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Isolation and Structural Determination of Aldose Reductase Inhibitor from Korean Fermented Soybean Paste

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Abstract Aldose reductase catalyzes the conversion of glucose into sorbitol. Inhibiting this enzyme in diabetes mellitus can delay or prevent pathogenic process. Aldose reductase inhibitor was screened from Korean fermented soybean pastes (Doen-jang) and purified via sequential processes of ethanol extraction, HP-20 column chromatography, ethyl acetate extraction, silica gel column chromatography, and crystallization. Aldose reductase inhibitor was identified as genistein with molecular weight of 270 Da and molecular formula of $C_{15}H_{10}O_5$ based on UV spectrometry, ^1H and ^{13}C NMRs, and mass spectrometry. Genistein inhibited aldose reductase of pig lens with IC_{50} level of 20 μM. Because genistein was effective against aldose reductase of animal source, it may be a potential therapeutic agent for diabetic complications.

Keywords: aldose reductase inhibitor, genistein, fermented soybean paste, diabetes

Introduction

Diabetes mellitus is a group of metabolic disorders with a common manifestation of hyperglycemia. Chronic hyperglycemia causes damage to eyes, kidneys, nerves, heart, and blood vessels. Diabetes mellitus is classified into four types, including type 1 (insulin-dependent diabetes mellitus, IDDM, and juvenile diabetes), type 2 (non-insulin-dependent diabetes mellitus, NIDDM, and adult-onset), “other specific types” (diabetes mellitus with various etiologies), and gestational diabetes mellitus (1, 2). Although the lifespan of diabetic patients can be prolonged by administration of insulin, means of preventing debilitating and late-onset complications associated with this disease have not yet been developed. Diabetic complications occur in many tissues and affect various sensory organs, nervous system, circulation, and renal excretion (3-6). The mechanism through which diabetic complications are generated remains yet unknown. Diabetic complications generally appear in tissues possessing an insulin-independent glucose transport system. The onset and severity of diabetic complications appear to be related to the management of blood glucose levels. There is much evidence that aldose reductase provides a common biochemical link in the pathogenesis of many diabetic complications (7-13).

Aldose reductase converts glucose into sorbitol, which is further processed into fructose. The enzyme has a low affinity for glucose. Under normal conditions metabolic fluxes through the pathway are generally low in most tissues. However, increasing either the glucose concentration in some tissues with diabetes mellitus or the amount of aldose reductase involved in pathogenesis of diabetic complications can alter this trend (14-16).

Several animal studies and preliminary clinical trials indicate that inhibition of aldose reductase can prevent cataracts and renal diseases (15), suggesting that aldose reductase inhibitors are useful for the prevention of certain diabetic complications.

In this study, aldose reductase inhibitors were screened as agents for the prevention of diabetic complications. Aldose reductase of pig lens was chosen after screening of inhibitors. Various sources including plants, animals, mushrooms, microbes, and foods were tested for inhibitory activities against aldose reductase. Ultimately, aldose reductase inhibitor was isolated and purified from fermented soybean paste, and its chemical structure and biological properties were characterized.

Materials and Methods

Reagents and materials Silica gel for column chromatography, silica TLC plate (Kiesel gel 60 F254) and cellulose TLC plate (cellulose F) were purchased from Merck & Co., Inc. (Whitehouse Station, New Jersey, USA). HP-20 resin was a product of Mitsubishi Chemical Co. (Tokyo, Japan). GR grade (Duksan Pharmaceutical Co., Ltd., Seoul, Korea) and HPLC grade (Mallinckrodt Inc., Phillipsburg, New Jersey, USA) organic solvents were used. L-Glyceraldehyde and NADPH were obtained from Sigma-Aldrich Corp. (St. Louis, Missouri, USA). Korean fermented soybean pastes (Doen-jang) manufactured by “P” Food Company were used.

