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Article

Urea-induced change in subsite structure of glucoamylase from *Rhizopus neveus*: Evaluated by steady-state kinetics with substrate malto-oligosaccharides

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Subsite structure, number of subsites and their affinities Ai for glucose residue, of glucoamylase from *Rhizopus niveus* was analyzed under the presence of urea by means of the steady-state kinetics for the substrate malto-oligosaccharides Gn (degree of polymerization, n = 2~7). Urea was found to effect on (decrease in) the subsite affinities A_2 and A_6, suggesting that hydrophobic interactions between substrate and these subsites (2 and 6) are decreased in the presence of urea. Based on the experimental results, tryptophan residues are supposed to locate around the two subsites, 2 and 6.

Keywords
steady-state kinetics, rate parameters, subsite structure, glucoamylase, urea, *Rhizopus niveus*, hydrophobic effect.

Enzyme-catalyzed reactions are usually carried out in the dilute solution system at the most laboratories, in fact we have studied the steady-state kinetics on the enzyme-catalyzed hydrolysis reaction of several kinds of amylase including glucoamylase and evaluated their subsite structures on the basis of the steady-state kinetics in the dilute solution systems [1-9].

However, in the cytosol of biological cells and also in the reaction vessel of the manufacture process for making materials, almost all the reactions cannot proceed in the dilute solution

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system. The enzyme-catalyzed reactions must be investigated in the unusual (not dilute) conditions to get information on the structure and reaction, thus we are interesting in effects of some chemicals for the enzyme-catalyzed reaction under the unusual conditions. Urea is very worthy, because urea has no OH group, then urea would not be an acceptor for the transfer/condensation reactions, if possible, under high concentration in the reaction mixture. Urea is known to be one of the reagents for denaturation of proteins and inactivation of enzymes. Here, no denaturation and no change in conformation of glucoamylase was confirmed to be caused by urea under the experimental conditions employed. Thus, urea could be effect on the enzyme function without change in conformation. Another reagent, popular for protein denaturant, guanidine HCl has dissociating ionic group (electric charge) in the molecule, whereas urea does not. Urea does not concern an electrostatic interaction with the enzyme, thus urea is an excellent reagent for investigattion of effect on the enzyme function. For the basic and applied fields, it is interesting and important to investigate the effect of urea on the glucoamylase-catalyzed reaction for substrate malto-oligosaccharids.

In this study, the steady-state kinetics was carried out in the presence and absence of urea for evaluation and characterization of the subsite structure of glucoamylase from *Rhizopus niveus*.

EXPERIMENTAL

Materials

An enzyme preparation glucoamylase from *Rhizopus niveus* (1,4-*D*-glucan glucohydrolase, EC 3.2.1.3.), a pure (crystalline) grade, was purchased from Toyobo Co., Osaka, and was used without further purification.

Concentration of the enzyme solution was determined spectrophotometrically assuming $A^{1\%}_{1cm}$ at 280 nm to be 16.3 and a molecular weight of 58,000. Malto-oligosaccharides Gn (abbreviation G_2-G_7 represents a degree of polymerization n=2~7) were given from Nihon Shokuhin Kako Co., Ltd., Fuji City, and confirmed to be pure by an HPLC analysis. An enzymatic glucose-determination kit, Glucose C-II Test Wako, which is consisted with GOD, was obtained from Wako Pure Chemicals Co., Osaka, and used after 1:3 dilution with 0.06 M phosphate buffer pH 7.1. Urea was crystallized from water before use.

Other chemical reagents in guaranteed grade were purchased from Nakalai Tesque Inc., Kyoto, and used without further purification.

