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Isolation and Purification of Polyhydroxylated Alkaloids from Silkworm (*Bombyx mori* L.)

Hyun-Su Kim[1], Hyun-Jung Ko[2], Yong Seak Cho[2], Jae Yeon Lee[2], Kyo Yeol Hwang[2], Jin-Won Kim[3], Heui-Sam Lee[3], Iksoo Kim[3], Kang-Sun Ryu[3], Young-Ho Kim[2] and Su Il Seong[1,2]*

[1]*Biotopia Co., Ltd. Hwasung city, Kyounggido 445-743, Korea.*

[2]*Department of Biology, The University of Suwon, Suwon P.O. Box 77, Kyounggido 440-600, Korea.*

[3]*Department of Sericulture and Entomology, The National Institute of Agricultural Science & Technology, RDA, Suwon 441-100, Korea.*

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Several polyhydroxylated alkaloids were isolated from the extracts of freeze-dried silkworm powder, and purified by ion exchange chromatographic analysis. Through the HPLC analysis, we could identify 1-deoxynojirimycin (DNJ) and a kind of calystegin B_2 (HS-58) as well as a noble compound (HS-74) from the purified polyhydroxylated alkaloids. In order to know the characteristics of these isolated alkaloids as enzyme inhibitors, glycosidase inhibition activities of these identified alkaloids including other two non-purified alkaloids (SWP 3-1 and SWP 3-2) were investigated.

Key words: Polyhydroxylated alkaloids, Glycosidase inhibitor, *Bombyx mori*, 1-deoxynojirimycin, DNJ

Introduction

These days, there are many people who suffer from life-style related diseases such as diabetes mellitus, heart trouble, *etc.*, as they advance in years. Especially, diabetes is now spreading increasingly worldwide as a major global disease. In an attempt to pave the way for treating the disease, some researchers have paid attention to the glycosidase inhibitory activities of the naturally-occurring polyhydroxylated alkaloids (Yagi *et al.*, 1976; Schmidt *et al.*, 1979; Asano *et al.*, 2000). Especially, Asano and his colleagues were successful in isolating many polyhydroxylated alkaloids including 1-deoxynojirimycin (DNJ) from mulberry root extracts, and discovered potentiality of the alkaloids as an inhibitor of mammalian α-glucosidase.

Later, Asano *et al.* (2001) found that silkworm powder extracts also have a potential for α-glucosidase inhibition by showing an evidence of significant anti-hyperglycemic effect on both alloxan-induced diabetic mice and high carbohydrate-diet fed mice. Also, they found that the main anti-hyperglycemic substance exerting α-glucosidase inhibition in the silkworm powder extracts is DNJ. As for the hyperglycemic activity of the DNJ, Jacob (1995) suggested that it reduces glucose uptake by inhibiting the breakdown of complex carbohydrates in the gut and, resultantly, the postprandial rise of the blood glucose level, which is a characteristic of diabetes, is minimized.

In this study, we isolated several polyhydroxylated alkaloids from the extracts of freeze-dried silkworm powder, and successfully purified three kinds of alkaloids by ion exchange chromatographic analysis. These purified alkaloids were then analyzed through HPLC, and one compound turned out to be a novel alkalids. With the interest of industrialization of these alkaloids, we investigated the inhibitory effects of these isolated alkaloids on several glycosidase such as α-glucosidase, β-glucosidase, β-galactosidase, and crude porcine intestinal maltase.

Materials and Methods

Isolation of polyhydroxylated alkaloids from the silkworm powder

The freeze-dried silkworm powder (10 kg) was treated with 50% EtOH (100 *l*), and the ethanol extract was filtered through the Celite 545. The filtrate was applied to a column

*To whom correspondence should be addressed.
Biotopia Co., Ltd. Hwasung city, Kyounggido 445-743, Korea.
Tel: +82-31-220-2483; E-mail:sseong@mail.suwon.ac.kr

chromatography of Amberlyst 15 (10 cm × 120 cm, H^+ form), and eluted with 0.5 N NH_4OH. The ammonium eluate was concentrated, and the brown oil-like concentrate was then applied to another column chromatography of Dowex 1 × 2 – 100 (10 cm × 60 cm, OH^- form). The column was eluted with water to remove anionic compounds, and the water eluted was concentrated to be yellowish oil. The yellowish oil (about 10 g) were then applied to a column chromatography of Amberlite CG-50 column (4 cm × 90 cm, NH_4^+ form), and thereafter the column was eluted with water (15 ml of fraction size). The water elution was then followed by elution with 0.5 N NH_4OH. We collected five fractions from water eluants were applied to a successive column chromatography of Amberlite CG-50 column (2 cm × 80 cm, NH_4^+ form) and CM-Sephadex C-25 column (2 cm × 80 cm, NH_4^+ form) with 0.01 N NH_4OH elution. The polyhydroxylated alkaloids were obtained from the final water fractions of Dowex 1 × 2 – 100 column (2 cm × 40 cm, OH^- form) chromatography. The obtained alkaloids fractions were kept in state of freeze-dried powder.

