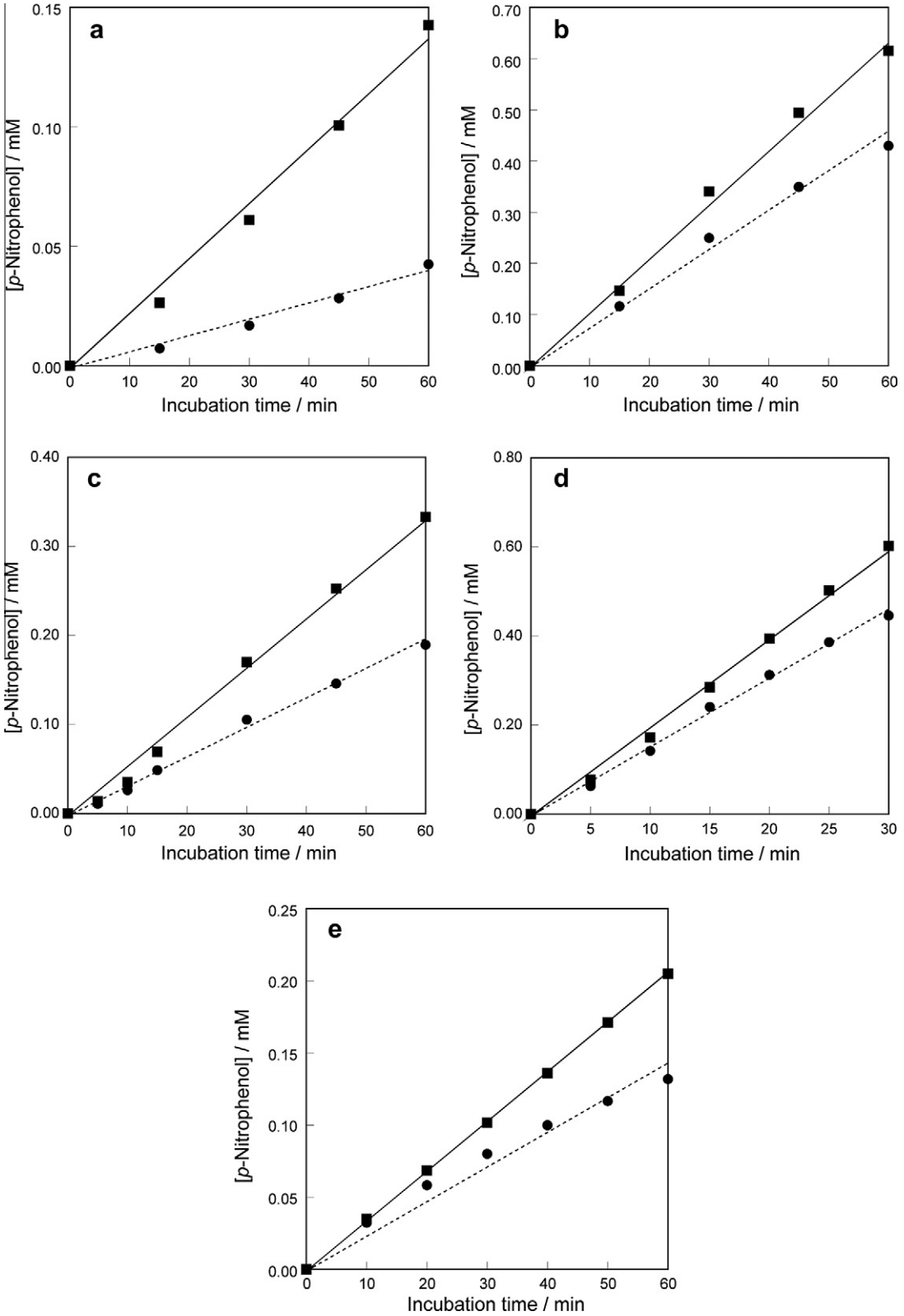

Figure 2. Plots of *p*-nitrophenol concentration produced during hydrolysis as a function of incubation time in the absence and presence of solute **6** (50 mM). (a) α-Glucosidase from from *Bacillus stearothermophillus*; (b) α-glucosidase from *Saccharomyces cerevisiae*; (c) α-glucosidase from yeast; (d) β-glucosidase from almond; (e) alkaline phosphatase from calf intestines. Closed circle (dashed line): absence of solute, closed square (solid line): presence of solute **6**. Conditions: [solutes **6**] = 0 or 50 mM, [enzyme] = 2.5×10^{-5} mg/mL, [substrate] = 2.0 mM and [phosphate buffer (pH 7.0)] = 0.10 M at 37 °C.

decreased and the solution turned turbid at concentrations above 1000 mM, suggesting that α-glucosidase was aggregated by the addition of solute **6**. These results indicate that the enhancement of enzymatic activity of α-glucosidase requires a metabolite

