α -Glucosidase Inhibition Assay in an Enzyme-immobilized Amino-microplate

Toshiro MATSUI,[†] Mayu SHIMADA, Nozomi SAITO, and Kiyoshi MATSUMOTO

Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University,
6-10-1 Hakozaki, Higashi, Fukuoka 812-8581, Japan

α -Glucosidase (AGH) from the small intestine of rat was immobilized onto a glutaraldehyde (GA) activated NH₂-96 well microplate to establish a convenient and rapid AGH inhibition assay system. After AGH immobilization, remaining GA groups were blocked by β -alanine to induce a negative charge on the surface of the well. The AGH-plate showed an enzyme activity of 444 nU/well under an assayed condition at 37°C for 2 h using 0.3 mM 4-methylumbelliferyl- α -D-glucopyranoside as a fluorogenic substrate. Inhibitory powers of voglibose and acarbose as therapeutic AGH inhibitors were successfully evaluated to have IC₅₀ values of 13 and 114 nM, respectively.

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Introduction

Recent studies regarding the prevention or improvement of noninsulin-dependent diabetes mellitus (NIDDM) provide useful information that the long-term intake of α -glucosidase (AGH, *exo*-type α -D-glucoside *O*-linkage hydrolase, EC 3.2.1.20) inhibitor, acarbose, to achieve moderate blood glucose level (BGL) was effective for improving hyperglycemia in borderline NIDDM subjects.¹ This indicates that achieving a high BGL close to as normal as possible through the retardation of AGH activity or inhibition of the digestive process of carbohydrates in the gut could be useful for treating diabetic patients. In this respect, we have clarified natural AGH inhibitors, including acylated anthocyanins,^{2,3} caffeoylquinic acids⁴ and theaflavins⁵ as an alternative medicinal food compound.

Along with screening studies of natural AGH inhibitors, we have also proposed a new immobilized AGH (*i*AGH) assay system to mimic the membrane-bound condition⁶ since AGH in mammalian intestine is anchored in the membrane by the polypeptide chain spanning the bilayer only once in the manner of N(in)/C(out) orientation⁷ to form a sucrase-isomaltase (SI) complex.⁸ The use of the *i*AGH assay system allowed the prediction of an *in vivo* antihyperglycemic effect of a given compound with *i*AGH inhibitory activity; compounds with less *i*AGH inhibitory activity were no longer candidates responsible for postprandial BGL lowering, even if they showed apparent AGH inhibitory activity by a conventional free AGH assay.^{9,10} However, the proposed *i*AGH assay still provided great effort, because it requires each set of a reaction column containing AGH-immobilized support for each assay sample. In this study we, thus, undertook to develop a convenient *i*AGH-microplate assay capable of successive evaluation of the AGH inhibitory activity in one run-in assay.

Experimental

Materials

Rat intestinal acetone powder was purchased from Sigma Chemical Co. (St. Louis, MO). Papain (14 U/mg-solid, from papaya latex, EC. 3.4.22.2) was a product of Nacalai Tesque (Kyoto, Japan). 4-Nitrophenyl- α -D-glucopyranoside (*p*NPG) and 4-methylumbelliferyl- α -D-glucopyranoside (MUG) were products of Sigma Chemical. Voglibose (BASEN®, 0.2 mg/tablet) and acarbose (Glucobay®, 50 mg/tablet) as synthetic AGH inhibitors were obtained from Takeda Medical Co. (Osaka, Japan) and Bayer Medical Co. (Leverkusen, Germany), respectively. A 96 well-microplate attached with a NH₂ group (0.78 nmol cm⁻², SUMILON-8FA, Aminoplate) or COOH group (0.44 nmol cm⁻², SUMILON-8FC, Carboxylplate) was purchased from Sumitomo Bakelite Co. (Osaka, Japan). Other reagents were of analytical grade and used without further purification.