Screening and extraction of aldose reductase inhibitors To screen for an effective aldose reductase

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inhibitor, 34 kinds of plants, animals, mushrooms, microbes, and foods (Table 1) were investigated. Samples were extracted with either methanol or hot water. Ten volumes of 80% methanol was added to the samples, then the mixtures were incubated for 3 hr at room temperature. The extraction was repeated three times. Samples were centrifuged to obtain supernatants, which were concentrated and dried under reduced pressure. After dry extracts were dissolved in methanol, the solutions were diluted with water. The methanol content of the final mixtures was 20 %. Extraction with hot water was performed in the same manner as those used with methanol except that 80°C distilled water was used instead of methanol.

Preparation of aldose reductase Pig lenses were cut into small pieces and homogenized in three volumes of 20 mM sodium phosphate buffer (10 mM 2-mercaptoethanol, 250 mM sucrose, 2 mM EDTA, and 0.1 mM PMSF; pH 7.4). The homogenates were centrifuged at 20,000 x *g* for 20 min at 4°C, and the supernatants were collected. Ammonium sulfate was added to the supernatants in 40% saturation. To the supernatants obtained after centrifugation, ammonium sulfate was again added in 75% saturation. The first and second precipitates were collected, and dissolved in 20 mM sodium phosphate buffer (5 mM 2-mercaptoethanol 0.5 mM EDTA, pH 7.5) to obtain a crude enzyme solution of aldose reductase (17).

Measurement of aldose reductase activity Aldose reductase activity was assayed using a spectrophotometer on a thermomax microplate reader. The activity was estimated by measuring differences in absorbance values of NADPH at 340 nm for 10 min with D, L-glyceraldehyde as a substrate. Enzyme reactions were performed at 37°C in 200 μl of 0.2 M sodium phosphate buffer (pH 7.0) containing 10 mM D, L-glyceraldehyde, 600 μM NADPH, and an enzyme solution (0.198 mg ml^{-1}) (18-20). The effects of inhibitors against aldose reductase were observed by measuring differences in absorbance values of reaction solutions with and without an inhibitor. The concentration of test sample giving 50% inhibition of the enzyme activity (IC_{50}) was estimated from curve-fitting of the logarithmic concentration plotted against the inhibitory activity.

Purification of aldose reductase inhibitors One hundred grams of fermented soybean paste was extracted three times with 1 L of 80% ethanol. After the extracts were centrifuged, the supernatants were concentrated under reduced pressure. The mixtures were suspended in distilled water, and concentrated again under reduced pressure to completely remove the ethanol. Acidity of the ethanol extracts was adjusted to pH 4.0, and the solutions were adsorbed on an HP-20 column and eluted sequentially with isopropyl alcohol (IPA). Active fractions were obtained by elution of 50% IPA, followed by concentration under reduced pressure. After adjusting the acidity of the solutions to 3.0, the solutions were then extracted three times with an equal volume of ethyl acetate. The extracts were dehydrated with sodium sulfate (Na_2SO_4), filtrated through Whatman paper (No. 1), and concentrated under reduced pressure.

The concentrated samples were coated with a silica gel and loaded onto a silica gel column filled with a mixture of *n*-hexane/ethyl acetate (9:1). Stepwise chromatography was performed by eluting the mixed solvents with 9:1 and 6:4 ratios of *n*-hexane/ethyl acetate. Subsequently, the active fraction was obtained by elution with a mixture of *n*-hexane/ethyl acetate (6:4). The solution was concentrated under reduced pressure and purified via crystallization (Fig. 1).

