A New Screening Method for Glucosidase Inhibitors and its Application to Algal Extracts

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A screening method for α- and β-D-glucosidase inhibitors was developed by using agar plates. An enzyme agar plate containing either α- or β-D-glucosidase was used in combination with a substrate agar plate containing p-nitrophenyl α- or β-D-glucopyranoside. Paper disks impregnated with algal extracts were placed on the enzyme agar plate, then preincubated for 2.5 h at 25°C. The substrate agar was layered on the enzyme agar. Inhibitory zones appeared colorless in the yellow background of p-nitrophenol. Extracts of algae collected in southern Hokkaido were screened for α- and β-D-glucosidase inhibitors by the proposed agar plate method. Among nine algal species examined, seven species inhibited α-D-glucosidase, whereas five species inhibited β-D-glucosidase.

Key words: screening, glucosidase, inhibitor, algae

α-D-Glucosidase (EC 3.2.1.20) and β-D-glucosidase (EC 3.2.1.21) are hydrolytic enzymes of α- and β-glucosidic linkages of glucosides, respectively. Nutritionally, small intestinal α-D-glucosidase hydrolyzes maltose to glucose which is taken up into blood. Therefore, inhibition of α-D-glucosidase is effective for diseases caused by high blood glucose level, such as diabetes and obesity, by controlling the kinetics of intestinal carbohydrate digestion. β-D-Glucosidase in plants hydrolyzes coexistent β-glucosyl conjugates of poisonous compounds such as cycasin. Inhibition of β-D-glucosidase should be useful for suppressing hydrolyzation of the toxic β-glucosyl conjugates.

Materials and Methods

Materials

α-D-Glucosidase of Saccharomyces cerevisiae and β-D-glucosidase of almond were obtained from Wako Pure Chemical Industries, Ltd. and Oriental Yeast Co., Ltd., respectively. p-Nitrophenyl α-D-glucopyranoside and p-nitrophenyl β-D-glucopyranoside were purchased from Tokyo Kasei Kogyo Co., Ltd. and Nacalai Tesque, Inc., respectively. Deoxynojirimycin and glucono-δ-lactone were obtained from Cambridge Research Biochemicals, Inc. and Wako Pure Chemicals Industries, Ltd., respectively.

Collection and Extraction of Algae

Five species of brown algae, Hizikia fastiforme, Sargassum thunbergii, Leathesia diffconvia, Pelvetia cneorum, Saccorhiza polyschides, and four species of red algae, Gloiopeltis furcata, Rhodoglossum japonicum, Corallina pilulifera, and Calliarthron yessoense, were collected along the coasts of Toi-cho and Shiriu-cho, Hokkaido, Japan, in June and July 1992. The algal specimens were individually weighed, cut into small pieces, and extracted with acetone-water (85:15). The concentrated extract was suspended in water, then successively extracted with ethyl acetate and n-butanol. The ethyl acetate and n-butanol soluble fractions were dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The water soluble fraction was directly evaporated.

α-D-Glucosidase Inhibition Assay

A pair of agar plates, an enzyme agar plate and a substrate agar plate were used in this agar plate method. The enzyme agar plate was prepared by the addition of 0.15 ml of 0.1 mg/ml α-D-glucosidase in 10 mM phosphate buffer (pH 7.0) and 0.3 ml of 0.1 mM p-nitrophenyl α-D-glucopyranoside. The substrate agar plate was prepared by dissolving 5% agar in 10 mM phosphate buffer (pH 7.0) containing 5 mM p-nitrophenyl α-D-glucopyranoside. Algal extracts (20 mg/disk) and known glucosidase inhibitors, deoxynojirimycin and glucono-δ-lactone, were impregnated onto each paper disk of 8 mm diameter. These paper disks were buried in the holes (8 mm) made by a cork borer on the enzyme agar. The enzyme agar with the paper disks was preincubated at 25°C for 2.5 h and covered with the substrate agar. After incubation at 25°C for 30 min, diameters (mm) of inhibitory colorless circles around the paper disks against the yellow background were measured. The procedure of the agar plate method is summarized in Fig. 1.

β-D-Glucosidase Inhibition Assay

The enzyme agar plate was made of 1.5% agar in 10 mM citrate buffer (pH 5.0, 20 mM) and 0.4 mg/ml β-D-glucosidase (0.15 mM), while the substrate agar plate was prepared by dissolving 3% agar in 10 mM citrate buffer (pH 5.0, 10 mM) containing 5 mM p-nitrophenyl β-D-glucopyranoside. Subsequent procedures were the same as for the α-D-glucosidase inhibition assay. The enzyme agar covered with the substrate agar was left at 25°C for 30 min, then placed in a container saturated with ammonia vapor. The procedure is summarized in Fig. 1.
Fig. 1. Procedure of the agar plate method.

