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# Catalytic Mechanism of β-Amylase from *Bacillus cereus* var. *mycoides*: Chemical Rescue of Hydrolytic Activity for a Catalytic Site Mutant (Glu367→Ala) by Azide

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**The hydrolytic activity of β-amylase from *Bacillus cereus* var. *mycoides* was lost on replacement of either of the catalytic residues (Glu172 or Glu367) with an alanyl residue. When maltopentaose and 2 M azide existed together mutant, E367A cleaved the glucosidic linkage of maltopentaose and produced maltose at pH 7.0 and 25°C, but the other mutants (E172A and double mutant E172A/E367A) did not. This indicates that azide acts as a general base instead of E367 and Glu172 acting as general acids, and that the hydroxide ion generated from a water molecule activated by azide attacks a reactive pyranose nucleophilically so that β-maltose is produced.**

**Key words: β-amylase, azide, catalytic mechanism, chemical rescue, site-directed mutant.**

Glycosyl hydrolases are classified into retaining and inverting ones, according to the mechanisms giving the overall retention and inversion of the anomeric configuration of the substrate, respectively. As for a retaining enzyme which lacks a catalytic residue due to site-directed mutagenesis, it has been reported that the catalytic activity is recovered with a nucleophile added externally, and the mechanisms of the chemical rescue have been investigated: in 1994, MacLeod *et al.* found that a catalytic site mutant (Glu127→Ala or Gly) of β-1,4-glycanase Cex from *Cellulomonas fimi*, a retaining enzyme, formed a new product, β-cellobiosyl azide, in the presence of azide (*1*), and Wang *et al.* have reported that a catalytic site mutant (Glu358→Ala) of β-glucosidase, a retaining enzyme from *Agrobacterium faecalis*, formed α-glucosyl azide as a product in the presence of azide or formate as an alternative nucleophile (*2*). In 1998, Viladot *et al.* reported that a mutant (Glu138→Ala) of a retaining 1,3-1,4-β-glucanase from *Bacillus licheniformis* formed β-glucosyl azide through a nucleophilic attack by azide of the glycosyl-enzyme intermediate, but the mutant (Glu134→Ala) formed α-glycosyl azide *via* single inverting displacement (*3*). In the case of a mutant (Glu387→Gly) of β-glucosidase from hyperthermophilic Archaeon *Sulfolobus solfataricus*, the mode of chemical rescue depended on the externally added nucleophile: Moracci *et al.* have reported that azide restored the catalytic activity of the mutant by attacking the α-side of the anomeric carbon of the substrate, thereby yielding an inverting glycosidase, and that sodium formate induced the opposite behavior, producing a 3-*O*-β-linked disaccharide derivative of the substrate (*4*). All enzymes, for which chemical rescue by an externally added nucleophile has been observed, are retaining β-glycosidases, but the chemical rescue of an inverting enzyme has not previously been reported.

β-Amylase [EC 3.2.1.2] is a typical inverting enzyme that catalyzes the hydrolysis of the α-1,4-glucosidic linkage of a substrate such as starch, and liberates β-maltose from the non-reducing end of a substrate (*5*). The three-dimensional structures of β-amylases from soybean (*6, 7*), sweet potato (*8*), a sevenfold mutant of barley (*9*), and *Bacillus cereus* (*10, 11*) have been reported, and the structures are very similar to each other. In this study, we create three catalytic site mutants of β-amylase from *Bacillus cereus* var. *mycoides* (abbreviated as BCM β-amylase) and show that the chemical rescue of the mutant occurs with the coexistence of azide, and discuss the roles of the catalytic residues of this enzyme.