Identification of aldose reductase inhibitor Identification of aldose reductase inhibitor was performed as described previously (21). Purity of the sample was analyzed using reverse-phase high performance liquid chromatography (Sep-Pak C18 column, Waters 510 pump system and Waters™486 UV detector, Waters, Milford, MA, USA). UV-visible Beckman DU series 600 spectrophotometer (Beckman Instruments, Brea, CA, USA) was used to observe the pattern of UV-VIS absorbance by scanning from 190 to 800 nm. Mass Spectrum was analyzed using

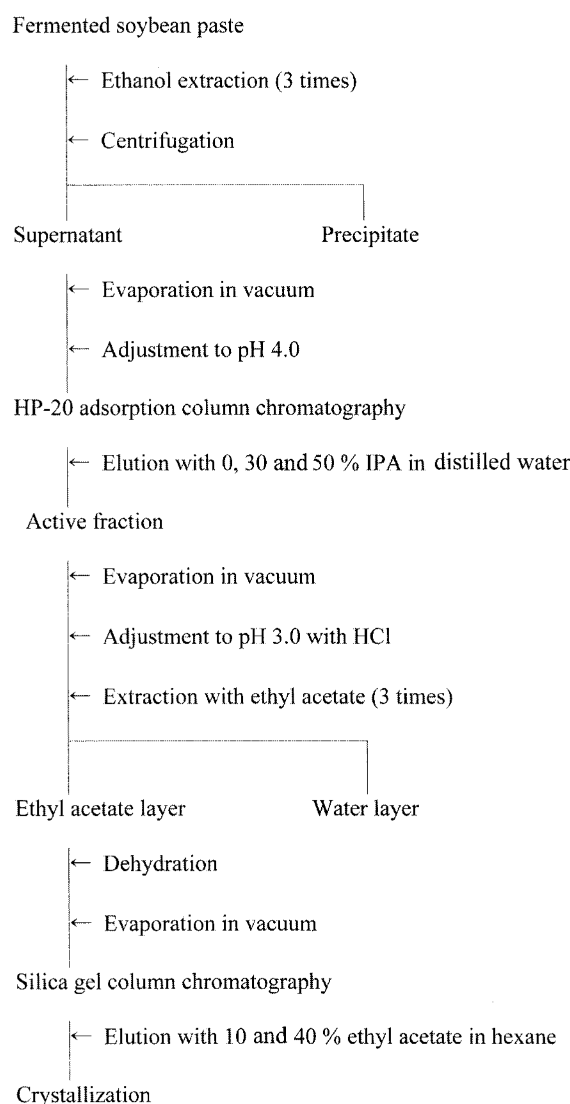

Fig. 1. Purification scheme for an aldose reductase inhibitor from fermented soybean paste.

EI-MS with a JMS-AX505WA mass spectrometer (JEOL Ltd. Tokyo, Japan), and NMR spectra were recorded on a Varian Unity 400 NMR (Varian Inc., Palo Alto, CA, USA) at 399.65 and 100.40 MHz for ^{1}H-NMR and ^{13}C-NMR, respectively.

Results and Discussion

Screening of aldose reductase inhibitors and purification of a selected inhibitor Thirty-four samples from plants, animals, mushrooms, microbes, and foods were extracted with either 80% methanol or hot water, and the inhibitory activities of the extracts against aldose reductase were tested. *Crataegus pinnatifida* (plant), fermented soybean paste, Japanese flowering apricot of maesil, and green tea showed strong inhibitory activities against aldose reductase (Table 1). Because fermented soybean paste showed not only high but also stable aldose reductase inhibitory activity, it was chosen for further experiments.

