Protein Glycation Inhibitors from the Fruiting Body of *Phellinus linteus*

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To characterize active principles for prevention and treatment of diabetic complications, the isolation of protein glycation inhibitors from the fruiting body of *Phellinus linteus* was conducted *in vitro* using the model systems of hemoglobin-Δ-glucosone (early stage), bovine serum albumin-methylglyoxal (middle stage), and N*-acetyl-glycyl-lysyl methyl ester-α-ribose (last stage) assays. Nine compounds were isolated from the active ethylacetate fraction of the fruiting body and identified as protocatechuic acid (1), protocatechualedehyde (2), caffeic acid (3), ellagic acid (4), hispidin (5), davallialactone (6), hypholomine B (7), interfungins A (8), and inoscavin A (9) by spectroscopic analyses. At the early stage of protein glycation, compounds 6, 8, and 9 exhibited inhibitory activity on hemoglobin AIC formation. For the middle stage, compounds 2, 6, and 9 showed a significant inhibitory effect on methylglyoxal-mediated protein modification and their IC₅₀ values were 144.28, 213.15, and 158.66 μM, respectively. At the last stage of glycation, compound 8 was found to be a potent inhibitor of the cross-linking of proteins, which was more effective than that of aminguanidine, a well-known inhibitor for advanced glycation end products. Consequently, compound 8 showed the most potent inhibitory effects at each stage of protein glycation. This mechanism may help to provide a protective effect against hyperglycemia-mediated protein damage.

Key words *Phellinus linteus*; diabetic complication; protein glycation inhibitor; hispidin; hemoglobin AIC; advanced glycation end product

Diabetes and diabetic complications are recognized as degenerative diseases that frequently occur with age, oxidative stress, non-enzymatic glycation, and extensive protein crosslinkings are implicated in both diseases. Hyperglycemia may play an important role in the pathogenesis of diabetic complications by several mechanisms such as increased aldose reductase-related polyol pathway flux, increased advanced glycation end products (AGEs) formation, AGEs receptor overexpression, protein kinase C isoform activation, increased hexosamine pathway flux, and excessive oxidation stress including superoxide overproduction. In particular, during hyperglycemia, body proteins undergo increased glycation where glucose reacts non-enzymatically with protein amino groups to form a labile Schiff base that rearranges to a stable Amadori product. This Amadori product undergoes further reactions involving reactive dicarbonyl intermediates such as 3-deoxyglucosone and methylglyoxal to form complex, heterogeneous, fluorescent and crosslinked structures called AGEs. In the presence of oxygen and transition metals, glucose can undergo autoxidation (autoxidative glycation) as can Amadori products (glycoxidation) to produce free radicals capable of damaging proteins, lipids, and nucleic acids. Indeed, diabetes and ageing are associated with a build up of tissue AGEs, increased oxidative stress, and a decline in antioxidant status. Furthermore, circulating serum AGEs can interact with receptor for AGEs to activate nuclear factor-kappa B which in turn generates pro-inflammatory molecules and oxidative stress. Recently, many potent and active synthetic AGE inhibitors have been presented to the drug market; however, these have limits in terms of use, and/or were withdrawn from clinical trials due to relatively low efficacies, poor pharmacokinetics, and unsatisfactory safety. Although these synthetic agents are proposed as the prototype for new and promising drugs, many researchers have been working to find new, potent, and safe AGE inhibitors from nature.

Mushrooms are universally accepted as nutritionally functional foods in East Asia, particularly China, Japan, South Korea and important sources of physiologically beneficial medicines. They produce various classes of secondary metabolites with interesting biological activities and thus have the potential to be used as valuable chemical resources for drug discovery. Interestingly, medicinal mushrooms such as *Phellinus linteus*, *P. igniarius*, and *Inonotus xeranticus* commonly produce a number of yellow antioxidant pigments that comprise hispidin derivatives and polyphenols. *P. linteus*, which is commonly referred to as Sangwhang in Korea, has been reported to have strong antioxidant activity. In our recent study, hispidin derivatives from *P. linteus* displayed therapeutic potential in the prevention and treatment of diabetic complications by inhibiting aldose reductase.

