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Isolation and Characterization of α-Glucosidase Inhibitor from the Fungus *Ganoderma lucidum*

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An α-glucosidase inhibitor, SKG-3, was isolated from the fruiting bodies of *Ganoderma lucidum* and its physico-chemical properties were characterized. It was a highly specific and effective reversible inhibitor of α-glucosidase. It showed very potent inhibitory activity against α-glucosidase with an IC_{50} value of 4.6 μg/ml, but no activity for any other glycosidases tested. Enzyme activity could be recovered upon dialysis, thus providing evidence for the reversibility of the inhibition. A Lineweaver-Burk plot indicated that the SKG-3 inhibition of α-glucosidase was competitive.

Key words: α-glucosidase inhibitor, *Ganoderma lucidum*, reversible, competitive inhibitor

Glycosidases located in the brush-border surface membrane of intestinal cells are the key enzymes of carbohydrate digestion. This is because only monosaccharides are readily taken up from the intestine and all other carbohydrates have to be broken down enzymatically in the intestine before they can be absorbed. The other cellular glycosidases are known to be vital for the processing of glycoproteins and glycolipids (Kornfeld and Kornfeld, 1985; Asano, 2003), which are involved in various biological reactions such as immune responses, metastasis of cancer and viral infections (Fischer *et al.*, 1995). No doubt, glycosidase inhibitors would be the most powerful tool for influencing the kinetics of intestinal carbohydrate digestion with immediate effects on glucose absorption, arise in blood sugar levels and insulin response. Also, these inhibitors might exhibit antiviral, antimetastatic and immunostimulatory activities through interference with the normal processing of glycoproteins and glycolipids.

While searching for α-glucosidase inhibitors, we found the active compound, designated SKG-3, in a methanol extract of the fruiting body of *Ganoderma lucidum*. This mushroom has been used in East Asia for the treatment of various kinds of diseases and has recently attracted much attention on account of its biological activities (Wasser *et al.*, 1999; Min *et al.*, 2000). Since nojirimycin has been discovered from the culture broth of the *Streptmyces* species, inhibitors of glycosidases such as castanospermine, swansonine, trehazolin, deoxynojirimycin and validamine have been isolated from plants and microorganisms (Watson *et al.*, 2001). These are all potent, but not specific inhibitors of glycosidases because they inhibit various glycosidases, including β-glucosidase, α-mannosidase, and β-galactosidase (Pan *et al.*, 1993). On the other hand, the SKG-3 compound potently inhibited α-glucosidase, but it did not show any significant inhibitory effects against β-glucosidase, α-mannosidase, and β-galactosidase. In the present study, the isolation and biological activities as well as the physico-chemical properties of this compound were described. The structure elucidation of the SKG-3 compound will be reported elsewhere.

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Materials and Methods

Materials

p-Nitrophenyl (PNP) -α-D-glucopyranoside, PNP-α-D-mannopyranoside, PNP-β-D-glucopyranoside and PNP-β-D-galactopyranoside were purchased from Sigma, USA. Brewers yeast α-glucosidase, almond β-glucosidase, *Escherichia coli* β-galactosidase, jack beans α-mannosidase were also obtained from Sigma, USA. The fruiting bodies of *Ganoderma lucidum* were purchased from a local herbal drug store.

Enzyme assays

The enzymatic activities of α-, β-glucosidase, α-mannosidase and β-galactosidase were determined colorimetrically by monitoring the release of *p*-nitrophenol from the appropriate *p*-nitrophenol glycoside substrate. The buffer systems, temperatures and substrates employed for each enzyme assay were as follows: brewers yeast α-glucosidase, 10 μmol sodium phosphate, pH 6.8, 37°C; α-mannodidase, 10 μmol citrate buffer, pH 4.5, 25°C; β-glucosidase, 10 μmol citrate buffer, pH 5.0, 37°C; β-

galactosidase, 10 μmol phosphate buffer, pH 7.3, 37°C.

The assay mixtures for these experiments contained 5 μmol of the appropriate *p*-nitrophenyl glycoside, 10 μmol of sodium phosphate or sodium citrate buffer, enzyme solution (1 U) and the various concentrations of the SKG-3 compound, all in a final volume of 0.5 ml. Following an incubation of 30 min at 37°C, the reaction was terminated by the addition of 3 vol of NH_4OH solution. The liberated *p*-nitrophenol was determined from the absorbance at 405 nm in a spectrophotometer.