Table 1. Various samples used for screening of aldose reductase inhibitors

Samples		Inhibition
Plant Sources		
Kuzu vine	*Pueraria thunbergiana*	++
Licorice	*Glycirrhizia glabra*	-
Cinnamon	*Cinnamomi cortex*	-
Tobacco	*Nicotiana tobacum* L.	++
Garlic	*Allium sativum* L. var. *pekinense* MAKINO	++
Crataegus pinnatifida	*Crataegus pinnatifida*	+++
Mulberry leaves	*Mori folium*	++
Ginger	*Zinglber officinale*	+
Coriolus versicolor	*Coriolus versicolor*	+
Turmeric	*Curcuma longa* L.	+
Siberian Ginseng extract	*Acanthopanax seticosus*	+
Gentiana scabra buergeri	*Gentiana scabra buergeri*	-
Ginkgo leaf	*Ginkgo biloba* L.	++
Peony	*Paeonia lactiflora* PALL	+
Korean Angelica	*Angelica gigas*	++
Balkal Skullcap	*Scutellaria baicalens*	++
Animal Sources		
Chondroitin		-
Chondroitin		-
Oyster extract	*Crassostrea gigas*	-
Mushroom Sources		
Reishi mushroom	*Ganderma lucidum*	++
Phelinus linteus	*Phelinus linteus*	+
Coriolus versicolor	*Coriolus versicolor*	+
Lentinus edodes, Shiitake	*Lentinus edodes*	+
Microbes		
Brewer's yeast	*Saccharomyces cerevisae*	-
Glutathion yeast	*Saccharomyces cerevisae*	-
Spirulina	*Arthrospira platensis*	-
Food Materials		
Fermented soybean paste		+++
Tofu		+
Maesil juice	*Prunus mume*	+++
Plum juice	*Prunus salicina*	++
Plum paste	*Prunus salicina*	++
Green tea	*Camellia sinensis*	+++
Oolong tea	*Camellia sinensis*	++
Black tea	*Camellia sinensis*	++

Inhibition degree: -, < 25%; +, 25-50%; ++, 50-70%; +++, > 70%

After the fermented soybean paste was extracted with ethanol, a supernatant was obtained by centrifugation of the mixture and concentrated under reduced pressure to remove ethanol. Crude inhibitor solutions were treated with an HP-20 absorbent resin and silica-gel column chromatography, resulting in the isolation of an aldose reductase inhibitor. The active fraction was further purified via crystallization.

Structural analysis of the aldose reductase inhibitor UV scanning of the purified inhibitor was performed. The maximum absorbance values were obtained at 208 and 258 nm, indicating the presence of a benzene ring. An IR spectrum for the analysis of functional groups was obtained (Fig. 2). A peak corresponding to a hydroxyl group was observed at 3412 cm^{-1}, and those at 1652 and 1500 cm^{-1} corresponded to a carbonyl group and a carbon double bond, respectively. A peak at 1200 cm^{-1} indicated the presence of a C-O bond in an ether group (Table 2). The inhibitor was thought to include an OH group, a C=O group, a C-O group, and an aromatic ring structure.

From ^{1}H-NMR analysis of the inhibitor (Fig. 3), a double resonance was detected in the range of 6.5~7.4 ppm, indicating different proton conditions in an aromatic ring. Resonance in the range of 3.3~4.0 ppm indicated a hydroxy group. From ^{13}C-NMR spectrum data, an aromatic ring was observed in the range of 100~160 ppm, and a resonance in the range of 180~200 ppm was thought to be that of carbons in a ketone group. Approximately 13 carbons with different conditions were thought to exist (Fig. 4). Fast atom bombardment mass spectra analysis (FAB-MS) of the inhibitor showed major peaks of 55, 136,

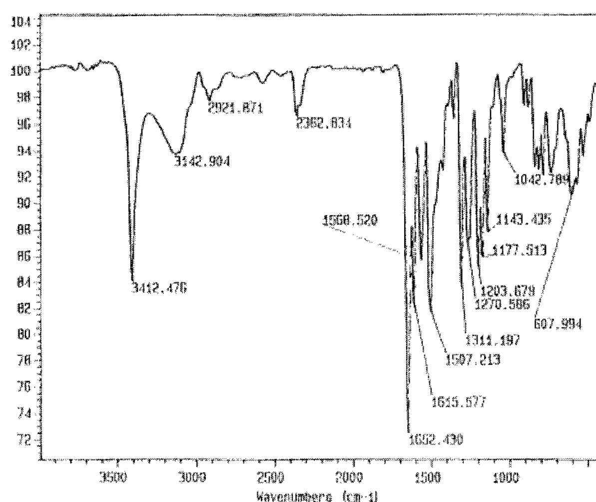

Fig. 2. IR spectrum of the aldose reductase inhibitor isolated from fermented soybean paste.