HPLC analysis

Purified polyhydroxylated alkaloids were identified with HPLC analysis (Asano *et al.*, 2000). HPLC analysis was carried out mainly followed by Kim *et al.* (2003).

Preparation of porcine intestinal enzyme

Brush border membranes prepared from porcine small intestine were suspended with 0.1 M phosphate buffer (pH 6.8). The suspended solution was centrifuged at 15,000 g for 30 min to obtain supernatant. The supernatant was added with 80% ammonium sulfate and centrifuged at 15,000 g for 20 min so as to precipitate enzyme proteins. The precipitate was subjected to dialysis for deionizing at 4°C for 12 hrs, and finally freezing dried. The protein content of porcine intestinal enzyme was measured by the modified method of Bradford (1976). The activities of disaccharides in porcine intestinal enzyme were determined by TLC Silica Gel F_{254} after the reaction between the porcine enzyme and several disaccharides such as maltose, sucrose, cellobiose and lactose. The released glucose on TLC plate was detected by spraying with Aniline-dephenylamine reagent. The activity of α-glucosidase in porcine intestinal enzyme was also detected on the Native-PAGE gel after reaction between the porcine enzyme and 4-Methllumbelliferyl-α-D-glucose as substrate.

Glycosidase inhibition activities of isolated alkaloids

The reaction of glycosidases was performed by using an appropriate *p*-nitrophenyl glycoside as a substrate at the optimum pH of each enzyme. In order to know the inhibition effects of alkaloids, each isolated alkaloids was also added in a sequence of concentrations at the beginning of the enzyme-substrate reactions. The reaction was continued for 45 min and stopped by adding 200 mM Na_2CO_3. The glycosidase inhibition activities of alkaloids were determined by measuring released *p*-nitrophenol spectrometerically at 405 nm. The enzyme inhibition activities of alkaloids were shown as $E.C._{50}$, which is the concentration of alkaloids inhibiting the glycosidases at 50 percent level (Scofield *et al.*, 1995).

Result and Discussion

We successfully isolated three kinds of alkaloids with over 98% purity as well as other two compounds after ion exchange chromatographic analyses from the freeze-dried silkworm powder. The isolated alkaloids were then identified by HPLC, comparing with the alkaloids, which had already been identified in the silkworm powder (Asano, 2001). From the chromatographic analyses of the three highly purified compounds, one turned out be DNJ (Fig. 1), and another was a kind of calystegin B_2. Although we were able to assign the compound as a kind of calystegin B_2, we could not identify the compound decisively at this time, because there are at least 14 iso-compounds in calystegin B_2, depending on the position and configuration of the hydroxyl groups comprising the alkaloid. Thus, further effort to identify exact isoforms is required. The calystegin B_2 compound isolated by us is named HS-58 (Fig. 2). However, the remaining one did not coincided with any alkaloid previously shown in the silkworm powder on HPLC. The compound might be a novel alkaloid, isolated for the first time in this study by us. The unknown compound is named here HS-74 (Fig. 3).

With the interest for industrial utilization of the isolated

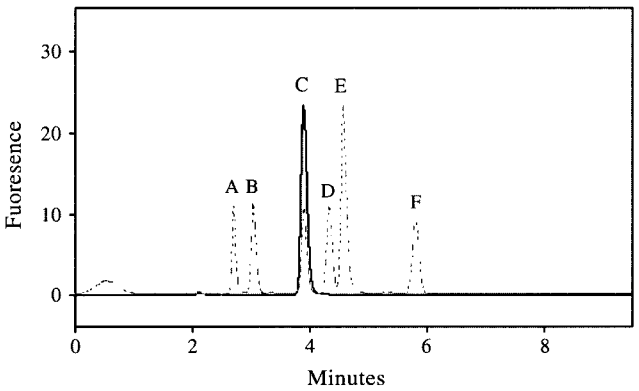

Fig. 1. Chromatogram of HPLC of the purified 1-deoxynojirimycin(DNJ) from silkworm powder. Several alkaloid compounds are shown on the some chromatogram in order to compare with DNJ. A, Gal-DNJ; B, Glc-DAB; C, Me-DNJ; D, 3-epi-FAG; E, FAG/DAB; and F, Caly-B_2 (Asano *et al.*, 2001).

