Isolation and Identification of Succinic Semialdehyde Dehydrogenase Inhibitory Compound from the Rhizome of *Gastrodia elata* Blume

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In our search for the anticonvulsant constituent of *Gastrodia elata* repeated column chromatographies guided by activity assay led to isolation of an active compound, which was identified as gastrodin on the basis of spectral data. Brain succinic semialdehyde dehydrogenase (SSADH) was inactivated by preincubation with gastrodin in a time-dependent manner and the reaction was monitored by absorption and fluorescence spectroscopic methods. The inactivation followed pseudo-first-order kinetics with the second-rate order constant of 1.2×10^3 $M^{-1}min^{-1}$. The time course of the reaction was significantly affected by the coenzyme NAD^+, which affected complete protection against the loss of the catalytic activity, whereas substrate succinic semialdehyde failed to prevent the inactivation of the enzyme. It is postulated that the gastrodin is able to elevate the neurotransmitter GABA levels in central nervous system by inhibitory action on one of the GABA degradative enzymes, SSADH.

Key words : *Gastrodia elata*, Anticonvulsant, Gastrodin, Brain GABA shunt, Succinic semialdehyde dehydrogenase

INTRODUCTION

GABA (γ-aminobutyric acid) is produced from glutamate in a reaction catalyzed by glutamate decarboxylase (EC 4.1.1.15) and further metabolized to succinate by the successive action of GABA transaminase (EC 1.2.1.24). The carbon skeleton of GABA enters the tricarboxylic acid cycle in the form of succinate. GABA metabolism has been well characterized in the mammalian central nervous system where GABA functions as a major inhibitory neurotransmitter. The observation that inactivation of GABA degradative enzymes, GABA transaminase or succinic semialdehyde dehydrogenase, in brain tissues increase the concentration of neurotransmitter support the fact that these enzymes exert a controlling influence on GABA levels (Fletcher and Fowler, 1980). Moreover the irreversible inhibition of these enzymes by inhibitory compounds is the basic mechanism of action of drugs used in the treatment of convulsive disorders (Lippert *et al.*, 1977). In view of the importance of succinic semialdehyde dehydrogenase in the metabolism of GABA, we have been searching for enzyme inhibitors involved in GABA shunt as anticonvulsant from *Gastrodia elata*, because it has shown anticonvulsant effects (Huang, 1989; Huh *et al.*, 1995).

Gastrodia elata Blume (Orchidaceae) is a perennial parasitic herbaceous plant, and its rhizome has been traditionally used as a tonic, a sedative and an antispasmodic (Soka, 1985). Even though several phenolic compounds have been isolated from *Gastrodia elata* (Lin *et al.*, 1996, Noda *et al.,* 1995, Ruan *et al.*, 1988, Taguchi *et al.*, 1981, Zhou *et al.,* 1980), its antispasmodic principle has not been reported.

In this paper, we will describe the isolation of succinic semialdehyde dehydrogenase inhibitor.

MATERIALS AND METHODS

Materials

Bovine brains were obtained from the Majangdong

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Packing Company in Seoul, Korea. Succinic semialdehyde, NAD^+, NADH were purchased from Sigma Chemical Co. (St. Louis, U.S.A.) CM-Sepharose, Blue-Sepharose, 5'-AMP-Sepharose were purchased from Pharmacia/LKB (Uppsala, Sweden), Ltd. Rhizoma Gastrodiae was purchased at the market in Seoul and identified by Dr. Hyunggyu Lee, Korea Research Institute of Bioscience and Biotechnology, Taejon. The reference specimen has been deposited in the laboratory of Natural Products Chemistry, Kyunghee University, Suwon.

Isolation of active compounds, gastrodin (1) and parishin (2)

The dried powder (1.2 kg) of the drug was extracted with 80% aqueous MeOH (4 L×2) at room temperature overnight and filtered through No. 2 Whatman filter paper. The filtrate was evaporated under reduced pressure to yield MeOH extracts (187 g). The 150 g of the extracts was poured into H_2O (700 mL) and extracted with EtOAc (700 mL×2) and *n*-BuOH (500 mL×2), successively. Each solutions were evaporated under vacuum to afford EtOAc (32 g), *n*-BuOH (86 g) and aqueous (32 g) fractions, respectively.