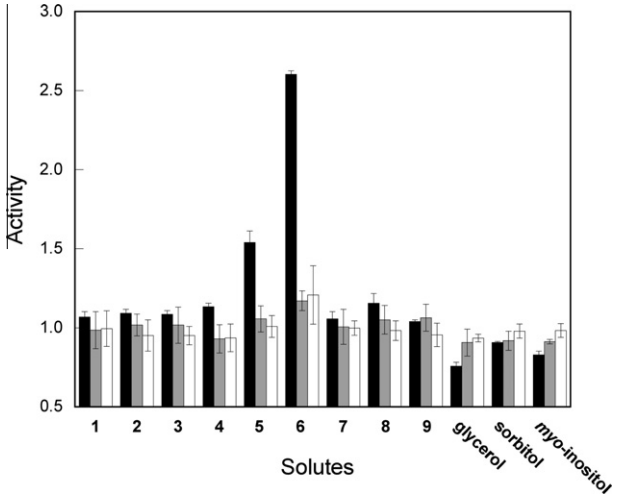

Figure 3. Comparison of the activity of hydrolysis reaction by α-glucosidases (black bar: from *Bacillus stearothermophillus*, gray bar: from *Saccharomyces cerevisiae*, white bar: from yeast) in a 100 mM phosphate buffer (pH 7.0) containing 2.0 mM substrate, 50 mM solutes, and 2.5×10^{-5} mg/mL α-glucosidase at 37 °C. Activity = initial velocity in the presence of solute/initial velocity in the absence of solute.

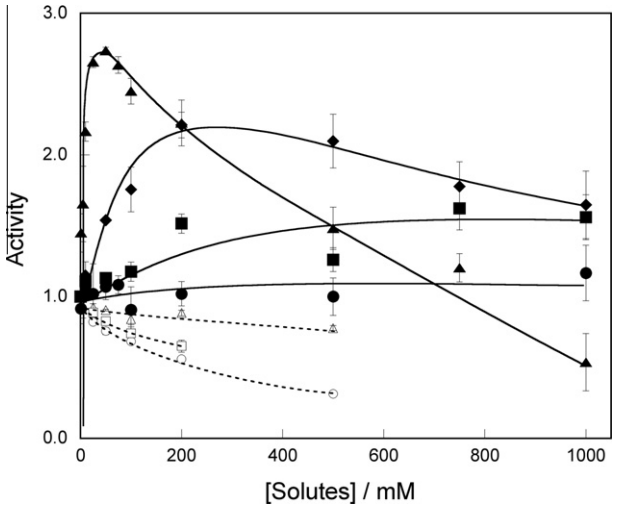

Figure 4. Plot of activities of α-glucosidase from *Bacillus stearothermophillus* as a function of solute concentrations. Experiments were performed in a 0.1 M phosphate buffer (pH 7.0) containing 2.0 mM substrate, 0– 1000 mM solutes **1** (closed circle), **4** (closed square), **5** (closed diamond), **6** (closed triangle), glycerol (open circle), *myo*-inositol (open square), or sorbitol (open triangle) and 2.5×10^{-5} mg/mL α-glucosidase at 37 °C.