Methods

Steady-state kinetics of the glucoamylase-catalyzed reactions for malto-oligosaccharides as substrates

Five minutes after mixing urea with enzyme solution (20 mM acetate buffer pH 4.5, 5.0°C), the substrate solution containing urea in 20 mM acetate buffer pH 4.5, 5.0°C was added to the enzyme-urea solution to initiate the enzyme-catalyzed reaction. After the start of the reaction, at 0, 2, 4, 6, and 8 min, an appropriate volume of the reaction mixture was taken out and was added into a 50 mM sodium hydroxide solution to stop the catalyzed reaction. The amount of glucose released in the catalyzed reaction was measured spectrophotometrically using a glucose assay-kit, Glucose C-II Test Wako to observe the initial velocity.

The steady-state kinetic parameters, the Michaelis constant K_m and the molar activity k_0, were determined on the basis of those initial velocities obtained from the catalyzed-reaction time-curves for malto-oligosaccharide substrates and evaluated by the steady-state kinetic procedures using an NEC PC and a Bio-graph software (Kyoto Soft Co.) [10].

Examination of urea-induced change in conformation of glucoamylase

The enzyme and urea solutions were mixed just prior to add the substrate to initiate the reaction. The reaction mixture was subjected to measure the difference UV-absorption spectrum of the enzyme, where the final concentration of the enzyme and urea was fixed to be 13.1 µM, and 5.4 (or 2.7) M, respectively. The molar difference absorption at 290 nm ($\Delta\varepsilon_{290}$) was evaluated on the time curve obtained from the reaction between the enzyme and urea by using a Shimadzu UV-1600 spectrophotometer at 5.0°C in an 0.02 M acetate buffer, pH 4.5 as the same procedures employed previously [11].

Viscosity in the mixture of the enzyme and urea was measured using an Ostwald viscometer at 5.0°C in 0.02 M acetate buffer, pH 4.5.

RESULTS AND DISCUSSION

Steady-state kinetics on the glucoamylase-catalyzed reaction for malto-oligosaccharides as substrates

Before beginning of the enzyme-catalyzed reaction, the urea solution was combined with the enzyme solution in a final concentration of 5.4 M and after 5 min the substrate solution in the presence of 5.4 M urea was added to the enzyme-urea mixture for start of the enzyme-catalyzed reaction at 5.0°C and pH 4.5. The product glucose was measured at

Table 1. Steady-state kinetic parameters for the glucoamylase-catalyzed reaction in the presence and absence of 5.4 M urea.

Substrate	Urea (M)	K_m (mM)	k_0 (sec^{-1})	k_0/K_m ($mM^{-1}sec^{-1}$)	$\Delta\ k_0/K_m$
G2	0	0.93±0.06	0.68±0.03	0.73	4.6
	5.4	2.70±0.07	0.43±0.03	0.16	
G3	0	0.31±0.11	1.50±0.28	4.8	4.0
	5.4	0.38±0.01	0.47±0.01	1.2	
G4	0	0.49±0.03	4.01±0.29	8.2	2.9
	5.4	0.83±0.14	2.30±0.11	2.8	
G5	0	0.15±0.05	4.20±0.13	28	3.5
	5.4	0.17±0.01	1.80±0.03	11	
G6	0	0.04±0.01	3.40±0.21	85	12
	5.4	0.30±0.03	2.10±0.14	7.0	
G7	0	0.03±0.003	2.90±0.15	97	11
	5.4	0.27±0.051	2.30±0.20	8.5	

Substrate; malto-oligosaccarides Gn, at pH 4.5 and 5.0℃,
± means the standard deviation.

the appropriate time intervals to observe the reaction time-course. The initial velocities obtained for the substrate malto-oligosaccharides G_2 ~ G_7 are plotted against the initial concentration of substrate; some typical examples of the results are illustrated in Fig. 1, where the solid lines are obtained theoretically using the rate parameters summarized in Table 1. It is clearly shown that an addition of urea affects on the enzyme-catalyzed reactions for all kinds of the substrate. The rate parameters, the Michaelis constant K_m and the molar activity k_0, were evaluated using a linear plot $v_0/[S]_0$ versus $[S]_0$ and the least squares method, where v_0 and $[S]_0$ are the initial velocity and the initial concentration of the substrate, respectively. The rate parameters evaluated for the substrates G_2 ~ G_7 together with their standard deviations are summarized in Table 1, where k_0/K_m was obtained based upon