Preparation of immobilized AGH-aminoplate

A 96 well-aminoplate was subjected to a glutaraldehyde (GA) activation treatment. The surface of each well was activated with 150 μ l of 2.5% GA coupling buffer (0.2 M phosphate buffer, pH 8.0) for 2 h at 20°C. After being rinsed with deionized water and 50 mM phosphate buffer (pH 8.0), 150 μ l of purified AGH (0.1 mg/ml) in the coupling buffer was added to the GA-activated well. The purification of AGH from rat intestinal acetone power was done according to our previous report.⁶ After incubation at 20°C for 2 h, the well was washed with 50 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl, followed by the addition of 150 μ l of a 0.1 M blocking reagent. In this experiment, four reagents (β -alanine, 2-aminoethanol, *n*-propylamine and ethylenediamine) were used to block any remaining active aldehyde groups to introduce carboxyl (COOH), hydroxyl (OH), methyl (CH₃) or amino (NH₂) group onto the well, respectively. After blocking at 20°C for 2 h, the support was thoroughly rinsed with 50 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl, 0.1 M citrate buffer (pH 4.0) containing 0.5 M NaCl and subsequently 50 mM phosphate buffer (pH 6.8). The *i*AGH-aminoplate was kept at 4°C in the

[†] To whom correspondence should be addressed.
E-mail: tmatsui@agr.kyushu-u.ac.jp

buffer. Immobilization of AGH (0.1 mg/ml) onto carboxylplate was performed by direct condensation with 75 μ l of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (40 mg/ml) in 20 mM phosphate buffer (pH 6.0) at 4°C for 24 h.

Inhibition assay for immobilized AGH

The immobilized AGH activity was assayed as follows. The *i*AGH-plate was directly suspended in 200 μ l of a substrate solution (50 mM phosphate buffer, pH 7.4). *p*NPG (0.7 mM) or MUG (0.3 mM) was used as a synthetic AGH substrate for measuring the overall *i*AGH activity. After incubation at 37°C for 2 h, the activity was determined by measuring the change in the fluorescent intensity on a Wallac 1420 Multilabel counter (Perkin Elmer Co., Tokyo, Japan). Excitation and emission wavelengths were set at 390 and 410 nm for *p*NP detection or at 355 and 460 nm for MU detection, respectively. During a kinetic study, the substrate concentration was set at 0.1–1.0 mM for measuring K_m and V_{max} from the Lineweaver-Burk plots. One unit of *i*AGH activity was defined as the amount of enzyme to hydrolyze 1 μ mol of substrate per min per well under the above-assayed conditions.

For an *i*AGH inhibition study, to the well of *i*AGH-microplate, 20 μ l of an inhibitor solution and 180 μ l of a substrate solution were added directly. After incubation at 37°C for 2 h, the *i*AGH inhibitory activity was determined by the difference in the *i*AGH activity with or without an inhibitor. The concentration of the AGH inhibitor required to inhibit 50% of the AGH activity under the assayed condition was defined as the IC_{50} value.

Stability test of prepared *i*AGH-microplate

The stability of the prepared *i*AGH-microplate activity was assessed as a function of the storage times of 1, 4, 7, 21 and 37 days after preparation. The well or *i*AGH-microplate was immersed in 50 mM phosphate buffer (pH 6.8) at 4°C until the assay.

Animal experiment

Male 7-wk-old Sprague-Dawley rats (SPF/VAF Crj; SD, Charles River Japan, Kanagawa) were fed a laboratory diet (CE-2, Clea Japan, Tokyo) and given water *ad libitum*. All rats were housed for 1 week at $21 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ humidity under controlled lighting from 8:30 to 20:30. Single oral administration of voglibose (dose; 0.07, 0.34, 0.76 mg kg^{-1}) or acarbose (dose; 1.5, 2.0, 3.0 mg/kg) was conducted in SD rats withheld for 16 h by gavage. After 5 min, 2 g/kg of a substrate (maltose) solution was administered to each rat. Control rats were administered with the same substrate solution without an inhibitor. At each time point to 120 min, about 20 μ l of blood sample was collected from the tail vein, being immediately subjected to a BGL measurement by a disposable glucose sensor (Glutest Pro, Sanwa Chemical Research, Co., Tokyo, Japan). The ED_{50} value, which is the effective dose required to achieve 50% suppression of BGL rise, was interpolatively estimated. Rat experiments in this study were carried out under the guidance of the Animal Experiment Committee of the Faculty of Agriculture and the Graduate Course of Kyushu University and the Law (No. 105, 1973) and Notification (No. 6, 1980 of the Prime Minister's Office) of the Japanese Government.