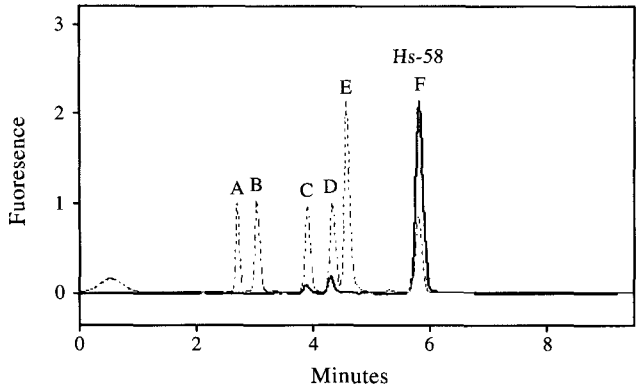

Fig. 2. Chromatogram of HPLC of HS-58 compound from silkworm powder. Several alkaloid compounds are shown on the some chromatogram in order to compare with DNJ. A, Gal-DNJ; B, Glc-DAB; C, Me-DNJ; D, 3-epi-FAG; E, FAG/DAB; and F, Caly-B_2 (Asano *et al.*, 2001).

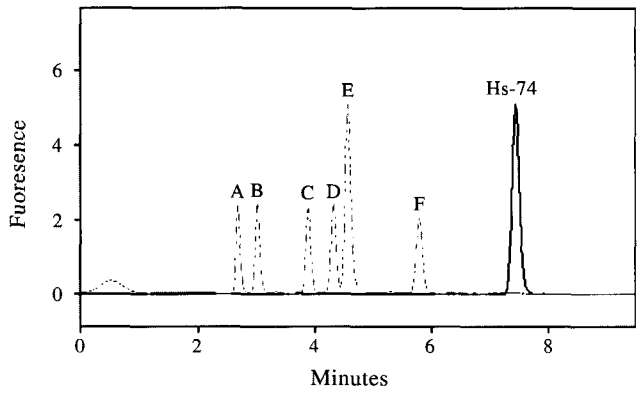

Fig. 3. Chromatogram of HPLC of HS-74 compound from silkworm powder. Several alkaloid compounds are shown on the some chromatogram in order to compare with DNJ. A, Gal-DNJ; B, Glc-DAB; C, Me-DNJ; D, 3-epi-FAG; E, FAG/DAB; and F, Caly-B_2 (Asano *et al.*, 2001).

alkaloids, we first investigated glycosidase inhibition activity of these alkaloids. It has been already well known that the naturally-occurring polyhydroxylated alkaloids including DNJ, have potent inhibition activity to the various α-glucosidasespecific disaccharides involved in mammalian digestion. Thus, some inhibitors of these enzymes, such as DNJ could be used therapeutic drugs for non-insulin-dependent diabetes mellitus (Yagi *et al.*, 1976; Schmidt *et al.*, 1979). We also detected the glycosidase inhibition activities of the isolated alkaloids.

Enzymes used in the inhibition test were α-glucosidase, β-glucosidase, β-galactosidase, and crude porcine intestinal maltase. The protein content of freeze-dried porcine intestine was 30 ug/mg, and maltase activity was clearly confirmed by thin layer chromatography and native polyacrylamide gel electrophoresis (Fig. 4 and 5).