To date, however, there have been no studies of the effect of the mushroom’s constituents on protein glycation inhibitory activity. The aim of the present study was to search for potential protein glycation inhibitors from the fruiting body of *P. linteus* and for application in the treatment of diabetic complications.

MATERIALS AND METHODS

**Instruments and Chemicals** ¹H- and ¹³C-NMR spectra
were recorded on a Bruker DPX 400 spectrometer (Karlsruhe, Germany) at 400 and 100 MHz, respectively. Chemical shifts are given in ppm (δ) using tetramethylsilane as an internal standard. Aminoguanidine hydrochloride (AG), methylglyoxal (40% aqueous solution), bovine serum albumin (essentially fatty acid free), δ-gluconolactone, N-acetyl-glycyl-lysine methyl ester acetate salt (G.K. peptide), and α-ribose were purchased from Sigma (St. Louis, MO, U.S.A.). Sephadex LH 20 (GE Healthcare Bio-Science AB, Sweden) and cosmosil 75C18-OPN (Nacalai Tesque, Kyoto, Japan) were used as column packing materials.

**Plant Materials** The fresh mushroom of *P. linteus* (1 kg) was provided by Samsung Herb Medicine Co., Ltd., Chuncheon, Korea and the voucher specimen (No. RIC-021) was deposited and maintained at the Herbarium of Regional Innovation Center, Hallym University, Republic of Korea.

**Isolation and Identification** The lyophilized fruiting body of *P. linteus* (1 kg) was extracted three times with methanol (MeOH) for 5 h. After removal of MeOH under reduced pressure, a dark brown residue (50 g, 5%) was obtained. The residue was suspended in water and then partitioned sequentially with n-hexane (7 g, 0.7%), methylene chloride (5.9 g, 0.59%), ethylacetate (EtOAc, 24 g, 2.4%), n-butanol (12.5 g, 1.3%), and water residue (1 g, 0.1%). Active compounds were purified from the EtOAc soluble portion by a bioassay-guided separation. A portion of the active EtOAc fraction was applied to a Sephadex LH-20 column chromatography which was eluted with MeOH to obtain 12 fractions. From fractions 1—3, compounds 1 (10 mg), 2 (9 mg), and 3 (11 mg) were isolated using a preparative HPLC (JAI Analytical Industry Co., Ltd., Tokyo, Japan) with 15% acetonitrile (MeCN), respectively. Fraction 4 was recrystallized to obtain compound 4 (15 mg). Compounds 5 (12 mg) and 6 (10 mg) from fractions 5 and 6, respectively, were purified using medium pressure preparative liquid chromatography over a reversed-phase Si gel (C-18) that was eluted with increasing MeCN (20 to 45%) in water and subsequent preparative HPLC with 25 or 30% MeCN. Compounds 7 (11 mg), 8 (11 mg), and 9 from fractions 7 and 8 were isolated using the above-mentioned process. Their structures were identified by spectral analyses.

**Hemoglobin-δ-Gluconolactone Assay** Evaluation of early stage protein glycation was determined with a δ-gluconolactone assay. The assay is specific for investigation of inhibitors to the formation of early glycation Amadori products. Briefly, samples were prepared by mixing 200 μl of fresh human blood with 40 μl of phosphate buffer (pH 7.4) as the blank or 40 μl of δ-gluconolactone (50 mM) as control. Test samples contained 200 μl of blood plus 40 μl of δ-gluconolactone and 10 μl of sample. After incubation at 37 °C for 16 h, the percentage of glycated hemoglobin present was determined using a HLC-723 G7 (Tosoh Corporation, Tokyo, Japan), an automatic ion-exchange HPLC system for the assay of hemoglobin A1c (HbA1c). Tosoh’s G7 separates hemoglobin into six fractions, including stable HbA1c, in only 2.2 min. Hemoglobin separation was achieved by a cation exchange nonporous polymer column (TSK gel GLYCO Hsi, Tosoh Corporation). Each hemoglobin fraction was eluted from the column by a step gradient of three buffers with different salt concentrations and was monitored at 415 nm. Blood samples were analyzed in triplicates. The percentage inhibition of HbA1c formation was calculated as [(HbA1c of the control−HbA1c of the test group)/(HbA1c of the control−HbA1c of the blank)]×100%. AG was used at a final concentration of 10 mM as a positive control.