structure possessing a zwitterion with a bulky ammonium cation. Furthermore, the structural effect is so drastic that excess amounts of the metabolite may induce aggregation of α-glucosidase. Judging from the differences in the effective concentration ranges of the different synthetic solutes on the activity enhancement of α-glucosidase, it is apparent that the chemical structure of zwitterionic metabolites plays an important role in determining the level of the effect that solutes have on α-glucosidase reactivity.

2.4. Kinetic parameters of α-glucosidase in the presence of synthetic solutes

To understand the acceleration mechanism, kinetic parameters (K_m, V_{max}) in the presence of solute **6** were estimated by Hanes–Woolf plots.[18] Figure 5(a) and (b) shows plots of K_m and V_{max}

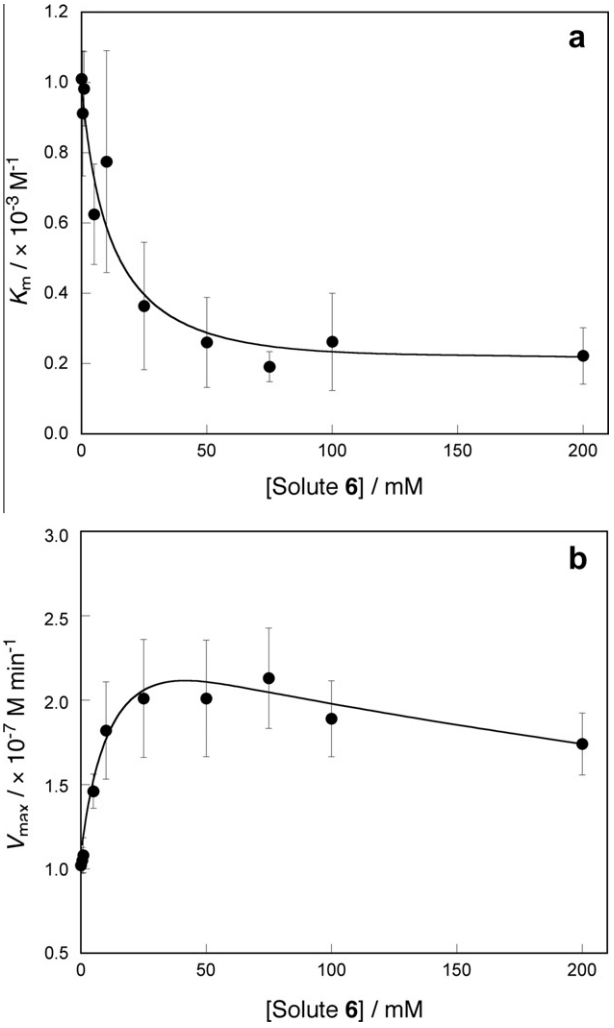

Figure 5. Plot of K_m values as a function of solute **6** concentration and (b) plot of V_{max} values as a function of solute **6** concentration.

values, respectively, as a function of solute **6** concentration. In Figure 5(a), the K_m drops by one-fifth when the concentration of solute **6** increases to 50 mM, and then reaches a plateau, indicating that substrate binding to α-glucosidase increases in the presence of solute **6**. V_{max} increases by 2.1-fold when the concentration of solute **6** increases to 30 mM and then slightly decreases (Fig. 5(b)). Judging from the minor CD spectral change of α-glucosidase in the presence of solute **6** (Fig. 6), the decrease in V_{max} in this concentration range may be related to a slight conformational change in the active site of α-glucosidase.

