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The *Rhizopus niveus* glucoamylase-catalyzed reaction for substrate maltose in the absence or presence of acetonitrile

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In the presence or absence of acetonitrile CH_3CN, the molecular mechanism on the *Rhizopus niveus* glucoamylse (GA)-catalyzed reaction was studied by the steady-state kinetics for a substrate maltose (G2). At high concentration of G2, the GA-catalyzed reaction was observed not to obey the Michaelis kinetics and was reasonably explained with a mechanism of the substrate-inhibition involving the ternary complex ESS. Moreover, CH_3CN was found to effect on (decrease in) the dissociation constants *Ks* (for ES) and *Ks'* (for ESS). No transglucosylation was confirmed for the G2 reaction. These findings will support the hydrophobic-driven mechanism proposed in a previous study.

Key words:
Rhizopus niveus, glucoamylase, substrate-inhibition, hydrophobic-driven mechanism, acetonitrile,

Glucoamylase(GA) catalyzes the hydrolytic reaction for the non-reducing-end glucosidic bond of substrate α-glucans including starch and malto-oligosaccharides. Based on the steady-state kinetic observations with a series of linear substrate malto-oligosaccharides Gn (degree of polymerization n = 2～7), the subsite theory has been successsfully applied to GA from *Rhizopus niveus*[1,2]. It is concluded that its active site is constructed with 7 subsites in total and the catalytic site is located at between subsites 1 and 2[1-12]. Based on the subsite structure of GA, the ligand binding to the enzyme is explained by the two-step mechanism as follows: at the first step, which is the fast association process, a ligand is bound to subsite 2, then moves to

subsite 1 at the successive second step, which is slow isomerization process. Thus the isomerizaiton process refers to the productive binding, in which subsite 1 should be occupied by the ligand[3-12].

On the other hand, enzymes catalyze sometimes an interesting reaction under the presence of an organic solvent. It must be an excellent idea to employ a non-alcoholic solvent CH_3CN, which would not be concerned to the solvolysis. Because CH_3CN has no OH group in the molecule.

In this paper, the steady-state kinetics for a substrate maltose G2 in the presence or absence of CH_3CN was carried out to investigate the effect of CH_3CN on the GA-catalyzed reaction.

EXPERIMENTAL

Materials.

The enzyme sample GA from *Rhizopus niveus* (1,4-α-D-glucan glucohydolase, EC 3.2.1.3) was purchased from Toyobo Co., Osaka, and used without further purification. It was confirmed to be pure using an SDS-PAGE technique. Concentration of the enzyme was determined spectrophotometrically with the absorption unit $\underline{A}^{1\%}_{280nm}$ of 16.3 cm^{-1} and the molecular weight of 58,000[10]. G2, CH_3CN and other chemicals were purchased from Nacalai Tesque Inc., Kyoto, which were used without further purification. 1-Phenyl-3-methyl-pyrazolone (PMP) was purchased from Kisida Chemicals Co., and the other reagents and solvents for precolumn labeling were used of the highest grade in the commercially available reagents.

Steady-state kinetics on the GA-catalyzed hydrolysis for G2.

The time course of the GA-catalyzed reaction for G2 was observed to evaluate the initial velocity $\underline{v}$ in an 0.025M acetate buffer at pH 4.5 and 5.0 °C. The amount of a product glucose released by the catalyzed reaction was measured using a commercially available glucose-assay kit, Glucose C-II Test Wako.

High-performance liquid chromatography.

The High-performance liquid chromatography (HPLC) apparatus employed was composed of a Shimadzu Liquid Chromatograph LC-4A, a Hitachi 833 data processor, a Jasco UVIDEC-100-II UV detection spectrophotometer, and a Cosmosil Packed Column 5C18-AR-II(4.6-mm I.D. x 150-mm length). Elution was carried out with a mixture of 2.5mM ammonium dihydrogen-phosphate and 20% acetonitrile at flow rate 1.0ml/min and column temperature was 40 °C. Preparation of the precolumn labeling with 1-phenyl-3-methylpyrazolone (PMP) was carried out by the same procedures described elsewhere[13].

Examination of the CH_3CN-induced change in conformation of GA.

For examination of CH_3CN-induced change in conformation, UV-difference absorption-spectra of GA were observed using a Shimadzu UV-1600 spectrophotometer under the presence or absence of CH_3CN in an 0.025M acetate

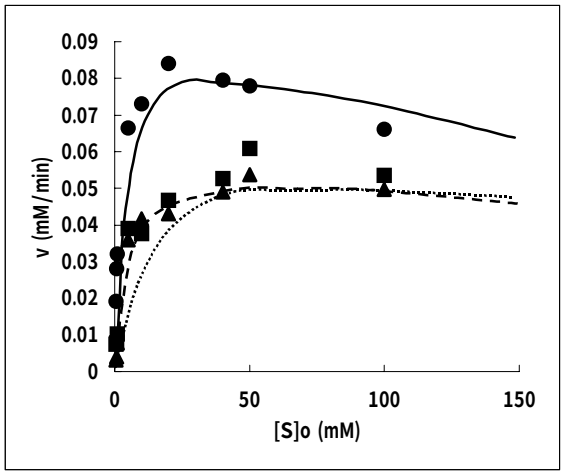

Fig.1. The v-[S]o plots of the GA-catalyzed reaction for G2

$[E]_o$, 3μM, at pH 4.5 and 5°C; closed circles; CH_3CN 0%, closed squares; CH_3CN 20%, closed triangles; CH_3CN 40%. Lines were obtained theoretically using Eqs.(1) and (2) with the values summarized in Table 1.

