Evaluation of $\alpha$-Glucosidase Inhibition by Using an Immobilized Assay System

Tomoyuki Oki, Toshio Matsui,* and Kiyoshi Matsumoto
Division of Bioresource and Bioenvironmental Sciences, Faculty of Agriculture, Graduate School Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan. Received March 9, 2000; accepted May 24, 2000

The inhibitory effects of natural and synthetic inhibitors on the intestinal membrane-bound hydrodase, $\alpha$-glucosidase (AGH), were evaluated by using an immobilized cyanogen bromide-activated Sepharose 4B support. Immobilized AGH (iAGH) inhibition study by synthetic inhibitors (acarbose and voglibose) revealed that the magnitude of inhibition differed from that in the free AGH (fAGH) study: IC$_{50}$ value of acarbose in iAGH-maltase assay system, 340–430 nM; fAGH, 11 nM. iAGH-maltase inhibition by both inhibitors was influenced by blocking reagents with different functional groups (COOH, OH, CH$_2$, and NH$_2$ groups). On the other hand, significant iAGH-sucrase inhibitory activity was observed only when using the negatively charged support induced by 0.1 M $\beta$-alanine. The $K_c$ values obtained in the iAGH assay system were similar to those from the fAGH method. With natural inhibitors, the iAGH-sucrase inhibitory activity of $\alpha$-xylene, with in vivo glucose suppression, increased twice compared to that in fAGH. Green tea extract gave almost the same inhibition for both AGH assay systems.

Key words $\alpha$-glucosidase; immobilization; inhibition; noninsulin-dependent diabetes mellitus

One of the most direct and beneficial types of therapy for noninsulin-dependent diabetes mellitus (NIDDM) is achieved by control of the blood glucose level after a meal by delaying glucose absorption. To date, certain synthetic inhibitors of $\alpha$-glucosidase (AGH), an exo-type $\alpha$-D-glucoside O-linkage hydrodase, have been developed and used for the therapeutic treatment of NIDDM. In our studies concerning prophylaxis of NIDDM using functional food components, Bacillus licheniformis alkaline protease hydrolyzate derived from sardine muscle had a relatively high inhibitory activity against baker's yeast AGH (IC$_{50}$=0.487 mg/ml) as did green and oolong teas (IC$_{50}$=0.111 and 0.113 mg/ml, respectively). However, the AGH inhibitory effect varied greatly according to origin; the hydrolyzate and its isolated bioactive peptides, showing inhibitory action against baker's yeast AGH, did not retard the action of rat intestinal AGH (IC$_{50}$>3.45×10$^{-3}$ mg/ml). This indicated that the use of mammalian or rat intestinal AGH may be essential for evaluating the inhibitory action of natural or synthetic compounds.

Odaka et al. reported that the in vivo AGH inhibitory effects of pharmaceuticals did not match with those obtained in in vitro experiments. Evidence for this discrepancy comes from in vitro estimation of AGH activity. According to Hunziker et al., AGH in mammalian intestine is anchored in the membrane by the polypeptide chain spanning the bilayer only once in a N(in)/C(out) orientation. This indicates that AGH inhibition studies should be performed with membrane-bound or immobilized AGH (iAGH) assay systems, and not with free AGH (fAGH). However, there is currently no report on an iAGH assay system. Thus, in this study, we have attempted to establish a rat intestinal iAGH inhibitory assay system to mimic the small intestinal membrane. The effect of surface charge of prepared support on iAGH activity was also investigated.

MATERIALS AND METHODS

Materials Rat intestinal acetone powder was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Papain (14 U/mg-solid, from papaya latex, EC. 3.4.22.2) was the product of Nacalai Tesque (Kyoto, Japan). Cyanogen bromide (CNBr)-activated Sepharose 4B support was purchased from Pharmacia Biotech AB (Upsala, Sweden). Voglibose (BASEN, 0.2 mg/tablet) and acarbose (Glucobay, 50 mg/tablet) as a synthetic AGH inhibitor were obtained from Takeda Medical Co. (Osaka, Japan) and Bayer Medical Co. (Leverkusen, Germany), respectively. Green tea was obtained from a commercial source, and its hot-water extract was prepared according to our previous paper. Other reagents were of analytical grade and used without further purification.