2.5. Optimum conditions of α-glucosidase in the presence of synthetic solutes

We then compared the optimum pH, temperature, and NaCl concentration for α-glucosidase[19] in the absence and presence of solutes **1** and **6** (Fig. 7). Figure 7(a) shows that the presence of the solutes did not alter the optimum pH of α-glucosidase, suggesting that the reaction mechanism is preserved even in the presence of solutes. On the other hand, the optimum temperature for α-glucosidase shifted upwards in the presence of both solutes **1** and **6**. According to previous studies,[15–17] naturally-occurring metabolites thermally stabilize various proteins, and our results are in good agreement (Fig. 7(b)). Moreover, we compared the optimum NaCl concentration of α-glucosidase in the absence and presence

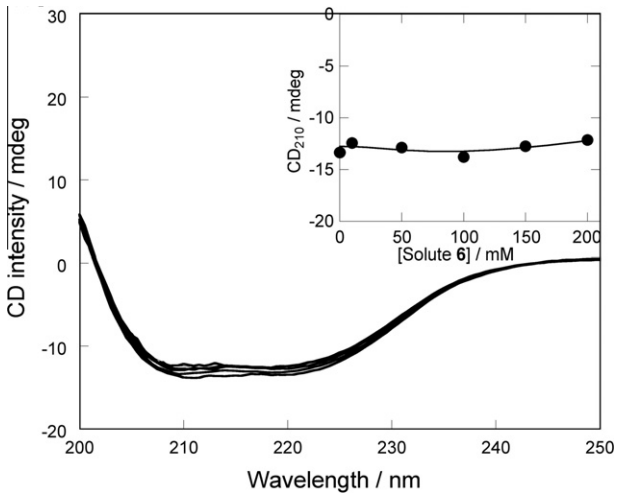

Figure 6. Comparison of CD spectra of α-glucosidase with increasing the solute **6** concentration. Inset: plot of CD_{210} values as a function of solute **6** concentration. Conditions: [solute **6**] = 0–200 mM, [α-glucosidase] = 0.5 mg/mL, [phosphate buffer (pH7.0)] = 0.1 M at 37 °C.

of solutes **1** and **6**. As shown in Figure 7(c), α-glucosidase from *B. stearothermophillus* possesses an optimum salt concentration of zero. Interestingly, however, the presence of solute **6** clearly increased the salt tolerance of α-glucosidase. The presence of solute **6** facilitated the hydrolysis reaction, and therefore the rate constant was still high even though the NaCl concentration increased to 1 M. However, solute **1**, a naturally-occurring zwitterionic metabolite, did not alter the salt tolerance of α-glucosidase. Thus, we found that these metabolite analogs not only facilitated enzymatic hydrolysis but also enhanced the thermostability and salt tolerance of α-glucosidase.

2.6. Substrate specificity of α-glucosidase in the presence of synthetic solute

Finally, the substrate specificity of α-glucosidase was investigated using *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, and *p*-nitrophenyl-α-D-mannopyranoside as substrate mimics. The initial velocities of the hydrolysis reactions in the absence and presence of solute **6** (50 and 750 mM) are summarized in Table 1. The velocity for *p*-nitrophenyl-α-D-glucopyranoside in the absence of solute **6** is six- to eight-fold faster than that for the substrate mimics. The addition of solute **6** to the solution containing *p*-nitrophenyl-α-D-glucopyranoside accelerated the hydrolysis velocity by a factor of 2.5 compared to the reaction carried out in the absence of solute **6**. On the other hand, the presence of solute **6** reduced the velocities for the mimics. The overall velocity for the substrate was 14–24 times faster than that for the substrate mimics, resulting in the enhancement of substrate specificity during hydrolysis. When the same experiment was performed using solute **6** at a concentration of 750 mM, no significant enhancement in the enzymatic activity was observed. Thus, the enhanced substrate specificity is related to the concentration range of the added solute, and furthermore, it can be attributed to changes in both the binding and catalytic properties of α-glucosidase to the substrate and the mimics.