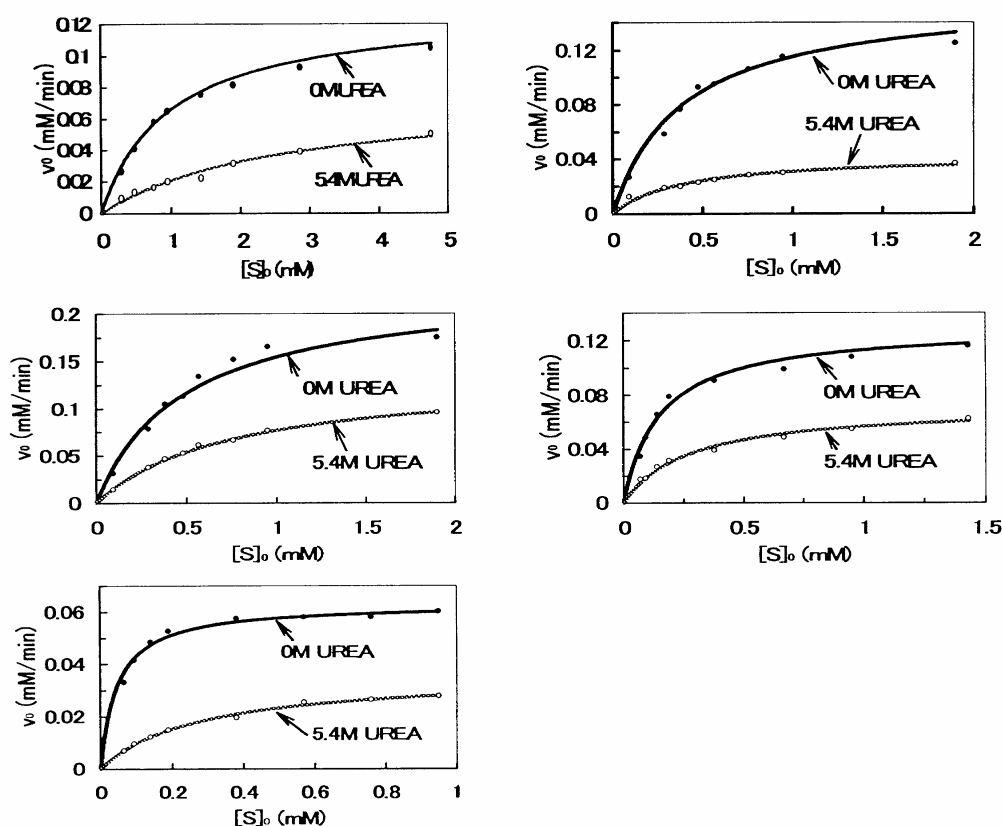

Fig. 1. Typical examples of s-v plot for the glucoamylase- catalyzed reaction in the presence and absence of 5.4 M urea.
Substrate: G2, G4 and G6 (left hand side from up to down), G3 and G5 (right hand side from up to down), Enzyme: for G2; 3.0 µM, for G3; 1.5 µM, for G4; 1.0 µM, for G5; 0.5 µM and for G6; 0.3 µM, at pH 4.5 and 5.0°C.

these rate parameters. An addition of urea results in an increase in K_m for all the substrates, especially a large increase was observed for G_6 and G_7, where is only a small difference was observed on k_0. For G_6 and G_7, $\Delta k_0/K_m$ a difference in k_0/K_m between in the presence and absence of urea, is 11 ~12 times. However, that is only 3 ~4 times for $G_3 \sim G_5$.