## MATERIALS AND METHODS

*Mutation of BCM β-Amylases*—Site-directed mutants of active site carboxylates (Glu172 and Glu367) of BCM β-amylase were generated by the megaprimer method (*12*). 7.9 kb pHBCW (*13*) was used as a template. The forward and reverse primers commonly used were 5′-TGACCATGGCTGTAAATGGAAAAGGAA-3′ containing an *Nco*I site and N-terminal Ala, and 5′-TTAAGCTTTTACCAACTACTTGTATGAGA-3′ containing a *Hin*dIII site and C-terminal Trp, respectively. The mutant primers used to generate the

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[2] The effect of azide on the activity of the wild enzyme was examined by reaction calorimetry and polarimetric measurement. The relative activity in the presence of 2 M azide decreased by 1/2. In the presence of azide, the degree of hydrolysis of G5 by E367A was determined from a densitogram of the reaction products on a TLC plate, and the amount of remaining G5 was calculated. As for the wild enzyme, the reaction time required to hydrolyze G5 to the same degree was calculated to be 4.1 s using an integrated Michaelis-Menten equation for competitive product inhibition. Comparing the time with 40 h, the relative activity of a mutant was evaluated.

[3] For β-galactosidase, it has been reported that β-galactosyl azide is hydrolyzed to galactose and azide (*20*).

Abbreviation: BCM, *Bacillus cereus* var. *mycoides*.

mutations were as follows, the positions of the mismatches being underlined: primer (Glu172→Ala), 5′-GATATCTTAGTGCACCAGCTGGTC-3′; and primer (Glu367→Ala), 5′-GTATTAAACGGTGCAAATGCTTTAAG-3′. In the case of mutant (Glu172→Ala), the first PCR was started by adding *Pfu* DNA Polymerase (Stratagene) to a mixture of a template DNA, and forward and reverse (mutant) primers, and conducted under the following conditions: 94°C, 1 min, 43°C, 1 min; 72°C, 3 min, for 25 cycles. The PCR product, megaprimer, was purified with a GENECLEAN II KIT (BIO 101, Inc.) to remove the forward primer. The second PCR was performed with the megaprimer and the template DNA, under the following conditions: 94°C, 1 min, 72°C, 1 min, for 5 cycles. After adding the reverse primer, the reaction was carried out under the same conditions as for the first PCR. In the case of mutant (Glu367→Ala), the reverse primer was used for the first PCR, and the megaprimer and forward primer were used for the second PCR. The final mutagenic 1.5 kb fragments for the construction of E172A and E367A were digested with *Nco*I and *Hin*dIII, and then inserted between the *Nco*I and *Hin*dIII sites of an expression vector, pET21d (Novagen), to create plasmids pET21-e172a and pET21-e367a, respectively As for a double mutant DNA, Glu172→Ala/Glu367→Ala, pET21-e172a was used as the template, and pET21-e172a/e367a was created in the same way as for pET21-e367a. The mutants were transformed, and their sequences were confirmed by DNA sequencing (Perkin Elmer). The plasmid DNA was transformed to *Escherichia coli* BL21 (DE3) for expression of a mutant β-amylase. Overexpression of the mutant β-amylase was performed by the following method: Each transformant was grown in 100 ml LB culture medium (1.0% polypepton, 0.5% yeast extract and 0.5% NaCl) containing 10 μg of ampicillin at 37°C with shaking. The culture was then transferred to 800 ml of 2× TY medium (1 6% polypepton, 1.0% yeast extract and 0.5% NaCl) containing 1% glucose and 80 μg of ampicillin, and incubated at 37°C until the medium turbidity reached $A_{600}$ 0.8. Isopropyl β-D-thiogalactopyranoside was added to the medium to a final concentration of 0.1 mM, followed by culturing at 18°C for 48 h. Bacteria were harvested and suspended in 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 100 mM NaCl. The cells were disrupted by ultrasonication and the soluble fraction of the mixture was collected by centrifugation at 12,000 ×*g* for 20 min at 4°C. The cell free extract was added to 200 g of raw starch (corn) for 1 day at 4°C, and then eluted with 200 ml of a 20% (w/v) maltose solution (*14*). After adding ammonium sulfate to the solution to 20% saturation, the solution was applied to a Phenyl-Toyopearl column (Tosoh) equilibrated with 50 mM sodium phosphate buffer 20% saturated with ammonium sulfate, pH 7.0. The column was washed with the same buffer and then eluted with 50 mM sodium phosphate buffer. The fractions were dialyzed against 50 mM Tris/HCl buffer, pH 9.0, and then applied to a DEAE-Toyopearl column (Tosoh) equilibrated with 50 mM Tris/HCl buffer, pH 9.0. After washing the column with the same buffer, elution was performed with a linear NaCl gradient, from 0 to 100 mM, in 50 mM Tris/HCl buffer, pH 9.0. The mutant enzymes at each purification step were monitored by SDS-PAGE. Approximately 100 mg of purified mutants was obtained from 3.2 liters of cell broth.