Results and Discussion

Assessment of *i*AGH-microplate assay

Considering that the possible immobilization amount of AGH would be extremely low towards the surface area of well

Fig. 1 Immobilized AGH activity of carboxylplate and aminoplate. 4-Methylumbelliferon (MU) from MUG as a substrate was determined for 4 h at 37°C for both *i*AGH-plates. The fluorescent intensity of MU was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Data are expressed as mean $\pm$ SD ($n = 6$).

(<1 cm^2), MUG with high fluorescent intensity at a level of pmol was used as a substrate throughout the study. Though data were not shown, the fluorogenic detection range of *p*NPG, a common substrate for carbohydrases, was as narrow as 0.2–1 μ mol/assay volume.

On the basis of our previous finding⁶ on preferential introduction of negative charges onto AGH-support by β -alanine to mimic the intestinal epithelium microvilli containing glycocalyx, such as sialic acid and uronic acid,¹¹ *i*AGH-microplate blocked by β -alanine was primarily used for optimizing the incubation time. As shown in Fig. 1, the production of 4-methylumbelliferon (MU) from MUG by *i*AGH action was linear with the incubation time to 4 h for both carboxylplate and aminoplate. Considering the apparent difference in the fluorescent intensity between the blank and the sample (>100000) and rapid incubation, we set at an incubation time of 2 h for both plates' assay in this study. The activities of *i*AGH-aminoplate and *i*AGH-carboxylplate were estimated to be 444 and 585 nU/well, respectively.

Catalytic property of *i*AGH-microplates

The effect of the blocking reagent on the *i*AGH activity was investigated. As summarized in Table 1, the kinetics of the prepared plates greatly differed from induced groups onto aminoplate; β -alanine and 2-aminoethanol-induced aminoplates showed similar K_m values as carboxylplate, whereas plates blocked by ethylenediamine and *n*-propylamine provoked less affinity with MUG. The difference in the kinetics for each prepared plate may be due to the accessibility of MUG to a charged plate. Among the prepared plates, β -alanine-aminoplate did show a high catalytic efficiency (v_{max}/K_m) of 0.08 as well as carboxylplate. This indicates that the introduction of carboxyl groups onto the surface of the plate was more favorable for the activation of immobilized AGH, being matched with an anionic environment of the intestinal epithelium microvilli by acid mucopolysaccharides.¹¹

Inhibition studies of *i*AGH-microplates

Table 2 summarizes the inhibition of acarbose and voglibose against *i*AGH-microplates with different blocking groups. Apparently, the prepared plates showed a variety of inhibitory activities with blocking groups. β -Alanine- and *n*-propylamine-induced plates showed potent IC_{50} values of around 20 nM for voglibose, while no, or poor, inhibitory activity was observed in

Table 1 Effect of blocking reagent on the kinetics of immobilized AGH onto amino- or carboxylplate^a

Plate	Blocking reagent	Functional group on the surface of plate	K_m /mM	V_{max} /nmol min ⁻¹	V_{max}/K_m
Amino	β -Alanine	-COOH	0.41	0.033	0.080
	Ethylenediamine	-NH ₂	ND ^b	ND	—
	<i>n</i> -Propylamine	-CH ₃	4.5	0.038	0.008
	2-Aminoethanol	-OH	0.14	0.0026	0.018
Carboxyl	—	-COOH	0.61	0.070	0.115

a. Kinetic parameters were determined from the Lineweaver-Burk plots using MUG as a substrate at a concentration range of 0.1 – 1.0 mM for 2 h at 37°C. b. ND, not detected.