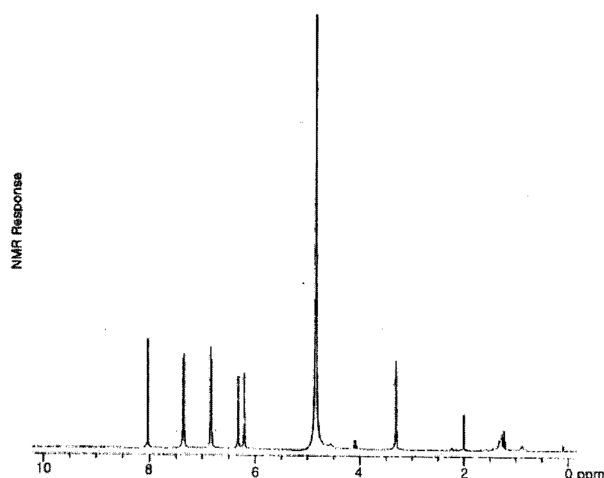

Fig. 3. ^{1}H-NMR spectrum of the aldose reductase inhibitor isolated from fermented soybean paste.

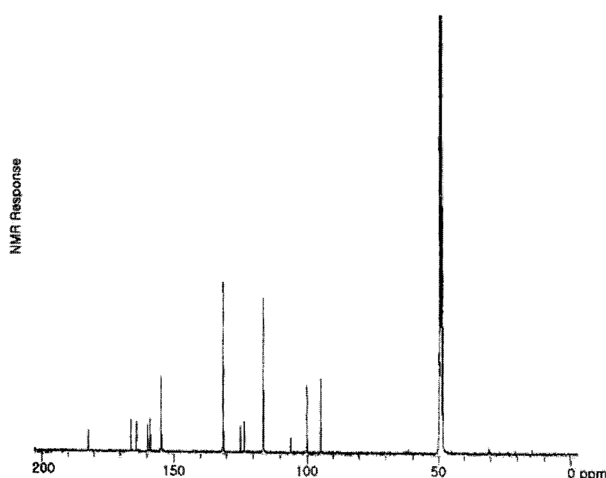

Fig. 4. ^{13}C-NMR spectrum of the aldose reductase inhibitor isolated from fermented soybean paste.

and 273 *m/z* M (Fig. 5). Thus, the molecular weight of the inhibitor was estimated to be 270 Da. Based on these results, the inhibitor was confirmed as genistein with a molecular formula of $C_{15}H_{10}O_5$ (Fig. 6, Table 2).

Table 2. Physico-chemical properties of the aldose reductase inhibitor isolated from fermented soybean paste

Properties	Analysis data
Appearance	Pale yellow powder
Melting point	297-298°C
Molecular formula	$C_{15}H_{10}O_5$
IR ν_{max} (cm^{-1})	3424, 3100, 1658, 1648, 1616
MS (m/z)	270
$UV_{\lambda max}$ MeOH	208, 258 nm
Solubility	
soluble	Methanol>Acetone>Ethyl acetate >Isopropyl alcohol
insoluble	Hexane, H_2O
Temperature stability(~70°C)	stable

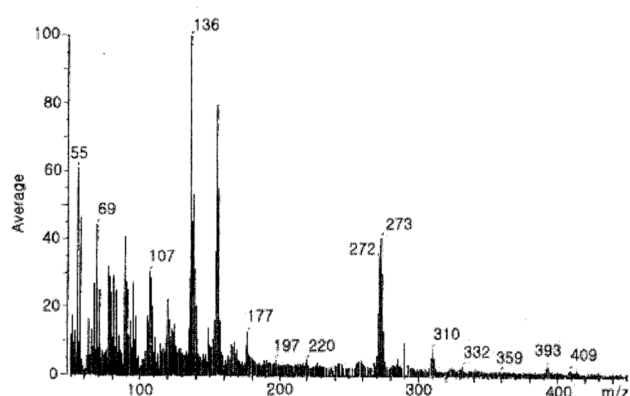

Fig. 5. FAB-Mass spectrum of the aldose reductase inhibitor isolated from fermented soybean paste.