The five N-terminal residues of the mutants were determined with a protein sequencer (Shimadzu PSQ-1), and CD spectra from 200 to 300 nm were measured with a J-720 CD spectropolarimeter (Jasco). These data for the mutants were the same as those for the wild enzyme.

*Assaying of the Reaction by TLC*—A mixture (1 ml) of 50 mM maltopentaose and enzyme with or without a nucleophile (2 M azide or formate) was incubated at pH 7.0 (25 mM phosphate buffer) and 25°C for 40 h. The concentrations of the enzymes used were 5.0 μM for the wild enzyme, 5.9 μM for E172A, 13.6 μM for E367A, and 1.1 μM for E172A/E367A, respectively. The reaction was terminated by heating at 100°C for 10 min, and the reaction mixture was then subjected to TLC with DC-Fertigplatten Kieselgel 60 (Merck) as the adsorbent. The solvent system was ethylacetate:methanol:water = 12:7:3, by volume. After development, the spots of sugars were visualized by heating the plate at 140°C for 5 min after spraying with vanillin-conc. $H_2SO_4$ in ethanol. For NMR measurement of the reaction product, the product was collected from the TLC plate and extracted with methanol.

*Measurement of Relative Activity*—The hydrolytic reaction of a substrate was measured at pH 7.0 (25 mM phosphate buffer) and 25°C. (i) The relative activities of the wild enzyme and mutant E367A in the presence or absence of 2 M azide were determined from a densitogram of the reaction products on a TLC plate (ii) The hydrolysis of maltopentaose catalyzed by the wild enzyme in the presence or absence of 2 M azide was followed by the reaction calorimetry method with an Omega Microcalorimeter (MicroCal, MA, USA) (*15*). The reaction was initiated by injecting 20 μl of a 375 nM enzyme solution into the sample cell (1.374 ml) filled with the substrate solution (2 mM). (iii) The hydrolysis of 1% soluble starch catalyzed by the wild enzyme in the presence and absence of 2 M azide was followed by the polarimetric method with a PM-70 automatic polarimeter (Union Giken) (*16*). The concentration of the enzyme used was 0.75 nM and the light pass of the reaction cell was 5 cm.

*Synthesis of β-Maltosyl Azide*—2′,3′,4′,6′-Tetra-*O*-acetyl-α-D-glucosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucosyl azide (hepta-*O*-acethyl-β-maltosyl azide) was synthesized by the method of Tóth *et al.* (*17*). The chemical structure of this compound was confirmed by inspection of proton and $^{13}C$ NMR spectra recorded with a JEOL α-500 spectrometer. Although the peaks of acetyl groups were not assigned because of peak overlapping, correlation peaks between glucose protons and acetyl groups except for the anomeric proton of the reducing end were clearly observed in the HMBC spectrum (5.36 H1′, 4.80 H2′, 5.30 H3′, 5.00 H4′, 3.90 H5′, 4.02–4.20 H6a′/H6b′, 4.66 H1, 4.73 H2, 5.21 H3, 3.98 H4, 3.74 H5, 4.20–4.47 H6a/H6b, $J_{12}$ = 8.6 Hz, $J_{1'2'}$ = 4.3 Hz, 95 56 C1′, 69.86 C2′, 69.10 C3′, 67.80 C4′, 68.50 C5′, 61.32 C6′, 87.32 C1, 71.34 C2, 74.93 C3, 72.21 C4, 74 09 C5, 62 39 C6). A MALDI-TOF-MS spectrum was obtained with a Shimadzu KOMPACT MALDI using 10 mg/ml 2,5-dihydroxybenzoic acid in water.ethanol (9:1) as the matrix. TOF-MS spectrum *m/z* 684 ($M+Na^+$). β-Maltosyl azide was obtained by deacetylation of hepta-*O*-acetyl-β-maltosyl azide (*18*) A FAB-MS spectrum was obtained with a JEOL JMS-AX500 mass spectrometer using glycerol as the matrix. FAB-MS spectrum *m/z* 390 ($M+Na^+$).