Various modifications of the standard assay mixture were made in order to test the effects of the SKG-3 compound. In some cases, the enzyme, buffer, and varying amounts of SKG-3 were preincubated at room temperature for 10 to 60 min, and the reaction was initiated by the addition of the substrate. In other experiments, the substrate concentration was gradually increased at fixed concentrations of the SKG-3 compound in order to determine the type of inhibition.

Isolation of the SKG-3 compound

The dried fruiting bodies of *Ganoderma lucidum* were sliced and extracted with methanol for 3 d at room temperature. The extract was concentrated at reduced pressure and was applied to the active carbon column chromatography. The column was washed with 50% methanol solution and then eluted by 70% acetone containing 0.1 N NH_4OH solution. The eluate was concentrated and partitioned between ethylacetate and water. The ethylacetate fraction was evaporated to yield a yellow residue. The residue was chromatographed on a silica gel column (2.5×45 cm) by stepwise elution, increasing the percentage of ethylacetate (2-10%) in hexane. The fractions containing an inhibitory compound were combined and subjected to Sephadex LH-20 chromatography, then eluted with methanol. Final purification was achieved by HPLC (C_{18} reverse phase column, methanol:acetonitrile:water (4 : 1 : 1.5) as an isocratic solvent system, UV detection at 205 nm) to give a single compound, SKG-3 tentatively named.

Dialysis

α-Glucosidase (0.5 ml) and the SKG-3 compound were incubated for 2 h at 37°C and then were dialyzed against the phosphate buffer (5 mM, pH 6.7) at 4°C for 24 h. Furthermore, the buffer was changed every 12 h. Another 0.5 ml aliquot was kept at 4°C for 24 h without dialysis and served as a control.

Results and Discussion

Isolation and purification of SKG-3

The α-glucosidase inhibitory compound, SKG-3, was isolated from *Ganoderma lucidum* by an activity based fractionation using various chromatography techniques. The isolation procedure is outlined in Fig. 1. The purity of the isolated compound was confirmed by TLC and HPLC. The purified SKG-3 compound showed a single spot on the TLC plate (*vide infra*) and was eluted as a single peak (13 min retention time) in HPLC.

Glycosidase inhibitory activity

The effects of SKG-3 concentration on the inhibition of the various glycosidases were tested and clearly demonstrated that SKG-3 was a potent inhibitor of α-glucosi-

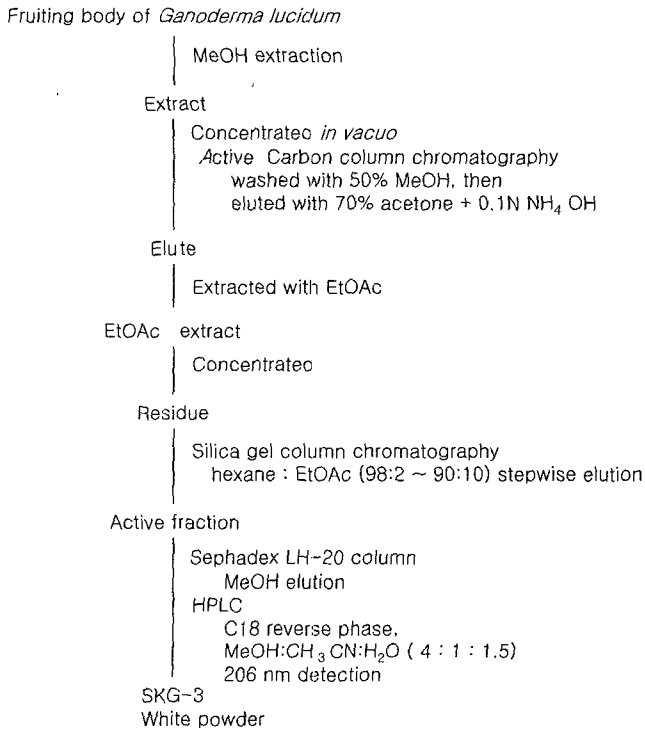

Fig. 1. Isolation of SKG-3 from the fruiting body of *Ganoderma lucidum*.