Partial Purification of AGH AGH was purified from rat intestinal acetone powder by a slight modification of the procedure reported by Cogoli et al. The acetone powder (2.5 g) was homogenized with 1% papain in 125 ml of PCC buffer (100 mM potassium phosphate, 50 mM potassium citrate, and 154 mM potassium chloride, pH 7) containing 5 mM EDTA and 10 mM $\varepsilon$-cysteine. After incubation at 37 °C for 1 h, the homogenate was centrifuged at 10000×g for 1 h. To the supernatant 40% satd. (NH$_4$)$_2$SO$_4$ was added. After removing the precipitate by centrifugation (10000×g, 30 min), the solution was adjusted to 60% saturation of (NH$_4$)$_2$SO$_4$, and the resultant precipitate was collected by ultracentrifugation (55000×g, 1 h). The precipitate was then dissolved in 10 mM potassium phosphate buffer (pH 6.8) and dialyzed against the same buffer at 4 °C overnight. The dialyze was ultrafiltered (M.W. <200000), and then the supernatant was lyophilized. The AGH preparation was stored at −10 °C until use.

Immobilization of AGH onto CNBr-Activated Sepharose 4B CNBr-activated Sepharose 4B (50 mg-dry gel) was immersed in 2 ml of 1 mM HCl for 15 min. After being rinsed with 1 mM HCl and a coupling buffer (0.1 M borate buffer containing 0.5 M NaCl, pH 7.5), 1 ml of the prepared AGH (2 mg) in the coupling buffer was added to the Sepharose and incubated at 20 °C for 2 h. After the incubation, the support was washed with the coupling buffer, followed by the addition of 1 ml of 0.1 M blocking reagent. In this experiment, the reagents, $\beta$-alanine, 2-aminoethanol, n-propylamine, and eth-
ylenediamine, were used to introduce carboxyl (COOH), hydroxyl (OH), methyl (CH₃), and amino (NH₂) groups onto the support, respectively. After the blocking, the support was rinsed thoroughly with 0.1 M citrate buffer (pH 4.0) containing 0.5 M NaCl and thereafter with the coupling buffer. As a result of the preparation, 2.5 mg of iAGH was immobilized on 1 g-wet gel of the support. The preparations were kept at 4°C in a model intestinal fluid (0.1 M phosphate buffer (pH 6.8)) advanced in the Japanese Pharmacopoeia (JP XIII).

**Assay for iAGH Activity and Inhibitory Activity**

iAGH activity was assayed as follows. The iAGH support (10 mg-wet gel) was taken in an end-capped ASSIST Mini-column with 45–90 μm of polyethylene filter (CC-07, 5 ml, ASSIST, Tokyo, Japan). The assay was started after adding 1.0 ml of the model intestinal fluid containing maltose (0.5–10 mM) or sucrose (2.5–45 mM) to it. After incubation with a rotating cultivator (4 rpm, RT-5, TAITEC, Saitama, Japan) at 37°C for 30 min (maltase assay) or 60 min (sucrase assay), the reaction was stopped by filtration of the solution in the column. Activity was determined by measuring the liberated glucose from maltose or sucrose in the filtrate by Glucose CII-Test Wako (Wako Pure Chemical Institute, Co., Osaka, Japan). One unit of maltase or sucrase activity was defined as the amount of enzyme to hydrolyze 1 μmol of substrate per min under the mentioned assay conditions.

For iAGH inhibitory assay, to the tube with the iAGH support (10 mg-wet gel), 10 μl of inhibitor solution and 990 μl of substrate solution were added. Final concentrations of maltose and sucrose were set at 10 mM and 45 mM, respectively. After incubation at 37°C for 30 min (maltase assay) or 60 min (sucrase assay), the liberated glucose was measured to evaluate the iAGH inhibitory activity. Inhibitory activity was estimated by the difference in the amount of glucose in the filtrate with or without inhibitor. The concentration of AGH inhibitor required to inhibit 50% of the AGH activity under the assayed condition was defined as the IC₅₀ value. In the kinetic and inhibitory measurements, the experimentally obtained data (average values from three determinations, C.V.: <5.0%) were processed in terms of Michaelis–Menten kinetics, and the plot of inhibition (%) versus logarithmic inhibitor concentrations (r > 0.997).

**Assay for fAGH Activity and Inhibitory Activity**

fAGH inhibitory assay was performed according to the procedure described in our previous paper. In this study, the same quantity (25 μg) of AGH as in the iAGH assay system (corresponding to 55 mU-maltase/assay and 16 mU-sucrase/assay) was used. The final concentrations of each substrate and the incubation time were the same as described above.