The *n*-BuOH fraction (30 g) was applied over silica gel (200 g) column eluting with $CHCl_3$-MeOH-H_2O [65:35:10 (lower phase)→6:4:1] to afford 8 fractions monitored by TLC. The fourth fraction was chromatographed on the silica gel column (70 g, $CHCl_3$-MeOH=3:1) to yield pure gastrodin (**1**, 232 mg).

Gastrodin (**1**): White powder (MeOH-H_2O), mp. 154~156°C, ^{1}H-NMR (400 MHz, CD_3OD) 7.31 (2H, d, *J*=8.7 Hz, H-2, 6), 7.10 (2H, d, *J*=8.7 Hz, H-3, 5), 4.97 (1H, d, *J*=7.5 Hz, H-8), 4.55 (2H, s, H-7), 3.93 (1H, br. d, *J*=8.0 Hz, H-13_a), 3.74 (1H, dd, *J*=8.0, 4.8 Hz, H-13_b), 3.58~3.45 (4H, H-9, 10, 11, 12); ^{13}C-NMR (100 MHz, CD_3OD) 157.94 (C-4), 136.44 (C-1), 129.89 (x2, C-2, 6), 117.79 (x2, C-3, 5), 101.94 (C-8), 77.61 (C-10), 77.39 (C-12), 74.57 (C-9), 71.01 (C-11), 64.70 (C-7), 62.17 (C-13).

The aqueous fraction (20 g) obtained previously was subjected to XAD-II (200 g) column chromatography eluting reverse gradiently in solvent polarity (H_2O→H_2O-MeOH→MeOH). The 50% aqueous MeOH eluent was chromatographed on the silica gel column [230 g, $CHCl_3$-MeOH-H_2O=65:35:10 (lower phase) → 6:4:1 →6:5:1] to afford 10 fractions, the sixth one of which was chromatographed on the silica gel column (60 g, $CHCl_3$-MeOH-H_2O=6:4:1) to give parishin (**2**, 3.1 g).

Parishin (**2**): ^{1}H-NMR (400 MHz, CD_3OD) 7.24 (4H, d, *J*=7.8 Hz, H-2, 2'', 6, 6''), 7.16 (2H, d, *J*=8.1 Hz, H-2' 6'), 7.07 (4H, d, *J*=7.8 Hz, H-3, 3'', 5, 5''), 7.05 (2H, d, *J*=8.1 Hz, H-3', 5'), 4.97 (2H, s, H-7'), 4.94 (3H, d, *J*=7.2 Hz, H-8, 8', 8''), 4.92 (4H, s, H-7, 7''), 3.88 (3H, br. d, *J*=11.6 Hz, H-13_a, $13'_a$, $13''_a$), 3.72 (3H, br. d, *J*=9.6, H-13_b, $13'_b$, $13''_b$), 3.45-3.57 (12H, D-glucopyranosyl moieties), 2.94 (2H, d, *J*=15.2 Hz, H-15_a, $15''_a$), 2.78 (2H, d, *J*=15.2 Hz, H-15_b, $15''_b$); ^{13}C-NMR (100 MHz, CD_3OD) 174.54 (C-14'), 171.18 (x2, C-14, 14''), 159.08 (x3, C-1, 1', 1''), 131.21 (x6, C-2, 2', 2'', 6, 6', 6''), 131.13 (x2, C-1, 1''), 130.74 (C-1'), 117.96 (x6, C-3, 3', 3'', 5, 5', 5''), 102.23 (x3, C-8, 8', 8''), 78.00 (x3, C-10, 10', 10''), 77.90 (x3, C-12, 12', 12''), 74.91 (x3, C-9, 9', 9''), 74.90 (C-15'), 71.38 (x3, C-11, 11', 11''), 68.46 (C-7'), 67.54 (x2, C-7, 7''), 62.60 (x3, C-13, 13', 13''), 44.85 (x2, C-15, 15'').