Because the enhanced substrate specificity may be caused by conformational perturbation of α-glucosidase, we measured the urea concentration necessary for deforming α-glucosidase as a function of solute **6** concentration (Fig. 8). At a 50 mM concentration of solute **6**, where the activity and substrate specificity were increased, the urea concentration was notably higher than at other solute concentrations, suggesting that the added solute inhibits

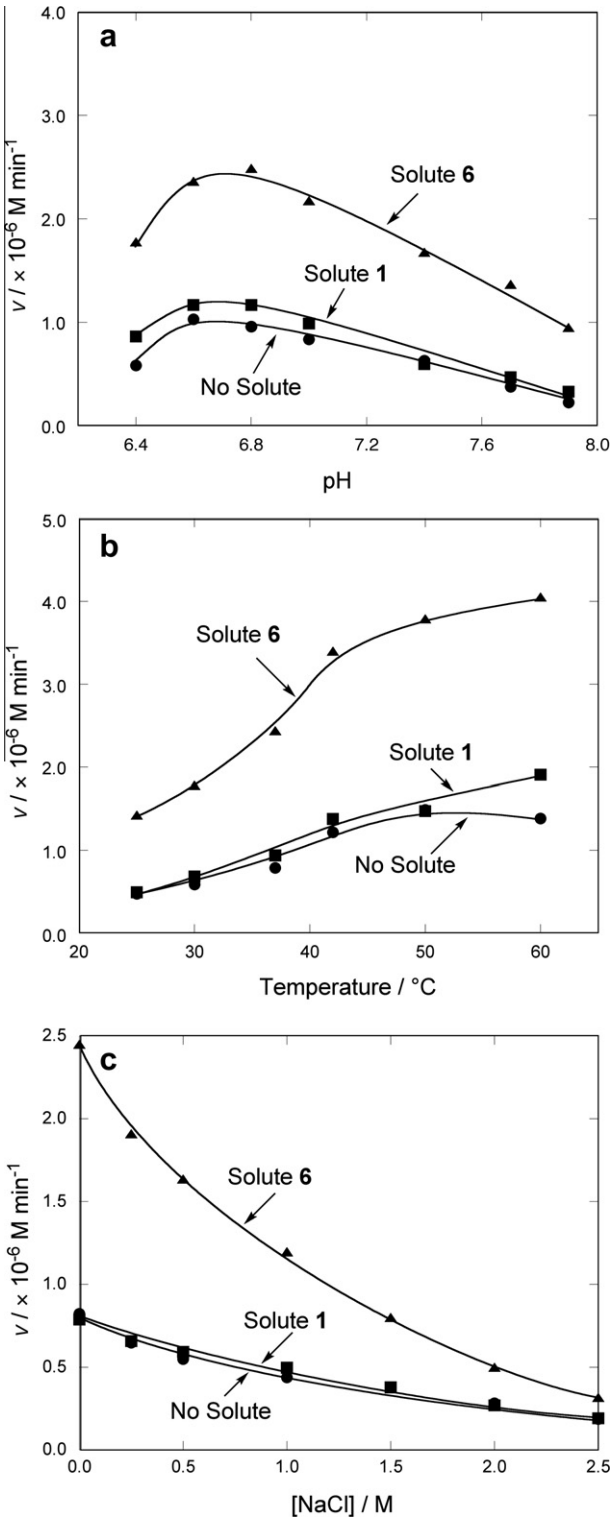

Figure 7. Comparison of optimum conditions [(a) optimum pH; (b) optimum temperature; (c) optimum salt concentration] of α-glucosidase in the absence and presence of synthetic solutes. Solid circle: no solute, solid square: solute **1**, and solid triangle: solute **6**. Conditions: [solutes **1** or **6**] = 0 or 50 mM, [α-glucosidase] = 2.5×10^{-5} mg/mL, [substrate] = 2.0 mM, [NaCl] = 0–2.5 M and [phosphate buffer (pH 6.4–7.9)] = 0.10 M.

conformational perturbation within its effective concentration range. Very interestingly, the concentration was considerably higher than that in the absence of solute **6**. This indicates that solute **6** effectively fixes the conformation of α-glucosidase to allow the substrate to bind to the active site.