**Bovine Serum Albumin-Methylglyoxal Assay** The modified procedure of Lee et al. was followed. Bovine serum albumin (10 mg/ml) was incubated at 3 °C for 7 d with methylglyoxal (5 mM) in sodium phosphate buffer (0.1 M, pH 7.4). Dimethylsulfoxide used for dissolving samples was found to have no effect on the reaction. All of the reagent and samples were sterilized by filtration through 0.2 μm membrane filters. The fluorescence intensity was measured at an excitation wavelength of 330 nm and an emission wavelength of 410 nm with a Luminescence spectrometer LS50B (Perkin-Elmer Ltd., Buckinghamshire, England). AG was tested as a known inhibitor. The concentration of each test sample giving 50% inhibition of the activities (IC50) was estimated from the least-squares regression line of the logarithmic concentration plotted against the remaining activity.

**Nα-Acetyl-glycyl-lysine Methyl Ester-α-ribose Assay** This test was used to evaluate the ability of samples to inhibit the cross-linking of G. K. peptide in the presence of α-ribose using the method described by Rahbar et al. and Nagaraj et al. G. K. peptide (80 mg/ml) was incubated with α-ribose (0.8 M) under sterile conditions in sodium phosphate buffer (0.5 M, pH 7.4) at 37 °C for 24 h. Compounds 1—9 were added to a final concentration of 1 mM except AG which was used at 10 and 50 mM. At the end of the incubation period, the fluorescence intensity was measured at an excitation wavelength of 330 nm and an emission wavelength of 415 nm.

**Statistical Analysis** Data are presented as means±S.E.M. Statistical comparisons between groups were performed using the Student’s t-test. The values at *p<0.05 and **p<0.01 were considered to be statistically significant difference.

**RESULTS AND DISCUSSION**

Conditions such as diabetes, which have a metabolic overload of reducing sugars, rapidly accelerate AGEs formation. As AGE levels rise during diabetes, interruption of normal function occurs via three distinct mechanisms, namely AGE induced cross-linking of extracellular matrices, intracellular formation of AGEs, and the chronic activation of specific receptors such as the receptor for AGEs. Due to the range of dysfunction produced by the accumulation of AGEs in diabetes, there is a growing need for early recognition and intervention in this process. Thus, interventions that reduce AGE accumulation appear to be protective against the development of the complications of diabetes.

To search active compounds from the fruiting body of *P. linteus*, its extract was systematically divided into five different solvent fractions. Those fractions were then evaluated for protein glycation inhibition. Of the fractions, the EtOAc fraction exhibited the strongest inhibitory activity against AGEs formation in the bovine serum albumin-methylglyoxal assay, and its IC50 value was 65.86 μg/ml (Table 1). Since this result suggests the presence of many AGEs inhibitors in the EtOAc fraction, special attention was focused on isolating the active principle from this fraction.
Table 1. Inhibitory Effects of Extract and Fractions from the Fruiting Body of P. linteus on Protein Glycation

<table>
<thead>
<tr>
<th>Extract and fractions (Ext./Fr.)</th>
<th>Bovine serum albumin-methylglyoxal assay (IC50, µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH Ext.</td>
<td>71.21</td>
</tr>
<tr>
<td>n-Hexane Fr.</td>
<td>&gt;196</td>
</tr>
<tr>
<td>Methylene chloride Fr.</td>
<td>&gt;196</td>
</tr>
<tr>
<td>Ethylacetate Fr.</td>
<td>65.86</td>
</tr>
<tr>
<td>n-Butanol Fr.</td>
<td>80.70</td>
</tr>
<tr>
<td>Water Fr.</td>
<td>&gt;196</td>
</tr>
</tbody>
</table>

![Fig. 1. Chemical Structures of Compounds 1—9 Isolated from P. linteus](image)