Alkaline hydrolysis of parishin (2)

Parishin (**2**, 30 mg) was dissolved in MeOH (5 ml) containing 10% aqueous KOH (0.5 ml) and stirred at room temperature. The reaction mixture was neutralized with Dowex 50 W×8 (H^+ form) and filtered through filter paper. The filtrate was evaporated and subjected to silica gel column chromatogrphy eluting with $CHCl_3$-MeOH (3:1) to give gastrodin (**1**, 21.5 mg).

Enzyme purification and assay

The purification of bovine brain succinic semialdehyde dehydrogenase was performed by a method previously described (Lee *et al.*, 1995). For precise kinetic data, the formation of NADH was measured by the increase in absorbance at 340 nm. All assays were performed in duplicate and the initial velocity data was correlated with a standard assay mixture containing 10 μM succinic semialdehyde and 5 mM NAD^+ in 0.1 M sodium pyrophosphate (pH 8.4) at 25°C.

Reaction of succinic semialdehyde dehydrogenase with gastrodin

Succinic semialdehyde dehydrogenase was preincubated with gastrodin in 0.05 M potassium phosphate buffer (pH 8.0) at 25°C. Aliquots withdrawn from the incubation mixture were assayed for enzymatic activity in various intervals. Protection experiments were performed in a similar manner except that the enzyme was preincubated with a substrate or coenzyme for 30 min before addition of gastrodin.

Spectroscopy

^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) were recorded on a Bruker ARX 400, and the IR spectrum was measured on Perkin-Elmer 599B. Melting point was taken with Fisher melting point apparatus (uncorrected).

Absorption spectroscopic measurements were carried out in Kontron UVIKON Model 930 double beam spectrophotometer. Fluorescence spectra were recorded in a Kontron SFM 25 spectrofluorometer.

The reaction of the enzyme with phenelzine was

monitored at 340 nm under pseudo-first-order conditions. The observed rate constant K_{obs} was obtained from a plot of log (A_t-A_∞) versus time according to the following equation (1):

$$A_t - A_\infty = (A_0 - A_\infty)_e^{-Kobs \cdot t} \quad (1)$$

where A_t, A_0 and A_∞ are the absorbance values at time t, zero, and ∞, respectively.

RESULTS

Isolation of gastrodin (1) and parishin from (2) *Gastrodia elata* Blume

The MeOH extracts of the crude drug was fractionated with EtOAc, *n*-BuOH and H_2O, successively, according to solvent polarity. Each fraction was bioassayed to show that the activity is concentrated in the *n*-BuOH fraction. By performing activity guided fractionation of the *n*-BuOH fraction, compound **1** was finally isolated.

Compound **1**, obtained as white powder in MeOH-H_2O (mp. 154~156°C), showed the absorbance band at 3480, 1880, 1495 cm^{-1} in the IR spectrum (KBr) indicating the presence of hydrogen-bonded hydroxyl and conjugated double bond. In the ^{1}H-NMR spectrum (400 MHz, CD_3OD) two doublet signals observed at δ 7.31 (2H) and δ 7.10 (2H), which were coupled to each other (*J*=8.7 Hz), led us to postulate the presence of *p*-disubstituted benzene ring. And a hemiacetal signal observed at δ 4.97 (1H, d, *J*=7.5 Hz) and several oxy-methine or oxy-methylene signals observed at δ 4.55-3.45 suggested the compound to be a glycoside. In the ^{13}C-NMR (100 MHz, CD_3OD) data, δ 129.89 (x2, d), δ 117.79 (x2, d) confirmed the *p*-disubstituted benzene structure. From the chemical shifts of two quaternary carbons of benzene, one was revealed to be linked to oxygen (δ 157.94) and the other to carbon (δ 136.44). The sugar moiety of the compound was determined to be D-glucopyranose from the chemical shifts of the carbon signals. Except previously referred carbon signals, an oxy-methylene signal was observed at δ 64.70 indicating the presence of *p*-hydroxybenzyl alcohol. The glycosylation position of the glucose was determined at 4-hydroxy from the chemical shifts of C-1 and C-7 carbon signals, and the anomeric configuration was elucidated to be β from the coupling constant (*J*=7.5 Hz) of the anomeric proton signal in the ^{1}H-NMR spectrum. The above results presented the chemical structure of compound **1** to be 4-β-D-gucopyranosyloxybenzyl alcohol, gastrodin (Taguchi *et al.*, 1981).