Based on the results obtained by the steady-state kinetics, we can reasonably refer K_m to the strength of binding between substrate and enzyme. Thus, change in free energy $-\Delta G$ for the binding is calculated based upon these K_m values according to an

equation $-\Delta G = RT\ln K_m$, where R and T are gas constant and temperature, respectively (Table 2). For substrates $G_3 \sim G_5$, $\Delta\Delta G$, a difference in $-\Delta G$ between the presence and absence of

Table 2. Binding free energy of Gn to glucoamylase.

Substrate	Urea (M)	−ΔG (kcal/mol)	ΔΔG (kcal/mol)
G2	0	3.8	0.5
	5.4	3.3	
G3	0	4.5	0.2
	5.4	4.3	
G4	0	4.2	0.3
	5.4	3.9	
G5	0	4.9	0.1
	5.4	4.8	
G6	0	5.6	1.1
	5.4	4.5	
G7	0	5.7	1.2
	5.4	4.5	

Based on the K_m values, the binding free energy −ΔG was evaluated in 0 M and 5.4 M urea, at pH 4.5 and 5.0ºC. ΔΔG is the difference between −ΔG in 0 M urea and −ΔG in 5.4 M urea.

urea, is small and almost identical; ΔΔG is 0.1 ~ 0.3 kcal/mol. However, for G_6 and G_7, ΔΔG value is pretty large; 1.1 ~ 1.2 kcal/mol.

Urea-induced change in the subsite structure of glucoamylase

Based on the subsite theory described previously [1,10,12,13], the kinetic parameters, the Michaelis constant K_m and the molar activity k_0, allow us to evaluate subsite affinity Ai, number of subsites, and the intrinsic rate constant k_{int}. The rate constant k_{int} is defined not to be dependent on the size of substrate saccharide (degree of polymerization n) as described elsewhere [12]. The subsite parameters, i, Ai, and k_{int}, are called “subsite structure”, as summarized in Table 3. It clearly shows that the second subsite (i =2, thus A2) has the largest affinity and the other Ai’s are much smaller than A2, nevertheless in the presence of urea. The subsite structures evaluated in the presence or

Table 3. Subsite structure (number of subsite, affinities of these subsite and k_{int}) of glucoamylase.

Subsite (i)		1	2	3	4	5	6	7
Subsite affinity (Ai)		A1	A2	A3	A4	A5	A6	A7
Ai (kcal/mol)	0 M Urea	-0.05	5.0	1.0	0.29	0.68	0.63	-0.01
	5.4 M Urea	-0.02	4.4	1.1	0.44	0.74	-0.20	0.11
k_{int} (sec^{-1})	0 M Urea; 12.0, 5.4 M Urea; 2.9							

Evaluated in the presence and absence of 5.4 M urea, kint; intrinsic rate constant.

absence of urea are schematically illustrated with histograms for good comparison of those between presence and absence of urea in Fig. 2.

It is interesting that an addition of urea to glucoamylase-catalyzed reaction decreases A2 and A6, whereas A1, A3, A4, A5, and A7 increase. Anyway, A2 and A3 are considerably larger than A4 ~ A7, then subsites 2 and 3 carry the essential role in the binding of substrate saccharides. Here the catalytic site must be adequately situated at between subsites 1 and 2, thus malto-oligosaccharides are exclusively bound in the two modes: one productive and one nonproductive binding modes. In the productive binding mode, maltose, for instance, is bound to occupy subsites 1 and 2, whereas its nonproductive mode occupies subistes 2 and 3. Thus an increase in A1 and a decrease in A2, of which findings are brought about by the presence of urea, have an important meaning for the enzyme-catalyzed reaction. Based on the kinetic parameters k_0 and K_m for n-mer substrates, a value k_0/K_m is given by the sum of the affinities Ai of the i-th subsites occupied by the binding of the substrate molecule, $\sum_{i}^{cov}$,

$$(k_0/K_m)_n = (0.018)\, k_{int} \sum_{p} \exp \left(\sum_{i}^{cov} Ai/RT\right)_{n,p} \quad (1)$$

where 0.018 arises from the contribution of mixing entropy in water (= 2.4 kcal/mol) and k_{int} is assumed constant irrespective of n and j, which is the total binding mode, then j conceives productive p and nonproductive q; j = p + q. Thus Eq.