## RESULTS AND DISCUSSION

BCM β-amylase is an inverting enzyme that hydrolyzes the α-1,4 glucosidic linkage of a substrate and produces the β-anomer of maltose. The catalytic residues of this enzyme have been found to be Glu172 and Glu367 by means of sequence alignment and structural analysis by X-ray crystallography (*10, 11*). To confirm that these residues act as catalytic ones, three catalytic site mutants: E172A [Glu172 →Ala], E367A [Glu367→Ala], and E172A/E367A [Glu172 →Ala/Glu367→Ala] were created by site-directed mutagenesis All had lost the activity. This suggests that they are catalytic residues.

It is conceivable that the catalytic reaction of the inverting enzyme proceeds through the SN1 or SN2 reaction (*19*). In either the SN1 or SN2 reaction, the carboxyl group (-COO⁻) of a catalytic residue, which acts as a general base, activates the coordinated water molecule Therefore, if an external nucleophile is able to occupy the space created on replacement of the catalytic residue with an alanyl residue, the activity of a mutant of which the general base is substituted would be restored. A mixture of a mutant and maltopentaose (G5) in the presence of azide or formate was incubated at pH 7.0 and 25°C for 40 h, and then the components of the mixture were analyzed by TLC (Fig. 1). In the case of E367A, new spots at positions corresponding to the mobilities of G2 and G3 in the presence of a nucleophile were detected in lane 5 [see plates (a) and (b) in Fig. 1], but no corresponding spots appeared in the cases of the other mutants (see lanes 4 and 6) The ratio of the relative activity of E367A to that of the wild enzyme in the presence of 2 M azide was estimated to be about 1/20,000 by densitome-

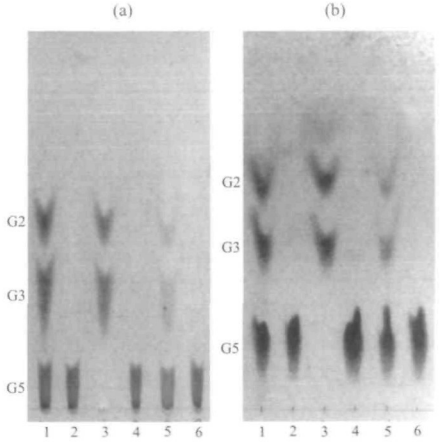
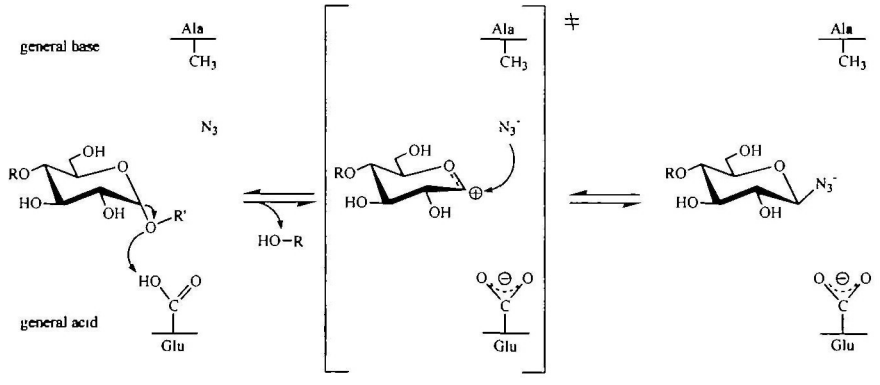