Table 1
Comparison of initial velocities of α-glucosidase for the substrate or the mimics in the absence and presence of solute **6** (50 mM or 750 mM)[a]

Substrate		[Solute **6**] = 50 mM		[Solute **6**] = 750 mM	
	$v_{absence}$ M min^{-1}	$v_{presence}$ M min^{-1}	$v_{presence}/v_{absence}$	$v_{presence}$ M min^{-1}	$v_{presence}/v_{absence}$
p-Nitrophenyl-α-D-glucopyranoside	$9.7 \pm 0.5 \times 10^{-7}$	$24.0 \pm 0.3 \times 10^{-7}$	2.5	$7.7 \pm 0.4 \times 10^{-7}$	0.8
p-Nitrophenyl-β-D-glucopyranoside	$1.7 \pm 0.1 \times 10^{-7}$	$1.4 \pm 0.2 \times 10^{-7}$	0.8	$0.8 \pm 0.1 \times 10^{-7}$	0.5
p-Nitrophenyl-α-D-galactopyranoside	$1.2 \pm 0.7 \times 10^{-7}$	$1.0 \pm 0.1 \times 10^{-7}$	0.8	$0.9 \pm 0.1 \times 10^{-7}$	0.8
p-Nitrophenyl-α-D-mannopyranoside	$1.7 \pm 0.2 \times 10^{-7}$	$1.6 \pm 0.2 \times 10^{-7}$	0.9	$1.0 \pm 0.1 \times 10^{-7}$	0.6

[a] Enzymatic reactions were performed in a 100 mM phosphate buffer (pH 7.0) containing 2.0 mM substrate, 50 or 750 mM solute **6** and 2.5×10^{-5} mg/mL α-glucosidase at 37 °C.

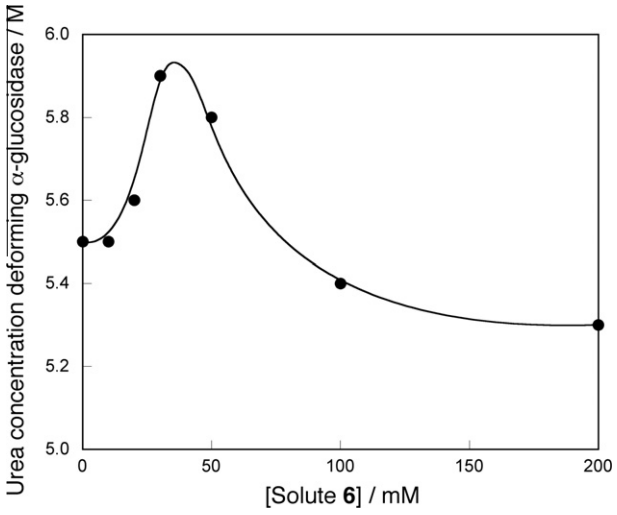

Figure 8. Urea concentration deforming α-glucosidase as a function of solute **6** concentration.

2.7. Speculated mechanism

According to previous studies,[15,20] glycine betaine (solute **1**) is preferentially hydrated in an aqueous solution and tends to be excluded from the biomolecular surface. This property is ascribed to both the strong hydrophilicity arising from the zwitterionic structure and the different density of the water molecules surrounding glycine betaine compared with that of bulk water.[20–22] The density difference arises from the cluster formation of water molecules on the hydrophobic surface of the aliphatic ammonium group.[22] These studies suggest that zwitterionic metabolites work as a solvactivator, not as an activator that specifically interacts with enzymes.