**RESULTS AND DISCUSSION**

**Catalytic Properties of iAGH toward Maltose and Sucrose**

Effect of immobilization of AGH on maltase activity was investigated with respect to blocking reagents with different functional groups. As shown in Fig. 1, the magnitude of maltase activity varied according to the type of blocking reagent (β-alanine > 2-aminoethanol > n-propylamine > ethylenediamine); iAGH support blocked with β-alanine (βAla/iAGH) gave the most potent maltase activity of 4.1 U/g-wet gel at pH 6.8. As a result of kinetic study (Table 1), the maximum velocity (Vₘₕₐₓ) increased in the order of supports blocked with ethylenediamine < n-propylamine < 2-amino-

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![Fig. 1. Effect of Blocking Reagents on Maltase and Sucrase Activities of Immobilized AGH onto CNBr-Activated Sepharose at pH 6.8](image)

Table 1. Kinetic Constant of Free and Immobilized AGHs, and Inhibitory Effect of Acarbose and Voglibose against Maltase Activity

<table>
<thead>
<tr>
<th>Assay system</th>
<th>Blocking</th>
<th>Kₘ (mM)</th>
<th>Vₘₕₐₓ (μmol/min/g-wet gel)</th>
<th>IC₅₀ (mM)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobilized AGH</td>
<td>β-Alanine</td>
<td>4.6</td>
<td>10.0</td>
<td>430</td>
</tr>
<tr>
<td></td>
<td>2-Aminoethanol</td>
<td>5.1</td>
<td>9.0</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>n-Propylamine</td>
<td>5.0</td>
<td>7.0</td>
<td>340</td>
</tr>
<tr>
<td></td>
<td>Ethylenediamine</td>
<td>4.0</td>
<td>5.3</td>
<td>350</td>
</tr>
<tr>
<td>Free AGH</td>
<td></td>
<td>3.1</td>
<td>N.D.²</td>
<td>11</td>
</tr>
</tbody>
</table>

² IC₅₀ value shows the concentration of inhibitor giving 50% inhibition of maltase activity. ³ N.D.; not determined.

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**Fig. 1. Effect of Blocking Reagents on Maltase and Sucrase Activities of Immobilized AGH onto CNBr-Activated Sepharose at pH 6.8**
Table 2. Kinetic Constant of Free and Immobilized\(^{a}\) AGHs, and Inhibitory Effect of Acarbose and Voglibose against Sucrase Activity

<table>
<thead>
<tr>
<th>Assay system</th>
<th>(K_m) (nm)</th>
<th>(V_{\text{max}}) (µmol/min/g-wet gel)</th>
<th>IC(50) (nm)(^{b}) Acarbose</th>
<th>Voglibose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobilized AGH</td>
<td>24.6</td>
<td>1.6</td>
<td>1200</td>
<td>62</td>
</tr>
<tr>
<td>Free AGH</td>
<td>20.0</td>
<td>N.D.(^{c})</td>
<td>890</td>
<td>7.1</td>
</tr>
</tbody>
</table>

\(^{a}\) Immobilized AGH was prepared by blocking with 0.1 µM \(\beta\)-alanine. \(^{b}\) IC\(50\) value shows the concentration of inhibitor giving 50% inhibition of sucrase activity. \(^{c}\) N.D.; not determined.

Table 3. Comparison of Inhibitory Activities of \(\alpha\)-Xylose and Green Tea Extract for Immobilized AGH\(^{a}\) with those for Free AGH

<table>
<thead>
<tr>
<th>Assay system</th>
<th>(\alpha)-Xylose</th>
<th>Green tea extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Sucrese</td>
<td>Sucrese</td>
</tr>
<tr>
<td>Immobilized AGH</td>
<td>0.18</td>
<td>0.43</td>
</tr>
<tr>
<td>Free AGH</td>
<td>0.36</td>
<td>0.52</td>
</tr>
</tbody>
</table>

\(^{a}\) Immobilized AGH was prepared by blocking with 0.1 µM \(\beta\)-alanine.

ethanol < \(\beta\)-alanine. Little significant difference in the apparent Michaelis constants \(K_m\) of AGHs (4.0—5.1 nm) was obtained, though the values of AGHs tended to be higher than for \(f\) AGH (\(K_m\) = 3.1 nm). Similar studies were performed with respect to sucrase activity. As shown in Fig. 1, iAGH-sucrase reactions were greatly influenced by the blocking reagents with the same order (\(\beta\)-alanine > 2-aminoethanol > \(\alpha\)-propylamine > ethylendiamine) as those of iAGH-maltase. However, higher sucrase activity was observed only for the \(\beta\)Ala-iAGH support (0.52 U/g-wet gel) with a \(V_{\text{max}}\) of 1.6 µmol/min/g-wet gel (Table 2), and not for the other iAGH supports (< 0.04 U/g-wet gel). Thus, the prepared \(\beta\)Ala-iAGH support, charged negatively (COO\(^-\)) in intestinal fluid (\(pK_{\text{a}}\) = 3.55\(^{11}\)), was found to be effective for the AGH inhibition study of both maltase and sucrase activities. It is known that glycoalkyl at the intestinal epithelium microvilli consists of glycoprotein containing acid mucopolysaccharides, such as sialic acid, uronic acid and so on.\(^{12}\) Therefore, \(\beta\)Ala-iAGH may be a good tool to mimic membrane-bound AGH or to evaluate AGH catalysis in the small intestine.