Fig 1 **Effects of an exogenous nucleophile on catalytic residue mutants of BCM β-amylase.** A mixture (1 ml) of 50 mM maltopentaose, 2 M nucleophile and a mutant or the wild enzyme was incubated at pH 7 0 (25 mM phosphate buffer) and 25°C for 40 h. The sample solutions for TLC contained sodium azide (a) and sodium formate (b) as the nucleophile, respectively Lanes 1 and 2 on each TLC plate contained standard sugars, lane 3 5 0 μM wild enzyme, and lanes 4–6 5 9 μM E172A, 13 6 μM E367A, and 1 1 μM E172A/E367A, respectively G2, G3, and G5 are maltose, maltotriose and maltopentaose, respectively

(Scheme 1)




(Scheme 2)

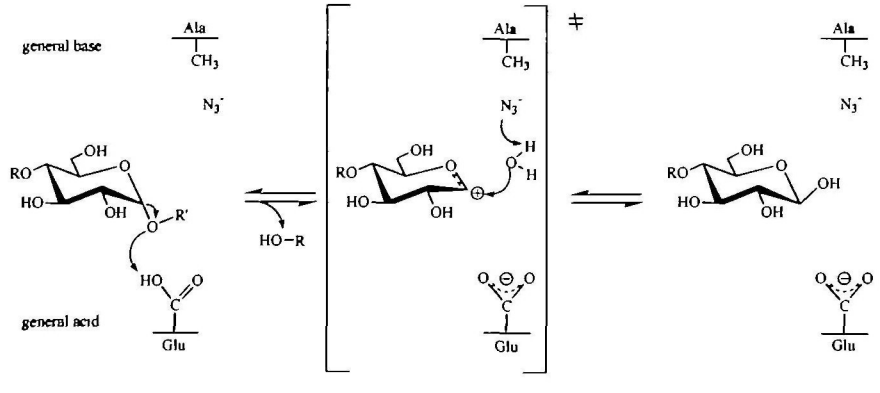



Fig 2 **Possible reaction mechanisms of chemical rescue by azide.**

try of the TLC spots, reaction calorimetry and polarimetric measurement,[2] thus rescue of the activity of E367A by azide was clearly observed. These findings suggest that Glu367 acts as a general base and Glu172, therefore, acts as the proton donor.

To investigate the reaction mechanism of E367A in the presence of azide or formate, the structure of the product exhibiting the greatest mobility in lane 5 in each of Fig. 1, (a) and (b), was analyzed by $^{1}$H-NMR and $^{13}$C-NMR spectroscopy. These spectra were completely identical to those of maltose, indicating the product is maltose. There are two possibilities that maltose is produced by E367A when 2 M azide exists in the reaction mixture (schemes 1 and 2 in Fig. 2). In the case of scheme 1, azide nucleophilically attacks an anomeric carbon of the reaction intermediate, β-maltosyl azide being yielded. After that, it is hydrolyzed to a maltose and an azide.[3] Another possibility is given in scheme 2. The azide pulls off the proton from a water molecule to produce an activated hydroxide ion, and the hydroxide ion of the water nucleophilically attacks the carbonium ion intermediate to produce β-maltose. To determine whether β-maltosyl azide is hydrolyzed or not, the compound was synthesized. A mixture containing synthesized β-maltosyl azide (50 mM), E367A (13.6 μM), and sodium azide (2 M) was incubated at pH 7.0 (25 mM phosphate buffer) and 25°C for 40 h, and then the mixture was analyzed by TLC. No products derived from β-maltosyl azide were detected (data not shown). This indicates that β-maltosyl azide is not hydrolyzed by E367A. Thus, it was found that the reaction mechanism of E367A in the presence of azide proceeds according to scheme 2 and not to scheme 1.

