2,2-Diphenyl-1-picrylhydrazyl Hydrate, a Stable Free Radical, Is an α-Glucosidase Inhibitor

Dong-Sun Lee,[a] Nam-Soon Kim,[b] and Sang-Han Lee*,[b]

Biophysical Chemistry Laboratory, The Institute of Physical and Chemical Research RIKEN Harima Institute,[a] Sayo, Hyogo 679–5148, Japan and Korea Research Institute of Bioscience & Biotechnology,[b] Taejon 305–333, Korea.
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Glycosidases play a pivotal role in processing of various glycoproteins and glycolipids. It is well known that glycosidases are also involved in a variety of degenerative metabolic disorders such as cancer and AIDS. In order to develop potent α-glucosidase inhibitors, we first screened 2,2-diphenyl-1-picrylhydrazyl hydrate as a candidate. 2,2-Diphenyl-1-picrylhydrazyl hydrate was found to inhibit α- and β-glucosidases as well as α- and β-mannosidases. It was also shown to be a non-competitive inhibitor of yeast α-glucosidase with a K_i value of 1.1×10^{-6} M. Taken together, we anticipate that 2,2-diphenyl-1-picrylhydrazyl hydrate may be a potent inhibitor for some incurable metabolic disorders including AIDS.

Key words 2,2-diphenyl-1-picrylhydrazyl hydrate; α-glucosidase inhibitor; enzyme kinetics; non-competitive inhibition

Glycosidases including glucosidases are not only essential to carbohydrate digestion but vital for the processing of glycoproteins and glycolipids. Glycosidases are also involved in a variety of human degenerative metabolic disorders and other diseases such as viral attachment[1] and carcinogenesis.[2] For these reasons, glycosidase inhibitors are important tools for studying their molecular modes of action and could be also prospective therapeutic agents.[3] α-Glucosidase inhibitors, such as nojirimycin, *N*-butyldeoxynojirimycin, nectricine, and castanospermine, are potent inhibitors of human immunodeficiency virus (HIV) replication and HIV-mediated syncytium formation *in vitro*.[4] Originally, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) was found to act as a potent stable free radical, showing an absorption spectrum at 517 nm with a violet color.[5,6] Because many proton-radical scavengers decolorize DPPH, it can be used for antioxidant assay.[7] In the course of our search for α-glucosidase inhibitors, DPPH was isolated and identified from various chemicals (data not shown). In this study, we first investigated the inhibitory mode of action of DPPH against α-glucosidase activity. We suggest a possibility that DPPH could be a potent tool for investigation of molecular mechanisms on α-glucosidase-related human disorders.

MATERIALS AND METHODS

Reagents *p*-Nitrophenyl (PNP) glycosides used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). α-Glucosidase (from bakers yeast, Sigma Chemical Co.), β-glucosidase (from almond, Sigma Chemical Co.), α-mannosidase (Jack beans, Sigma Chemical Co., St. Louis, MO, U.S.A.), and β-mannosidase (from snail acetone powder, Sigma Chemical Co.) were commercially available. A compound was screened out from various chemicals by an α-glucosidase inhibitor screening system. We finally confirmed that the compound was DPPH (data not shown), which was compared to authentic DPPH (Sigma Chemical Co.).

Enzyme Assays To evaluate whether or not DPPH inhibits various commercially available glycosidases, the assay for glycosidases was done as described previously.[8,9] In brief, α-glucosidase and the other glycosidases were assayed using 50 mM phosphate buffer at pH 6.7 and 50 mM Na-citrate buffer at pH 4.5, and the appropriate 1 mM *p*-nitrophenyl glycosides were used as substrates. The concentration of the enzymes is specified in each experiment. DPPH at the designated concentration was added to the enzyme solution in the buffer and incubated at 30 °C for 1 h, and the substrate was then added to initiate the enzyme reaction. When pretreatment was not specified, mixtures of substrate and DPPH at designated concentrations were prepared beforehand and added to the enzyme solution. The enzyme reaction was carried out at 30 °C for 30 min, and then 3 volumes of 1 M Na_2CO_3 were added to terminate the reaction. Enzymatic activity was quantified by measuring the absorbency at 405 nm. One unit of α-glucosidase and other glycosidases is defined as the amount of enzyme liberating 1.0 μmol of *p*-nitrophenol per min under the assay conditions specified.

Kinetics of Enzyme Inhibition The enzyme reaction was performed according to the above reaction conditions with inhibitors of various concentrations. Inhibition types for the inhibitors were determined by Dixon plot and its replot of slope *versus* the reciprocal of the substrate concentration.[10]

RESULTS AND DISCUSSION

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH), a stable free radical, has been used for detecting antioxidant activity in chemical analysis, because DPPH shows an absorption spectrum at 517 nm with a violet color.[5—7] In order to investigate potent α-glucosidase inhibitor, we, interestingly, screened out DPPH from chemicals. The chemicals included various antioxidants and radicals such as L-cysteine, superoxide dismutase, dimethyl sulfoxide, ascorbic acid, and apigenin.