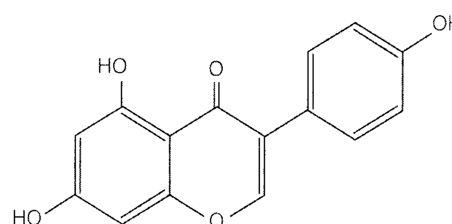

Fig. 6. Chemical structure of the genistein isolated from fermented soybean paste.

Inhibitory activity of genistein against aldose reductase Analysis of the inhibitory effect of genistein against an aldose reductase of pig lens revealed that genistein has an IC_{50} value of 20 μM for the aldose reductase (Fig. 7). Genistein has previously been reported to have anti-cancer, anti-oxidative, and anti-mutagenic activities (22-25). Recently, researchers in functional food areas have reported anti-cancer agents (26-28), protease inhibitors, phytic acid (29, 30), and isoflavone from soybeans (31, 32). Japanese fermented soybean pastes, miso, soy sauces, and shoyu have been reported to have anti-cancer (23, 24), anti-mutagenic (25) and anti-oxidant (26) activities. In addition, Korean soybean paste has been reported to have

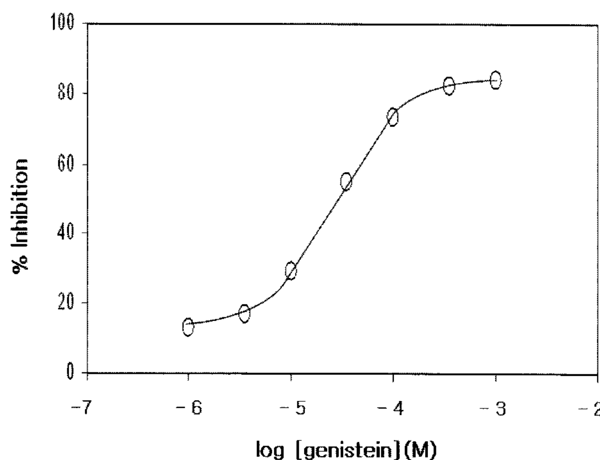

Fig. 7. Effect of genistein concentration on aldose reductase activity.

inhibitory activity against squalene synthase (21). However, the inhibitory activity of genistein against aldose reductase is reported here for the first time.

A variety of aldose reductase inhibitors have been developed for the prevention of diabetic cataract formation (14). Their inhibitory effects have provided evidences for the involvement of aldose reductase in diabetic complications. Tetramethyleneglutaric acid (TMG) was first reported as a compound modifying the cataract formation process via inhibition of aldose reductase (33). Recently, matteuorienates A, B, and C isolated from a rhizome of *Matteuccia orientalis* were reported to have strong inhibitory activities against aldose reductase (34). The IC_{50} values of matteuorienates A, B, and C were 1.0, 1.0, and 2.3 μM, respectively. As natural flavonoid, quercetin showed an inhibitory activity against aldose reductase with an IC_{50} value of 6.6 μM (35).

Genistein is the aglycone of genistin, whose D-glucose moiety is excluded, and genistin is one of the isoflavones existing in soybeans. Genistein has been found in miso, a traditional soy food (36). We believe that the genistein in fermented soy pastes was produced via hydrolysis of the genistin in soybeans during fermentation. The inhibitory activity of genistein against aldose reductase, which was screened by us from fermented soy pastes, is comparable to those of the previously reported inhibitors. Genistein showed an IC_{50} value of 20 μM against the aldose reductase of pig lens. Considering that the aldose reductase used in this study was obtained from an animal source, genistein has potential as a therapeutic agent for diabetic complications. Therefore, because genistein exists in fermented soy pastes, which are used daily as traditional foods by the Koreans and other Oriental peoples, it has a potential for use as a functional-food for the prevention of diabetes.

Acknowledgments

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