For the identification of active principles from the fruiting body, a portion of the active EtOAc fraction was subjected to a repeated chromatography on a Sephadex LH-20 and a reversed phase-Si gel to yield individual compounds 1—9. Their structures were elucidated based on 1-dimensional (1H- and 13C-NMR) and 2-dimensional NMR heteronuclear multiple quantum coherence (HMBC) spectral data analysis and by comparison with published spectral data. They were identified as protocatechuic acid (1), protocatechuoldehyde (2), caffeic acid (3), ellagic acid (4), hispidin (5), davallialactone (6), hypholomine B (7), interfungins A (8), and inoscavin A (9). The chemical structures of compounds 1—9 were shown in Fig. 1.

To determine the inhibitory effect of AGES formation, several assay methods have been proposed including assays based on inhibition of specific fluorescence generated during the course of glycation and AGES formation; assays based on the inhibition of AGES-protein crosslinks such as dimer and trimer formation; and specific ELISA assays using anti-AGES antibodies for quantitative measurement of AGES. However, none of these assays can accurately evaluate chemical intermediates and products generated during the early stages of glycation. We used a rapid assay method for evaluation of the early stage of glycation. This assay is based on the interaction of δ-glucosolactone, an oxidized analogue (ketoaldehyde) of glucose, with hemoglobin present in blood samples. It provides a relevant and physiological model to study glycation and potential inhibitors. Furthermore, it offers a means to differentiate between inhibitors of the early and late stages of glycation and provides a rapid method of screening large numbers of potential inhibitors of glycation.

Glucose reacts nonenzymatically with the NH2-terminal amino group of the β chain of human hemoglobin (Hb A; α2β2) to form an aldimine linkage, which subsequently undergoes an Amadori rearrangement to form the more stable ketoamine linkage, resulting in the formation of HbA1C which is formed slowly, continuously, and nearly irreversibly throughout the 120-d life-span of the red cell. Hence, the higher the ambient blood glucose, the greater the rate of glycation and the higher the HbA1C value. Each 1% change in HbA1C reflects a change in mean plasma glucose of about 35 mg/dl. About 90, 2.5, and 0.2% of the hemoglobin in the human red blood cells is Hb A (α2β2), Hb A2 (α2δ2), and Hb F (α2γ2). Figure 2 showed the inhibitory effect of compounds 1—9 at the early stage of protein glycation and representative HbA1C chromatograms. The level of HbA1C in the control was twice higher than the blank. This result indicated that compounds 6, 8, and 9 had the most potent inhibitory effects at 50.2, 63.1, and 45.7%, respectively, at a concentration of 1 and 10 µM.

In a bovine serum albumin–methylglyoxal system, methyl-
glyoxal readily reacts with protein lysine and arginine residues to produce high molecular weight, cross-linked, fluorescent products. The inhibitory effects of compounds 1—9 on methylglyoxal-mediated protein glycation were evaluated. Compounds 6, 8, and 9 exhibited significant inhibition, and their IC\textsubscript{50} values were 158.66, 213.15, and 261.30 μM, respectively, compared to AG (IC\textsubscript{50} 921.94 μM). Of compounds 1—4, with the exception of hispidin derivatives, compounds 2 and 4 showed strong inhibition with IC\textsubscript{50} values of 144.28 and 334.74 μM, respectively. Compounds 1 and 5 showed no inhibitory activity (Table 2). In a G. K. peptide–d-ribose system, a G. K. peptide containing a lysine residue was incubated with d-ribose for 24 h. This procedure was expected to generate peptides with advanced Maillard reaction product with dimerization through lysine–lysine cross-linking. Rahbar et al. pointed out that co-incubation of a G. K. peptide with d-ribose increased the formation of the late glycation products.23 On the basis of these interactions, we used this model system to evaluate the inhibitory effects of compounds 1—9 on protein cross-linking. As shown in Table 3, compound 8 exhibited substantial anti-cross-linking activities in a dose-dependent manner. At a concentration of 10 mM, the inhibitory effect of compound 8 and AG was over 82.5 and 41.67%, with IC\textsubscript{50} values of 11.93 and 1.15 mM, respectively (Fig. 3).