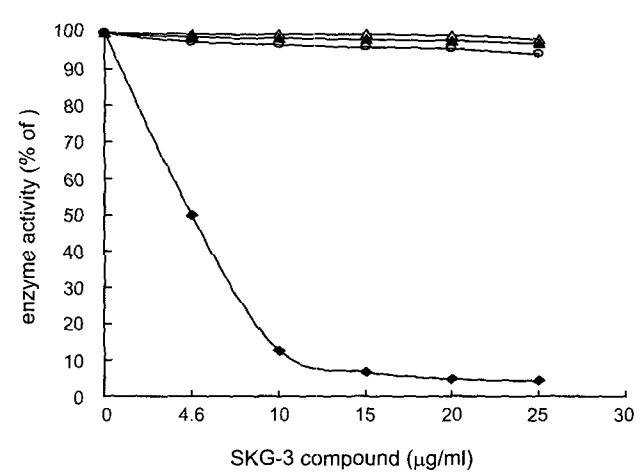

Fig. 2. Effects of the SKG-3 compound on the activity of various glycosidases. Incubation mixtures were as described in the text with the appropriate *p*-nitrophenol pyranoside. Inhibition of the enzymes (-◆-; α-glucosidase, -○-; β-glucosidase,-▲-; α-mannosidase, -△-; β-galactosidase) was expressed as a percent of enzyme control.

dase, showing 50% inhibition (IC_{50}) at 4.6 μg/ml, but not toward any other enzymes tested. The SKG-3 compound did not show any inhibitory activities toward β-glucosidase, β-galactosidase, or α-mannosidase when tested at a concentration of 100 μg/ml (Fig. 2).

The enzyme and SKG-3 solution were mixed and incubated at 37°C for a 1 h period. During the incubation, aliquots were removed at various time intervals and assayed for enzyme activity by mixing with the substrate solution. In these cases, controls were prepared without substrate to be certain that no color developed at 405 nm from the enzyme and inhibitor alone. The inhibition of α-glucosidase activity by SKG-3 was increased by the preincubation of the compound with the enzyme, indicating that this compound reacted with the enzyme slowly (Fig. 3).

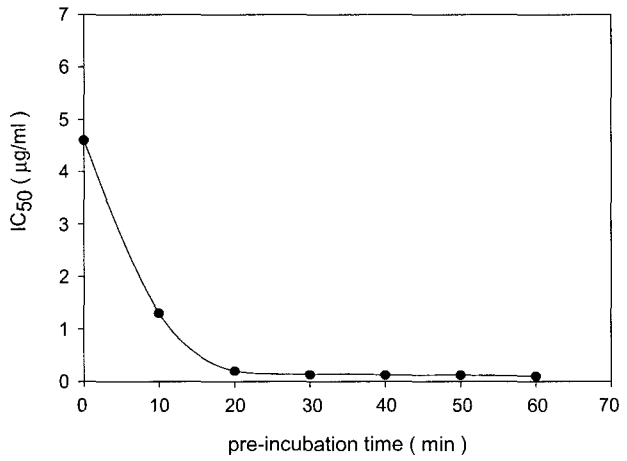

Fig. 3. The IC_{50} value for the inhibition of α-glucosidase depending on the time of pre-incubation with SKG-3. α-glucosidase was pre-incubated with various concentrations of SKG-3 for 0 to 60 min, and then PNP-α-glucopyranoside was added to the mixture to initiate the reaction. The IC_{50} value was estimated as the concentration showing 50% inhibition.