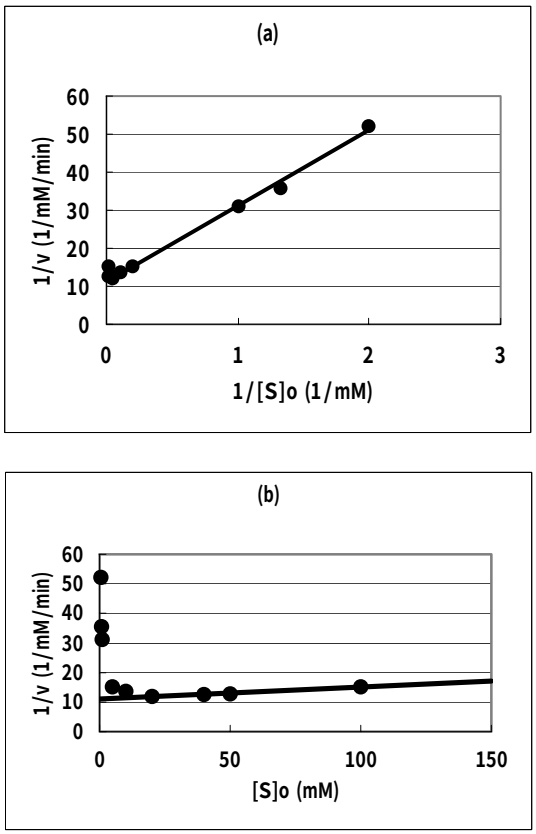

Fig.2. The linear plots of the GA-catalyzed reaction for G2.

Conditions were the same as described in Fig.1. (circles, CH_3CN 0%)

buffer, pH 4.5 at 5.0 °C. Changes in the molar difference absorption of the enzyme at 292nm ($\Delta\varepsilon_{292}$) were evaluated in the absence or presence of 40% and 20% CH_3CN.

RESULTS AND DISCUSSION

GA-catalyzed reaction for a substrate G2 in the presence or absence of CH_3CN.

In the presence or absence of CH_3CN, the GA-catalyzed reactions for G2 were observed and the v- $[S]_0$ plots were obtained as typical examples of the results are shown in Fig. 1., of which plots are rather characteristic for the enzyme-catalyzed reactions.

At higher concentration of G2, v does not increase but decreases with $[S]_0$. Thus for this case, a possible mechanism, substrate inhibition, was suggested as follows;

$$E + S \underset{Ks}{\rightleftharpoons} ES \longrightarrow E + P \quad (1)$$

$$ES + S \underset{Ks'}{\rightleftharpoons} ESS \quad (2)$$

Based on the reaction scheme in Eqs. (1) and (2), the rate equation is represented,

$\underline{v} = k_0[E]_0/(1+Ks/[S]_0+[S]_0/Ks')$ (3),

where the dissociation constants *Ks* and *Ks'* mean as follows: $Ks = [E]_0[S]_0/[ES]$ and $Ks' = [ES][S]_0/[ESS]$, respectively. Thus, the kinetic parameters *Ks*, *Ks'* and k_0 can be evaluated using a linear equation derived from Eq. (3)[14]. At low concentration of G2, the term $[S]_0/Ks'$ can be reasonably neglected then *Ks* can be evaluated, whereas at high concentration of G2, *Ks'* can be reasonably estimated. Typical examples of linear plot for low G2 and for high G2 in 0% CH_3CN are illustrated in Fig. 2(a) and Fig. 2(b), respectively. Based on these plots, the rate parameters were obtained as summarized in Table 1. Theoretical curves shown in Fig. 1 were obtained by Eq. (3) with the values listed in Table 1, according to the reaction scheme in Eqs. (1) and (2). The experimental points are in almost consistent with the theoretical curves, nevertheless in the absence or presence of CH_3CN, suggesting that the reaction scheme is compatible with the experimental results for G2.