These results insisted that for each stage of protein glycation compound 8 had the most potent inhibitory effect of all the compounds isolated from the fruiting body. These observations suggested that compounds 6 and 8 can potentially inhibit the glycoxidative modification of proteins. We previously reported that hispidin derivatives, especially hispidin dimer, had strong rat lens aldose reductase and human recombinant aldose reductase inhibitory activity.21 Hispidin derivatives with aldose reductase and protein glycation inhibitory activity were highly oxygenated and functionalized aromatic compounds and were composed of 6-[2-(3,4-dihydroxyphenyl)ethenyl]-4-hydroxy-2H-pyran-2-one (hispidin, 5) or/and 6-(3,4-dihydroxyphenyl)-4-hydroxy-3,5,hexadiene-2-one (hispolon) moiety.17 Lee et al. and Kim et al. reported that this class of compounds, in particular, davallialactone (6) and interfungins A (8) exhibited potent scavenging activity against superoxide radical anion, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical cation, and 1,1-diphenyl-2-picrylhydrazyl radical.16,17,19 Taken together, potential sites where compounds 6 and 8, the most effective active principles of P. linteus, may act to inhibit protein glycation were indicated in Fig. 4.

Recently there has been increased interest in natural products with anti-glycation properties and aldose reductase inhibitory activity or antioxidative activity. Polyphenolic compounds from Apocynum venetum, garcinol from Garcinia indica fruit, quercetin, eriodictyol, 5,6,4′-tri-hydroxy-7,8,3′-trimethoxy-flavone, and cirsilineol from Thymus vulgaris are known to be more potent than AG in inhibiting AGEs formation.30–32 Also, anthraquinones from Cassia tora and luteolin 6-C(6′-O-trans-cafeoylglucoside) from Phyllostachys nigra showed aldose reductase and protein glycation inhibitory activities.33,34 Flavonoids in particular have been

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**Table 2. Inhibitory Effects of Compounds 1—9 Isolated from the Fruiting Body of P. linteus on Protein Glycation**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Bovine serum albumin–methylglyoxal assay (IC\textsubscript{50} μM)</th>
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<tbody>
<tr>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>144.28</td>
</tr>
<tr>
<td>3</td>
<td>&gt;1089.33</td>
</tr>
<tr>
<td>4</td>
<td>334.74</td>
</tr>
<tr>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>158.66</td>
</tr>
<tr>
<td>7</td>
<td>428.00</td>
</tr>
<tr>
<td>8</td>
<td>213.15</td>
</tr>
<tr>
<td>9</td>
<td>261.30</td>
</tr>
<tr>
<td>AG</td>
<td>921.94</td>
</tr>
</tbody>
</table>

AG is used as a positive control.
recognized largely as beneficial antioxidants and AGEs inhibitors, and there are many reports on structure–activity relationships for the inhibitions of AGEs formation. The inhibitors, and there are many reports on structure–activity relationships for the inhibitions of AGEs formation.35,36) The following structural properties of flavonoids for the inhibition of AGEs formation have been reported: (1) as hydroxyl groups at the 3′-, 4′-, 5-, and 7-positions increased in number, the inhibitory activities became stronger; (2) the activities of flavones were stronger than those of the corresponding flavonols, flavanones, and isoflavones; (3) methylation or glucosylation of the 4′-hydroxyl group of flavonones, flavonols, and flavanones reduced activity; (4) methylation or glucosylation of the 3′-hydroxyl group of flavonols tended to increase activity; and (5) glycosylation of the 7-hydroxyl group of flavonols and isoflavones reduced activity.37)

We found out that hispidin derivatives from *P. linteus* possess strong protein glycation inhibitory activity, and the free radical scavenging activities of hispidin derivatives may contribute, at least in part, to their anti-glycation effects. This represents the possibility of the potential lead compounds for further development as natural drugs for diabetic complications. In this regard, further studies should be focused on the mechanism of action of hispidin derivatives for the development of new natural drugs.

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