We chemically modified the structure of glycine betaine. In particular, solute **6**, possessing the largest hydrophobic surface, showed a profound effect on enzymatic activation. Kinetic analysis showed that an increase in the solute **6** concentration reduced the K_m values between α-glucosidase and its substrates, in good agreement with previous studies. Furthermore, a comparison of solutes **4**, **7**, and **8**, which possess a similar surface area for the ammonium group, revealed that the hydrophobic surface plays important roles in effective solvactivation; that is, the degree of enzymatic activation by solute **7**, possessing a polar oxygen enabling interaction with bulk water molecules, was smaller than that of solutes **4** and **8**. These results strongly support the hypothesis that zwitterionic metabolite analogs also work as solvactivators in aqueous solutions, and that the enhanced surface area of the ammonium group intensifies the ability. Therefore, the analogs not only facilitate the activity of α-glucosidases, but also of β-glucosidase and alkali phosphatase at the same concentration range. Further mechanistic studies are in progress.

3. Conclusions

The zwitterionic metabolite analogs that we synthesized facilitated the hydrolysis reaction of various hydrolases. Mechanistic studies using α-glucosidase revealed that the chemical structure of the metabolite analogs played a significant role in enzymatic activation. In particular, solute **6**, which possesses an *n*-butyl chain in the ammonium group, had a profound effect on the acceleration of the hydrolysis reaction of α-glucosidase. Furthermore, the acceleration of enzymatic hydrolysis is characteristic of zwitterionic metabolite analogs and is not observed for polyols. Kinetic analysis revealed that the acceleration of the reaction was related to both K_m and V_{max} values, and depended on the structure and concentration of the metabolite analog added in the buffer solution. Furthermore, the addition of the metabolite enhanced not only the rate constant, thermostability, and salt tolerance, but also the substrate specificity of α-glucosidase through a reduction of conformational perturbation. To the best of our knowledge, this is the first known molecule that enhances the reaction rate, thermostability, salt tolerance, and substrate specificity of an enzyme simultaneously. We thus believe that these zwitterionic metabolite analogs have the potential to facilitate various enzymatic reactions used in diagnostic, industrial, and biological applications.

4. Experimental Section

4.1. Materials

Two α-glucosidases of different origin, from *B. stearothermophillus* and *S. cerevisiae*, and a commercially available α-glucosidase from yeast, a β-glucosidase from almonds; and an alkaline phosphatase from calf intestines were purchased from Sigma (Saint Louis, USA) and Wako Pure Chemicals Ltd. (Osaka, Japan) and were used without further purification. Zwitterionic solutes were synthesized according to a previously reported method.[14]

4.2. Enzymatic reactions

Enzymatic reactions were performed as follows: a 1 μL aliquot of α-glucosidase stock solution (2.5×10^{-5} mg/mL) was placed onto the wall of each well of a 96-well plate containing 199 μL substrate (0–200 mM) and 100 mM phosphate buffer solution (pH 7.0), and incubated at 37 °C for 3 min. The hydrolysis reaction was started by shaking the plate, thus mixing together the enzyme and buffer. The initial slopes of the absorbance at 405 nm against incubation time were converted into initial velocities using the molar extinction co-efficient of *p*-nitrophenol at 405 nm (10900 cm^{-1} M^{-1}). The initial velocities were estimated as the average of five measurements.

The hydrolysis of substrates during the incubation time in the absence of enzyme was negligible ($[p\text{-nitrophenol}]_{background}$ $< 6.5 \times 10^{-7}$ mM min^{-1}) even in the presence of solute **6** (0–1000 mM).

4.3. CD measurements

CD spectra were measured on a JASCO spectropolarimeter (Tokyo, Japan) connected to a thermo-programmer were performed at 37 °C. CD measurements were performed in a 100 mM phosphate buffer (pH 7.0) containing 0–200 mM solute **6** and 2.0 mg/mL α-glucosidase at 37 °C using a plate cell with a 0.01 mm pathlength.

Acknowledgments

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