Table 1. Kinetic parameters of the GA-catalyzed reaction for G2 in the presence and absence of CH_3CN

CH_3CN (%)	*Ks* (mM)	*Ks'* (mM)	k_0 (sec^{-1})
0	1.9	270	0.51
20	5.1	310	0.35
40	7.2	590	0.35

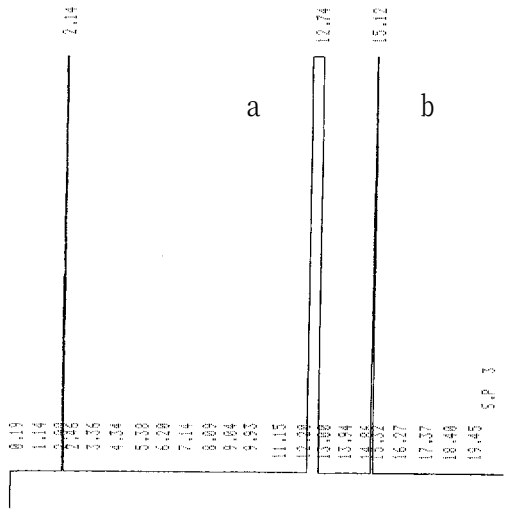

Fig.3. HPLC of the reaction mixture

$[E]_0$,3μM $[S]_0$,50mM at pH4.5, 20°C and 30min in the presence of CH_3CN 20%
Elutant, 20 v/v% CH_3CN in 2.5mM ammonium dihydrogenphosphate
Products, a:G2 b:glucose

Effect of CH_3CN on the mechanism of GA-catalyzed reactions.

In the presence of CH_3CN, the kinetic parameters, *Ks'* and k_0 for G2 are not so much different but almost identical to those in the absence of CH_3CN, whereas *Ks* is much different (see Table 1.). Thus, CH_3CN is supposed to effect on the binding of the substrate, where *Ks* may refer to the dissociation constant for the Michaelis complex. However, we do not have evidence on the *Ks* and *Ks'* processes of the substrate binding. Subsite structure of GA has been analyzed using the steady-state kinetics

with a series of malto-oligosaccharides as substrate[1-2]. Subsites 2 and 3 have binding affinities 4.9 and 1.6 kcal/mol, respectively, and other subsites have smaller affinity than these, thus G2 is mainly bound to occupy subsites 1 and 2, and to form the ES complex. According to the subsite theory, subsite affinity can give the *Ks* value[1-12]. The *Ks* value (1.9mM) in Table 1 is almost consistent with the value calculated with the affinities of subsites 1 and 2. When another G2 molecule is bound to subsites 3 and 4 and makes to form the ESS complex, the dissociation constant *Ks'* is calculated to more than 1000mM, however, the experimental result was obtained to be 270mM as shown in Table 1. It is suggested that another G2 molecule is bound at different subsites from subsites 3 and 4. In this stage of the investigation, we cannot conclude at what site the second substrate is bound to form the ESS complex.

The GA-catalyzed reaction was observed by the HPLC technique to confirm the hydrolytic reaction for the substrate G2. As an example of the results is illustrated in Fig.3, a product by transglucosylation was not found under the experimental conditions employed. Thus, in the presence of CH_3CN, the GA-catalyzed reaction for G2 will be explained by the mechanism "substrate inhibition" represented in the reaction scheme shown with Eqs.(1) and (2).

Examination of the CH_3CN-induced change in conformation of GA.

UV-difference absorption spectrum of the enzyme was observed in the presence of CH_3CN to examine the CH_3CN-induced change in conformation. The molar change in dfference aborption $\Delta\varepsilon_{292}$ was evaluated to be 450 in the presence of 20% CH_3CN. When one tryptophan residue is exposed from the inside of a protein molecule to its surface, $\Delta\varepsilon_{292}$, which is called denaturation-blue shift, is estimated to about 1,500[4,15-16]. Thus, the difference absorption observed here by the addition of 20% CH_3CN corresponds to 0.3 residue of tryptophan exposed. In the presence of 40% CH_3CN, 2/3 parts of a tryptophan residue are estimated to be exposed, then still not so much change in conformation can be induced by CH_3CN. In the case of 8M guanidine hydrochloride at 25 °C, 4.0 and more residues of tryptophan were exposed to the surface of GA molecule. Thus, it can be concluded that little change in conformation of GA was produced by the addition of CH_3CN under the experimental conditions employed.

The kinetic parameters *Ks* and *Ks'* obtained in the presence of CH_3CN are larger than those in its absence, whereas CH_3CN little effects on change in the conformation of GA. Thus, CH_3CN can be concluded to affect on the binding process of the complex formation between GA and G2. In the previous studies on the saccharide binding to an enzyme β-glucosidase, we have found that an organic

Table 2. Molar difference absorption o
at 292nm ($\Delta\varepsilon_{292}$) in the presence of Cl
at pH 4.5 and 5°C

CH_3CN(%)	20	40
$\Delta\varepsilon_{292}$($M^{-1}cm^{-1}$)	440	1060

solvent will weaken the hydrophobic interaction between a substrate and the enzyme, and have proposed a hypothesis, "hydrophobic driven" mechanism[14,17].

In this time, these experimental results with GA will support the hydrophobic-driven mechanism on the productive substrate binding proposed previously.

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