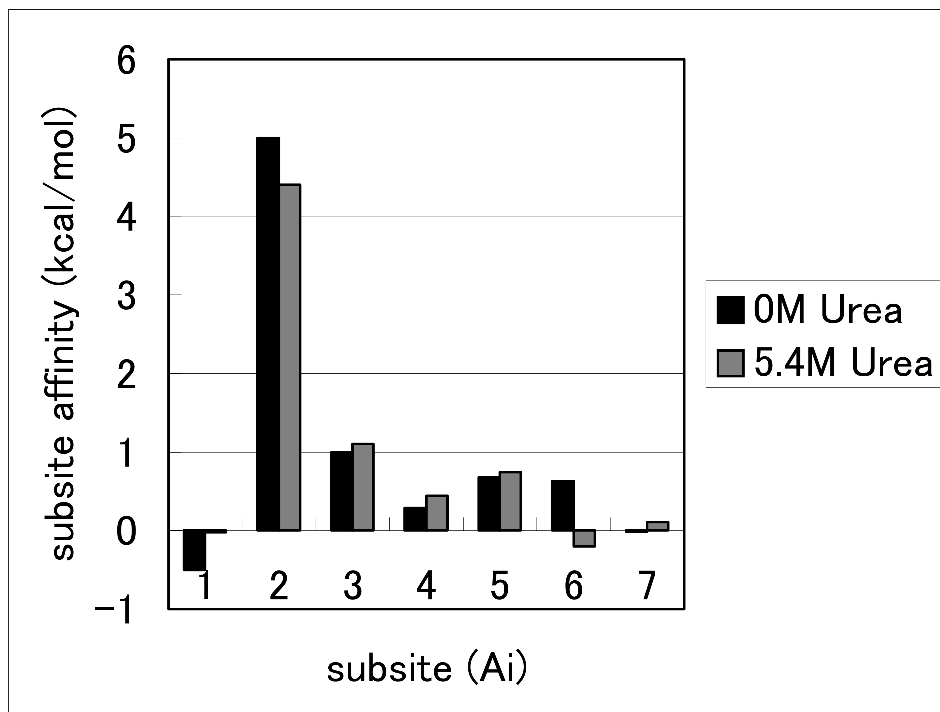

Fig. 2. Subsite structure of glucoamylase, evaluated in the presence and absence of 5.4 M urea, on the basis of the steady-state kinetic parameters obtained. The catalytic site; between subsite 1 and 2.

(1) indicates that only a productive binding (p) out of the total (j) is involved in a term k_0/K_m, which is proportional to k_{int} as seen in Eq. (1). A k_0/K_m is evaluated with the kinetic parameters K_m and k_0, which are obtained experimentally for the substrate malto-oligosaccharides Gn, as summarized in Table 1.

For malto-oligosaccharides G2~G5, a difference in k_0/K_m between the presence and absence of urea $(k_0/K_m)_{0Murea}/(k_0/K_m)_{5.4Murea}$ = around 3 ~ 4) should be reflected by k_{int}, as seen in Table 3, where the difference in k_{int} between the presence and absence of urea (around 4 times) is almost identical with that in k_0/K_m (3 to 4 times). On the other hand, the difference in k_0/K_m (= around 10 ~12) for G_6 and G_7 is obviously much larger than that in k_{int} (around 4 times), suggesting that urea results in much more effect on the productive binding of G_6 and G_7. Nozaki and Tanford have confirmed that the hydrophobic interaction between tryptophan residue and water is decisively weakened by urea [14]. Moreover, using kinetic methods a tryptophan

residue is suggested to locate at subsite 1 of the enzyme [4-6,10], thus it is possible that urea affects on weakening the hydrophobic interaction between the substrate saccharides and the tryptophan residue in the enzyme subsite.