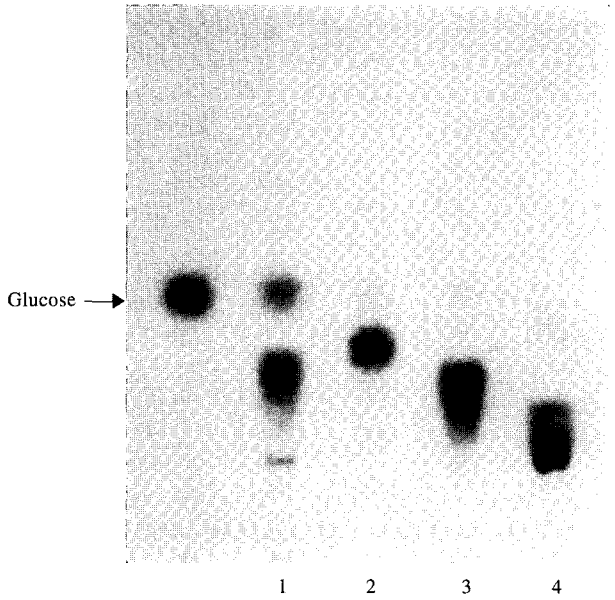

Fig. 4. Chromatogram of TLC after enzyme reaction between the porcine intestinal enzyme extract and four kinds of disaccharides. Glucose is appeared only in the enzyme reaction between maltose and porcine intestinal extract. 1, maltose; 2, sucrose; 3, cellobiose; and 4, lactose.

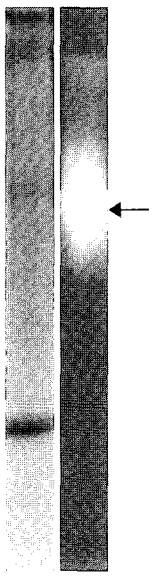

Fig. 5. α-Glucosidase zymogram of porcine enzyme extract on native polyacrylamide gel electrophoresis (right). Arrow shows the activity of α-glucosidase. Left lane is showing the proteins of porcine intestinal extract by staining with coomassie brilliant blu R-250 after native-PAGE analysis.

The $E.C._{50}$ values of DNJ, HS-58, HS-74 and other two non-purified alkaloids, SWP 3-1 and SWP 3-2 are shown in Table1. The enzyme inhibition effect of DNJ was most potent in α-glucosidase, followed by β-glucosidase, while any inhibition effect was not found in β-galactosidase. Fur-

Table 1. Enzyme inhibition activities of alkaloid compounds isolated from the silkworm powder

Enzymes Compound	α-glucosidase (E.C.$_{50}$)	β-glucosidase (E.C.$_{50}$)	β-galactosidase (E.C.$_{50}$)	Crude porcine enzymes (E.C.$_{50}$)
1-DNJ	2×10^{-5}M	2×10^{-3}M	NI	2×10^{-6}M
HS-58	70 ppm	3 ppm	NI	20 ppm
HS-74	NI	NI	NI	100 ppm
SWP 3-1	10 ppm	NI	NI	10 ppm
SWP 3-2	10 ppm	NI	NI	1 ppm

NI stands for the enzyme inhibition activity below 50%.

ther, DNJ showed very high potent inhibition effect to porcine intestinal maltase, showing about ten times stronger activity than α-glucosidase at the level of E.C.$_{50}$ (Table 1).

The enzyme inhibition activity of HS-58 was relatively low in most enzymes except for α-glucosidase, compared with DNJ. HS-58 turned out to be highly potent inhibitor of α-glucosidase with hundred times stronger activity than DNJ. As noted above, however, the exact identification of HS-58 has not been solved yet, though HS-58 coincides with the calystegin B_2 on HPLC. More detailed structural information of HS-58 will be fulfilled by means of GC-MS or NMR analysis in the future.

Unlikely our high expectation, the novel alkaloids, HS-74, did show very weak enzyme inhibition activity except for the marginal effect to porcine intestinal maltase. In spite of low enzyme inhibition activity, however, HS-74 is also expected as potent alkaloids candidate for industrial application in the future. Now, we are ready to identify the structural information of HS-74 by means of GC-MS or NMR analysis.

The two non-purified isolates from the silkworm powder, SWP 3-1 and SWP 3-2, were also subject to enzyme inhibition test. These two compounds exhibited almost same tendency of enzyme inhibition; relative potent inhibition effect to a α-glucosidase and porcine intestinal maltase, but almost no effect to β-glucosidase and β-galactosidase. Especially, inhibition effect of SWP 3-2 to porcine intestinal maltase was noteworthy. This result illustrates that the two isolates from the silkworm powder could be kinds of alkaloids. Purification and structural analyses of the two alkaloid candidates are required in the future.

Besides the use of alkaloids for the non-insulin-dependent diabetes mellitus, therapeutic applications of alkaloids are very wide and diverse like such as anti-cancer agents, immune stimulants, and anti-viral agents, etc. (Watson *et al.*, 2001). Recently, we also found DNJ is applicable for the suppression of multiplications of hepatitis-B and -C viruses. In this respect, the alkaloids isolated from the silkworm powder are expected as a possible candidate for various therapeutic agents.

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