Table 2 Effect of blocking reagent on AGH inhibitory activity of voglibose and acarbose for amino- or carboxylplate

Plate	Blocking reagent	Functional group on the surface of plate	IC ₅₀ /nM		Ratio of IC ₅₀ value (acarbose/voglibose)
			Acarbose	Voglibose	
Amino	β -Alanine	-COOH	114	13	8.5
	Ethylenediamine	-NH ₂	NI ^a	NI	—
	<i>n</i> -Propylamine	-CH ₃	40	25	1.6
	2-Aminoethanol	-OH	NI	NI	—
Carboxyl	—	-COOH	110	270	0.4

a. NI, no inhibition.

the other treated plates including carboxylplate. A similar inhibitory behavior in the plates was observed for acarbose. These results strongly suggested that the surface polarity of the plate induced by various blocking reagents greatly affected the inhibitory action of both inhibitors against AGH as well as the kinetic behavior (Table 1).

In the present SD rat experiments to evaluate *in vivo* antihyperglycemic potency of therapeutic AGH drugs (voglibose and acarbose), the intake of voglibose to 8wk-SD rats showed an ED₅₀ of 0.55 mg/kg in maltose-loaded rats, whereas a 5.6-times higher dose of acarbose was required for lowering the BGL rise (ED₅₀; 3.1 mg/kg) (Fig. 2). Odaka *et al.*¹² also reported a higher antihyperglycemic potency of voglibose, rather than acarbose by a factor of 14.6 times in sucrose (2.5 g/kg)-loaded SD rat (ED₅₀: acarbose, 5.42 mmol/kg; voglibose, 0.37 mmol/kg). These findings indicate that the magnitude of the *in vivo* antihyperglycemic power of voglibose would be potentially >5-fold stronger than that of acarbose. From the viewpoint of the antihyperglycemic potency of both inhibitors, we re-examined the IC₅₀ values of both inhibitors assayed by *i*AGH-plates (Table 2). As a result, the ratio of the IC₅₀ value of voglibose to acarbose greatly differed from induced blocking groups onto the plates; a comparable ratio of 8.5 with *in vivo* antihyperglycemic ED₅₀ value was observed for the β -alanine-*i*AGH plate. A lower ratio of the IC₅₀ value (0.4) by the carboxylplate than the β -alanine-*i*AGH plate suggested the importance of a spacer from the β -alanine skeleton to mimic the *in vivo* inhibition of intestinal membrane-bound AGH. The reproducibility of the *i*AGH activity for the β -alanine-*i*AGH plate was 4.5% of the correlation of the coefficient ($n = 8$).

Stability of *i*AGH activity of the β -alanine-induced microplate

The change in the *i*AGH activity of β -alanine-aminoplate stored at 4°C was examined up to 37 days after preparation. As shown in Fig. 3, the prepared plate retained its AGH activity of >60% during 21 days after preparation, while the activity disappeared at 37 days. This revealed that a reliable evaluation for AGH inhibition would be possible within 3 week-storage after preparation of the *i*AGH-plate, although attempts to improve

Fig. 2 Dose dependency of voglibose (A) and acarbose (B) on the reduction of glycemic responses in maltose-loaded (2 g/kg) SD rats. AUC_{0-120 min}, which is the area under the curve of incremental blood glucose levels up to 120 min, was used to calculate the BGL reduction ratio of each drug against control. Data are expressed as mean $\pm$ SD ($n = 4$).

the stability of the AGH activity of the plate are needed.

Achieving BGL close to normal as possible has been considered to be one of the major goals of NIDDM therapy, in which the inhibition of intestinal AGH could be useful for improving an excess postprandial BGL rise from dietary carbohydrates. In this study, we established an *i*AGH- β -alanine-aminoplate to evaluate the inhibition of intestinal membrane-

Fig. 3 Effect of the storage time on the immobilized AGH activity of β -alanine-aminoplate. The AGH activity of *i*AGH-plate was individually determined on 1, 4, 7, 21 and 37 day-storage at 4°C. Data are expressed as mean $\pm$ SD ($n = 6$).

bound AGH. The assay would overwhelm a conventional free AGH assay system, because of its possible estimation of an *in vivo* suppression effect of BGL rise by any inhibitors. The established assay was also preferable to our previous *i*AGH-Sephadex support assay,⁶ because of simultaneous determinations for evaluating AGH inhibition of many targeted samples in one-run-in assay.

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