A, preparation of the enzyme agar plate by dissolving 1.5% agar in buffer; B, preparation of the substrate agar plate by dissolving 3.0% agar in buffer containing p-nitrophenyl D-glucopyranoside; C, addition of glucosidase in buffer and spreading; D, placing paper disk in the hole made by a cork borer; E, preincubation at 25°C for 2.5h; F, placing the substrate agar plate on the enzyme agar plate and incubation at 25°C for 0.5h; G, measurement of diameter of colorless circle against yellow background, and in the case of α-glucosidase, placing the enzyme and substrate agars in a container saturated with ammonia vapor.

Results

Glucosidase Inhibition Assay of Known Glucosidase Inhibitors

Inhibitory activities of known glucosidase inhibitors, deoxynojirimycin and glucono-δ-lactone, by the agar plate method are shown in Table 1. In our experiments, minimum inhibitory doses (MIDs) of deoxynojirimycin against α-D-glucosidase and β-D-glucosidase were 50 and 100 μg/disk, respectively, while MIDs of glucono-δ-lactone against α-D-glucosidase and β-D-glucosidase were 500 and 250 μg/disk, respectively. The more the inhibitors were charged onto paper disks, the larger were the observed inhibitory zones.

Inhibitory Activities of Algal Extracts

Inhibitory activities against α-D-glucosidase of the ethyl acetate, n-butanol, and water soluble fractions of algal extracts are shown in Table 2. Extracts of seven species among the nine species examined showed inhibitory activities against α-D-glucosidase. The extracts of brown algae exhibited stronger inhibitory effects than the extracts of red algae. In particular, Hizikia fusiforme and Sargassum thunbergii were strongly inhibitory of the algal extracts examined.

Inhibitory activities against β-D-glucosidase of the ethyl acetate, n-butanol, and water soluble fractions of algal extracts are shown in Table 3.
acetate, n-butanol, and water fractions of algal extracts are shown in Table 3. Of the nine species of algae examined, the extracts of five species of brown algae showed inhibitory activities, while the four species of red algae showed no inhibitory activity.

**Discussion**

**Agar Plate Method as Glucosidase Inhibition Assay**

Glucosidase activity has so far been measured by the indirect colorimetric method based on a nitrophenol chromophore, a hydrolyzate of nitrophenyl glucosides as a substrate, or direct determination of glucose. The proposed agar plate method allows visual judgment of glucosidase inhibition assay. It may be possible to apply the present assay to other hydrolases, such as glycosidases, lipases and proteases, for which derivatives of visible chromophores can be used as substrates. This visual method must be applicable to water insoluble substances.

Using a spectrophotometrical method with p-nitrophenyl D-glucopyranoside as a substrate, deoxynojirimycin had $K_i$ values of 21 (pH 7) and 300 $\mu M$ (pH 5) for yeast $\alpha$-glucosidase and almond $\beta$-glucosidase, respectively, while glucosidase showed a $K_i$ value of 200 $\mu M$ (pH 6.2) for almond $\beta$-glucosidase. The sensitivity of this agar plate method is low. The more the inhibitors were charged, the larger the inhibitory zones became. However, inhibitory activity did not vary in proportion to the amount of inhibitor. The plate agar method is thus a semi-quantitative or quantitative method.

**Glucosidase Inhibitory Activities of Algal Extracts**

Most of the algal extracts examined showed inhibitory activities against $\alpha$-D-glucosidase. The ethyl acetate soluble fractions were especially stronger than the other fractions. The algal extracts examined showed inhibitory activities more frequently against $\alpha$-D-glucosidase than $\beta$-D-glucosidase. In the case of $\beta$-D-glucosidase, the water soluble fractions showed stronger inhibitory activity than the other fractions. These observations show that the inhibitory roles of $\alpha$- or $\beta$-D-glucosidase have different polarities. Brown algae examined showed inhibitory activities against glucosidases, while red algae showed weaker inhibitory activity. We can conclude that brown algae is a good source of glucosidase inhibitors.

Studies on the isolation and structural elucidation of glucosidase inhibitors from algal extracts are now in progress.

**References**