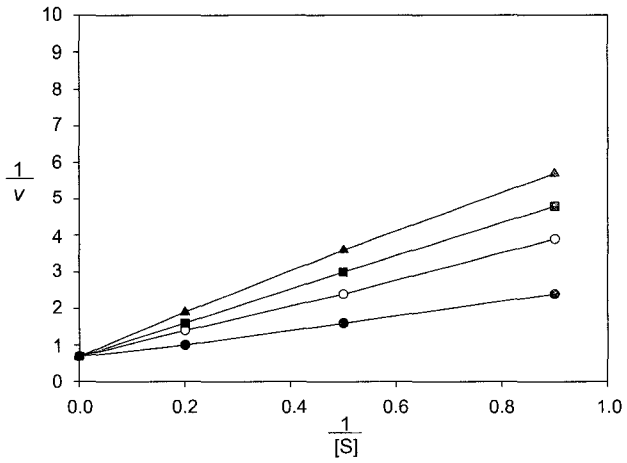

Fig. 4. Effect of substrate concentration on the SKG-3 inhibition of α-glucosidase. Reaction mixtures were described in the text but contained various amounts of substrate. Three different concentrations of SKG-3, 2 μg/ml, 5 μg/ml and 10 μg/ml (-●-; control, -○-; 2 μg/ml, -■-; 5 μg/ml, -▲-; 10 μg/ml)were added. The data were plotted by the method of Lineweaver and Burk.

In order to determine whether the SKG-3 compound was acting as a competitive or noncompetitive inhibitor of α-glucosidase, a series of experiments were carried out in which the substrate concentration was varied, and several different concentrations of the SKG-3 compound were used. When the data from these experiments were plotted by the method of Lineweaver and Burk, the intercept of $1/v$ versus 1/[S] was the same in the presence or absence of SKG-3, clearly indicating that the inhibition was of the competitive type with regard to the *p*-nitropheny-α-D-glucopyranoside substrate (Fig. 4).

The SKG-3 compound was removed from α-glucosidase by dialysis. When α-glucosidase was mixed with the amount of SKG-3 that was able to cause a 90% inhibition of enzyme activity and the mixture was placed in a dialysis bag, nearly all of the enzymatic activity was recovered after dialysis. In addition, α-glucosidase was mixed with an amount of SKG-3 sufficient to inhibit all of the enzymatic activity and this enzyme mixture was then subjected to serial dilution with buffer. When each dilution was assayed for enzyme activity, nearly all of the activity was recovered. The enzyme mixture without SKG-3 was diluted and assayed in the same way as a control. These results represented that the inhibition of α-glucosidase by SKG-3 was completely reversible.

The physico-chemical properties of the SKG-3 compound

The physico-chemical properties of the SKG-3 compound are summarized in Table 1. SKG-3 was soluble in meth-

Table 1. The physico-chemical properties of SKG-3 compound

Appearance	white powder
UV λmax in MeOH	230, 280 nm
GC-MS (m/z)	220
Solubility	soluble : MeOH, acetone, EtOAc, $CHCl_3$
	slightly soluble : hexane
	insoluble : H_2O, benzene
TLC (R_f)[a]	0.56
Color reaction	positive : Dragendorff's reagent
	Orcinol ferric chloride
	negative : Antimony trichloride
	Aniline-diphenylamine
	Ehrlich reagent
	Ninhydrine

[a]Kieselgel 60 F_{254}, Merck ; solvent ; hexane : diethyl ether (3 : 1)

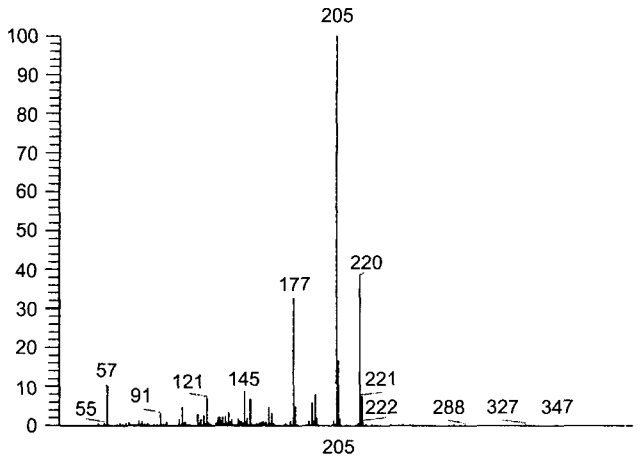

Fig. 5. Mass spectrum of SKG-3 compound.

anol, acetone, ethylacetate and chloroform, sparingly in hexane, and almost insoluble in water and benzene. In TLC on silica gel 60 F_{254} with hexane-diethylether (3 : 1) as the solvent, the R_f values were 0.56. Spraying the plates with Orcinol ferric chloride and Dragendorff's reagent produced a brown and yellow spot, respectively, indicating that the SKG-3 compound might be a sugar containing alkaloid. The molecular weight of the compound was estimated as 220 by GC-MS (Fig. 5). Detailed data will be published with structure elucidation. The UV spectrum of the SKG-3 compound in methanol exhibited maxima at 230 and 280 nm.