Inhibition Study of iAGH with Maltose and Sucrose

Tables 1 and 2 show the inhibition by acarbose and voglibose against AGHs and \(f\) AGH toward maltase and sucrase. For the inhibition study against sucrase, \(\beta\)Ala-iAGH support was used due to high sucrase activity, as shown in Fig. 1. Apparently, the influence of immobilization treatment of AGH on the maltase and sucrase inhibitions was large. For maltase retardation by synthetic AGH inhibitors, the AGH inhibition activity of acarbose estimated by iAGH assay was 1/50- to 1/40-fold lower than that by \(f\) AGH assay (IC\(50\) = 11 nm) (Table 1). In contrast, voglibose was evaluated to be a good iAGH inhibitor. Among the iAGHs, \(\beta\)Ala-iAGH showed the poor inhibitory activities (IC\(50\) of 430 nm for acarbose and 5.5 nm for voglibose. Sucrese inhibition by the two inhibitors was also evaluated with \(\beta\)Ala-iAGH (Table 2). As a result, the \(\beta\)Ala-iAGH inhibitory activities of both inhibitors were lowered, compared to those of \(f\) AGH. In particular, a 1/9-fold decrease in the IC\(50\) value of voglibose was observed in the \(\beta\)Ala-iAGH assay. These results strongly suggested that the inhibition power of an AGH inhibitor may be greatly variable depending upon whether the AGH was free or immobilized. Odaka et al.\(^{13}\) reported that the ED\(50\) value of acarbose, which is the quantity of AGH inhibitor required to suppress 50% of the blood glucose level, was 14.6 times higher than that of voglibose after oral administration of sucrose (2.5 g/kg) in Sprague-Dawley (SD) rats (acarbose; ED\(50\) = 5.42 mmol/kg, voglibose; 0.37 mmol/kg). In this study, the in vitro IC\(50\) value of acarbose (890 nm) by fAGH-sucrase assay was estimated to be 125 times higher than that of voglibose (7.1 nm) (Table 2). This revealed that the in vitro \(f\) AGH inhibitory assay system gave little information on the in vivo suppression effect of glucose absorption. On the other hand, under the \(\beta\)Ala-iAGH assay system, the ratio of IC\(50\) values of acarbose against voglibose was found to be 19.4 (acarbose; IC\(50\) = 1200 nm, voglibose; IC\(50\) = 62 nm), and almost agreed with the ED\(50\) ratio of 14.6. Based on this finding, the present \(\beta\)Ala-iAGH assay system was speculated to mimic the in vivo membrane-bound AGH hydrolysis reaction in the small intestine. However, a further in vivo study to verify the validity of the \(\beta\)Ala-iAGH assay system is needed, and is now under consideration.

Inhibition Study of iAGH by Natural Inhibitors

The \(\beta\)Ala-iAGH assay system was applied to natural AGH inhibitors, i.e., \(\alpha\)-xylose and green tea extract (Table 3), both of which have in vivo suppression effects on postprandial blood glucose level.\(^{15,16}\) \(\alpha\)-Xylose as a non-competitive sucrase-specific inhibitor\(^{19}\) inhibited sucrase activity in the \(\beta\)Ala-iAGH assay system twice as much as that by \(f\) AGH assay. On the other hand, there was little difference in the maltase and sucrase inhibitory activities of green tea extracts between the fAGH and \(\beta\)Ala-iAGH assay systems. Although there have been no comparative reports on the suppression effect of blood glucose levels between those natural AGH inhibitors, the present in vitro AGH inhibition data from the \(\beta\)Ala-iAGH assay system (Table 3) suggested that \(\alpha\)-xylose (IC\(50\) value against sucrase; 0.18 mg/ml) might have a 2.4-fold stronger glucose suppression effect than green tea extract (IC\(50\); 0.43 mg/ml).

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