To assess whether or not DPPH inhibits various commercially available glycosidases, we carried out glucosidase assay by the method of Tsujii *et al*.[8] with a slight modification. All glycosidases and PNP glycosides used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). As shown in Fig. 1, α-glucosidase was the most sensitive to DPPH, and the concentration required for 50% inhibitory concentration (IC_{50}) was calculated to be 5.2×10^{-6} M. At higher concentrations, DPPH inhibited the activi-

* To whom correspondence should be addressed. e-mail: sang@mail.kribb.re.kr

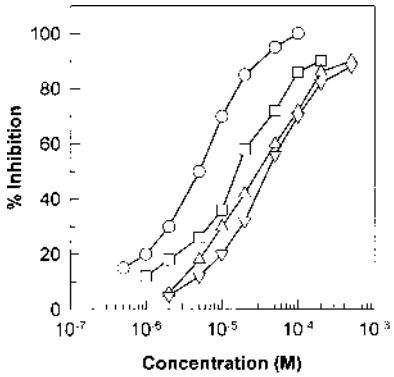

Fig. 1. DPPH Inhibits α-Glucosidase

Enzyme solutions were treated with various concentrations of DPPH. The amount of enzymes were as follows: 1 U/ml α-glucosidase (○), 0.5 U/ml β-glucosidase (□), 0.5 U/ml α-mannosidase (△), 0.1 U/ml β-mannosidase (▽). The mixtures of enzyme and DPPH were reacted at 30 °C for 1 h.

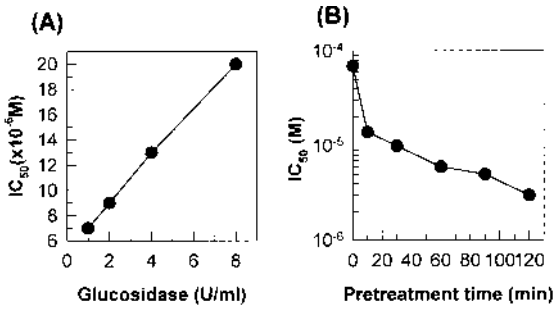

Fig. 2. The IC_{50} Value for the Inhibition of α-Glucosidase Varies Depending (A) on the Amount of α-Glucosidase and (B) on the Time of Pretreatment with DPPH (B)

(A), different amounts of α-glucosidase were treated with the indicated concentration of DPPH for 1 h, and in panel B, α-glucosidase (1 U/ml) was pretreated with the indicated concentrations of DPPH for the designated time in phosphate buffer (50 mM, pH 6.7) at 30 °C. After pretreatment of α-glucosidase with DPPH, PNP-α-glucopyranoside was added to the mixture to initiate the reaction. The IC_{50} values were determined by liberated PNP.

ties of α-mannosidase (from Jack beans, Sigma Chemical Co.), β-mannosidase (from acetone powder of snail, Sigma Chemical Co.) and β-glucosidase (from almond, Sigma Chemical Co.). The respective IC_{50} values were 1.3×10^{-5}, 3.1×10^{-5}, and 4.1×10^{-5} M, respectively. This result suggests that the activity of α-glucosidase (from bakers yeast, Sigma Chemical Co.) is reduced by DPPH in a dose-dependent response.

The effects of DPPH on α-glucosidase inhibition were examined by increasing the amount of the enzyme (Fig. 2A). The IC_{50} value increased from 7.1×10^{-6} to 2.2×10^{-5} M when the amount of α-glucosidase in the reaction mixture was raised from 1.0 to 8.0 units/ml (Fig. 2A). DPPH prevented α-glucosidase inhibition in a dose-dependent manner. The inhibitory activity of DPPH against α-glucosidase was increased by preincubation of the inhibitor with the enzyme (Fig. 2B). When the substrate and DPPH were added simultaneously, the IC_{50} was *ca.* 6.9×10^{-5} M. This value was decreased to about 12-times less (*ca.* 6.0×10^{-6} M) when α-glucosidase was treated with DPPH at 30 °C for 1 h before the initiation of enzyme reaction. With respect to this result, the observation that the IC_{50} value varies with the amount of the enzyme to be assayed may be related to the slow-binding nature of the inhibitor, because the time required to reach the binding equilibrium may vary with the enzyme concentration.

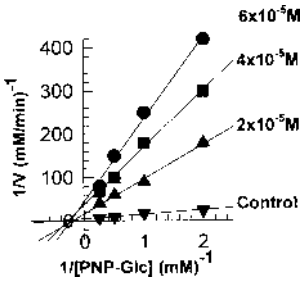

Fig. 3. Inhibition Kinetics of Yeast α-Glucosidase by DPPH

The same amounts of enzyme, substrate, and inhibitor were used, but the enzyme was treated first with DPPH for 1 h at 30 °C and then 50 μl of the substrate was added to initiate the enzyme reaction.

Double-reciprocal plots of α-glucosidase kinetics with DPPH are shown in Fig. 3. The enzyme activity was inhibited non-competitively when the enzyme was pretreated with DPPH for 1 h, showing that, at this point, the K_i value was 1.1×10^{-6} M (Fig. 3). This K_i value was calculated using the values of V_{max} obtained at 0 and 60 μM of DPPH. This value also may vary with enzyme concentration and preincubation time. At present, these inhibition studies provide useful information for the design of new potent inhibitors for glycosidases. Additionally, DPPH can be used as a treatment for metabolic disorders, although we know that DPPH may not reach the target enzyme before quenching with abundant proteins present in living tissues. To date, however, because the inhibitory activity of DPPH has not yet been studied at cellular levels, therefore, we are planning to examine the suppression of syncytium formation by DPPH. The yeast α-glucosidase is known to be very different from mammalian digestive enzymes,[11] suggesting that ongoing experiments should be focused on the inhibitory activity of DPPH against mammalian intestinal α-glucosidases. Further mechanism studies on how DPPH inhibits α-glucosidase using 3-dimensional analytical techniques such as crystallography would be rewarding, because the inhibition studies of glucosidases including α-glucosidase provide useful information for the molecular design of new potent inhibitors against glycosidases.

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