In this stage of the investigation, we have not explained why k_{int} decreases in the presence of urea and cannot deny the possibility that urea has another effect on the enzyme-catalyzed reaction including viscosity.

Anyway, we would like to conclude here that urea brings about a good result in the glucoamylase-catalyzed hydrolysis of malto-oligosaccharides having smaller degree of polymerization than that of G_6~G_7.

Effect of urea on the conformation of glucoamylase

In general, urea is one of the popular denaturation reagent for proteins and enzymes, then it is very essential to examine wheter there is or not an urea-induced change in conformation of the enzyme preparation. The examination was carried out using the UV-difference absorption spectrophotometry in the presence and absence of urea as can be seen in the previous paper [11]. A decrease in difference absorption ΔA at 290 nm ($-\Delta A_{290}$), which was produced by the urea-induced change in conformation, was found to be very dependent on reaction temperature and reaction time. ΔA_{290} at 5.0°C was found to be much smaller than that at 45°C. On the other hand, after an addition of urea, the difference absorption was caused within five minutes and after that the difference absorption was almost constant. Thus the difference absorption at 290 nm was recorded for about 30 min on the spectrophotometer and the reaction curves were extrapolated to time zero for evaluation of the ΔA_{290} value. Based on the difference absorption value ΔA_{290}, a molar difference absorption at 290 nm, $\Delta\varepsilon_{290}$ was evaluated to be around 700, of which value was produced by 5.4 M urea at pH 4.5 and 5.0°C. In case of a change in conformation, an exposure of one tryptophan residue from inner part to surface of a protein molecule refers to a decrease in 1,500 ~ 2,000 of $\Delta\varepsilon_{290}$, which corresponds to the denaturation blue-shift [15]. Thus, Δε value of 700 corresponds to a half (0.5 residue) of tryptophan exposed. It should be concluded that only a slight change in conformation is produced by addition of 5.4 M urea at 5.0°C, pH 4.5.

The urea-induced change in conformation of glucoamylase was also observed using the fluorescence difference-spectrophotometry, of which spectra have a peak at 290 nm when

excited at 280 nm as described elsewhere [10]. The experimental results concluded that little change in conformation was produced by an addition of 5.4 M urea at 5.0ºC and pH 4.5. Thus, the findings are consistent with the experimental results carried out with the difference absorption spectrophotometry as described above.

Viscosity of the experimental conditions employed for the glucoamylase-catalyzed reaction in the presence or absence of urea

Viscosity would be one of the environmental factors, which are highly affecting to its biological function, in fact, viscosity in cytosol of biological cells is generally 3~5 cP (centipoise). In this experiment, urea at the concentration of 2.7 or 5.4 M was employed to investigate its effects on the glucoamylase-catalyzed activity, of which reaction was observed by means of the steady-state kinetic methods in mixture of the enzyme and urea. It is postulated that viscosity could have a decisive influence on the catalyzed-activity of enzymes. Then, viscosity was measured for the solutions including urea at 0, 2.7, and 5.4 M at 5.0ºC, pH 4.5 . The results are as follows; 1.4 cP (0 M urea), 2.5 cP (2.7 M urea), and 5.7 cP (5.4 M urea). The glucoamylase-catalyed reaction-mixture including 5.4 M urea has not given so high viscosity, suggesting that urea does not affect so mach on the glucoamylase-catalyzed activity[16].

These experimental results indicate that urea effects on (decrease in) A2 and A6, suggesting that hydrophobic interactions between substrate and the Trp residues, which are supposed to locate around subsites 2 and 6, and substrate are decreased by an effect of urea molecule.

Nozaki and Tanford pointed out that hydrophobic interaction between Trp residue and water is weakened by urea [14,16]. Thus, our present findings may support the "hydrophobic-driven mechanism" for the productive formation of the substrate-enzyme complex [17].

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