Enzyme inhibitors have potential value in many areas of disease control and treatment. The control of the kinetics of carbohydrate digestion and monosaccharide absorption could be of value in the prevention and treatment of diabetes, obesity, hyperlipoproteinaemia and hyperlipidaemia (Murai *et al.*, 2002). In this respect, the inhibitors of α-glucosidase, a typical exo-type amylolytic hydrolase that releases α-glucose from the non-reducing end of a polysaccharide and oligosaccharide, are of particular interest. The potent α-glucosidase inhibitor Acarbose, a polysaccharide synthesized by *Actinomyces*, has been reported to retard or prevent the absorption of meal derived glucose into circulation and to blunt the postprandial rise in plasma glucose (O'Dea and Turton, 1985). However, its use has been limited by the side-effects such as flatulence and diarrhea due to colonic fermentation of nonabsorbed sugar. Small α-glucosidase inhibitors with a mol wt below 250, which can be absorbed appreciably from the gut into the bloodstream, are arousing great interest as therapeutic agents. Glycosidases are involved in a wide variety of functions. For example, digestive glycosidases break down large sugar-containing molecules to release monosaccharides that can be more easily taken up and used metabolically by the organism; lysosomal glycosidases catabolyze glycoconjugates intracellularly; and a wide range of glycosidases are involved in the biosynthesis of the oligosaccharide portions of glycoproteins and glycolipids which play vital roles in cellular structure and function. In order to be useful as an antidiabetic agent, the inhibitory activity should be more specific. That is, the inhibitor should be specific for intestinal brush border α-glucosidases but should have no other effect on the glycoprotein processing glycosidases (Jacob, 1995). Most of the presently available α-glucosidase inhibitors such as swainsonine, castanospermine, calystegine and deoxynojirimycin show broad activity spectra against various glycosidases (Pan *et al.*, 1993; Molyneux *et al.*, 1995) and have been shown to inhibit the processing and maturation of glycoproteins (Sunkara *et al.*, 1987). In addition to the inhibition of α-glucosidase, castanospermine also inhibited β-glucosidase (Saul *et al.*, 1985); swainsonine is a potent inhibitor of α-mannosidase (Cenci di Bello *et al.*, 1989); calystegine inhibits β-glucosidase and α-galactosidase (Molyneux *et al.*, 1996). Owing to their toxicities, these compounds were considered unsuitable for therapeutic use. Whether the toxic effects of these compounds were the result of the non-specific inhibition of the various glycosidase activities or changes in the structure of the oligosaccharide chains have not been determined.

The SKG-3 compound inhibited α-glucosidase but not any of the other glycosidases. It was reported that there are marked differences in the inhibition of isoenzymes of a given glycosidase in different species and even within the same cell (Scofield *et al.*, 1995). Due to the variations in specificity of isoenzymes, both between species and within the same cell, it is difficult to predict that SKG-3 will inhibit various forms of α-glucosidase. Therefore, the activities of SKG-3 *in vivo* against the various α-glucosidase-specific disaccharidases involved in mammalian digestion, such as sucrase, maltase, isomaltase, should be tested. However, the SKG-3 compound, when given in combination with a high carbohydrate diet orally, significantly reduced the postprandial plasma glucose levels in non-diabetic rats (unpublished data). SKG-3 was isolated from *Ganoderma lucidum*, which has long been regarded as an elixir in oriental medicine and confirmed no toxicity. This indicates that this compound might be useful in the oral treatment of diabetes and obesity. Although the reduction of the postprandial plasma glucose level by *Ganoderma lucidum* has been clinically recognized, no active principle has been reported. Consequently, the results presented herein suggest that the SKG-3 compound, the α-glucosidase inhibitor, might be an active principle. Furthermore, the SKG-3 compound appeared to be a valuable tool for investigating the mechanism and active site structure of α-glucosidase.

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