On the other hand, the reactions in the cases of mutants of β-1,4-glycanase Cex from *Cellulomonas fimi* (*1*) and *Bacillus* 1,3-1,4-β-D-glucan 4-glucanohydrolases (*3*) proceed *via* direct attack by azide of the intermediate, β-glycosyl azides being produced. Among the catalytic residue mutants of β-glycosidases, the only enzyme of which the three-dimensional structure with a substrate analogue has been reported is β-1,4-glycanase Cex from *Cellulomonas fimi* (*21*). At the final stage of the reaction mechanism, the role of azide for BCM β-amylase differs from that for β-1,4-glycanase Cex. In order to determine the configuration of the catalytic residue of each enzyme as to the reactive pyranose of a substrate, the reactive pyranoses in both enzyme-ligand complexes are superimposed in Fig. 3 The distance from the C1-atom of the reducing end glucose residue to the oxygen atom of the side-chain of the base catalyst Glu367 in BCM β-amylase (Oyama, T., Kusunoki, M., and Nitta, Y, unpublished data) is not so different from that of Glu127 in the β-1,4-glycanase Cex (*20*), however, Glu127 of β-1,4-glycanase Cex seems to be near C-1 of the reactive pyranose ring, but Glu367 of BCM β-amylase is near C-5: the distances from the C-1, C-3, and C-5 atoms of the pyranose ring to the oxygen atoms of the carboxyl group for the two enzymes are 4.7, 6.0, and 4.6 Å for Glu367, and 4.5, 5.6, and 5.9 Å for Glu127, respectively. In the case of the E127A mutant of the retaining β-1,4-glycanase Cex, it seems that an azide occupies the space created by the mutation and attacks the carbonium ion to produce β-cellobiosyl azide On the contrary, in the case of the E367A mutant of BCM β-amylase, the azide can not attack the carbonium ion directly and the reaction proceeds according to scheme 2 in Fig. 2.

For convenience's sake, we explained the mechanism of the chemical rescue of E367A by azide according to the scheme in which the addition of a hydroxide ion to the C-1 carbon proceeds through the SN1 reaction At present, however, we are able to explain the mechanism of the chemical rescue on the basis of either the SN1 or SN2 reaction, and the roles of azide and a water molecule in the reaction are the same in the cases of both SN1 and SN2. In this study, we found that a catalytic residue mutant, E367A, which had lost the hydrolytic activity, is reactivated by azide and produces maltose from maltopentaose. This is the first study on the chemical rescue as to the hydrolysis of substrate with an α-1,4-glucosidic linkage by an external nucleophile. We proposed a mechanism for the chemical rescue, and could confirm that Glu172 and Glu367 of BCM β-amylase are acid and base catalysts, respectively

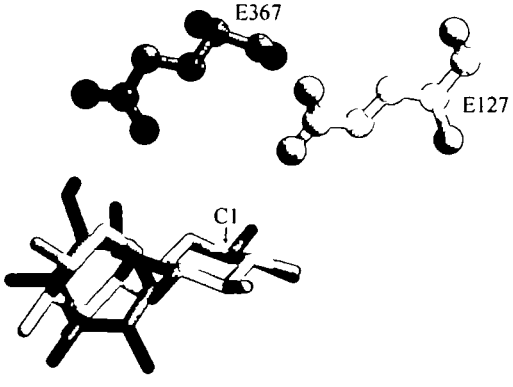



Fig 3 **The positions of E367 of inverting BCM β-amylase and E127 of retaining β-1,4-glycanase Cex.** The models show the configurations of the glutamyl residues and ligands in the enzyme-ligand complexes, where the pyranose rings ($^{4}C_{1}$-form) of the reducing-end residues of the ligands are superimposed upon each other at the catalytic site. The dark models are for BCM the β-amylase-maltose complex and the light ones for the β-1,4-glycanase Cex–fluorocellobioside complex. The anomeric form of the ligand binding to BCM β-amylase is the β-form

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