Meanwhile, we found a major component on the TLC of aqueous fraction showing similar characteristics to gastrodin including UV absorbance and coloring test. In order to isolate the compound aqueous fraction was applied to XAD-II column successively eluting with H_2O, 50% aqueous MeOH and MeOH. Because aqueous MeOH eluates were revealed to contain the compound, silica gel column chromatogrphy for the eluates was carried out eluting with $CHCl_3$-MeOH-H_2O [65:35:10 (lower phase)→6:4:1→6:5:1], gradiently, for the compound, parishin (**2**), to be purely isolated.

The ^{1}H-NMR spectrum (400 MHz, CD_3OD) of compound **2** showed the following signals due to three *p*-disubstituted benzene ring [δ 7.24 (4H, d), δ 7.16 (2H, d), 7.07 (4H, d), δ 7.05 (2H, d)], three hydroxyl methylene [δ 4.97 (2H, s), δ 4.92 (4H, s)] and three hemiacetal (δ 4.94, 3H, d) to indicate the presence of three gastrodin structures in the molecule. In addition, two allyl methylene protons were observed at δ 2.94 (2H, d) and δ 2.78 (2H, d). In the ^{13}C-NMR spectrum (100 MHz, CD_3OD) of compound **2**, three carbonyl [δ 174.54, δ 171.18 (x2)], one oxygenated quaternary (δ 74.90) and two allyl methylene carbon signals [δ 44.85 (x2)] as well as three gastrodin signals were observed to lead us to postulate the compound to contain one citric acid moiety in the molecule. Accordingly, the chemical structure of compound **2** was determined to be tris[4-(β-*D*-glucopyranosyl)benzyloxy] citrate, which was isolated from *Vanda parishii* and named as parishin (Dahmen and Leander, 1976).

Taguchi *et al.* (1981) isolated parishin from the rhizome of *Gastrodia elata* in acetylated form with low yield, 0.041%, but we isolated it as genuine form with much higher yield, 0.26%. And though they reported the isolation yield of gastrodin to be higher (0.146%) than that of parishin (0.041%), we isolated the parishin with higher yield (0.26%) than gastrodin (0.02%). Parishin will be easily hydrolyzed to produce gastrodin by not only alkaline treatment at room temperature but also hot water treatment. Therefore, parishin or its derivatives are probably hydrolyzed in the stomach or intestines to be absorbed in the form of gastrodin. We confirmed parishin to be hydrolyzed with the diluted solution of KOH (1%) to form gastrodin with high yield (89%).

Inactivation of succinic semialdehyde dehydrogenase by gastrodin

Inactivation of succinic semialdehyde dehydrogenase with increasing concentrations of gastrodin resulted in a progressive decrease in enzyme activity (Fig. 2).

The inactivation followed pseudo-first-order kinetics with gastrodin in the concentration range of 100 μM~400 μM. The pseudo-first-order rate constants obtained at each gastrodin concentration were replotted as a function of gastrodin concentration (Fig. 1, inset). The second-order rate constant for the inactivation of the enzyme by gastrodin was 1.2×10^3 $M^{-1}min^{-1}$ as determined from the slope of this plot. The inactivation

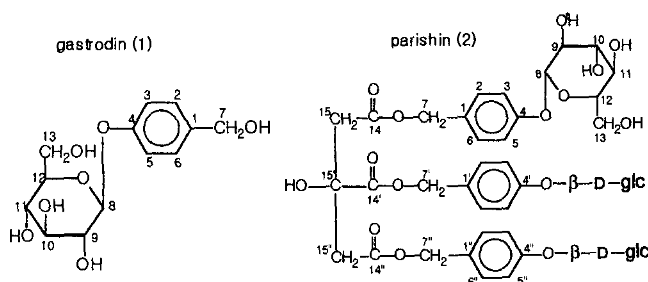

Fig. 1. Chemical structures of gastrodin (**1**) and parishin (**2**).

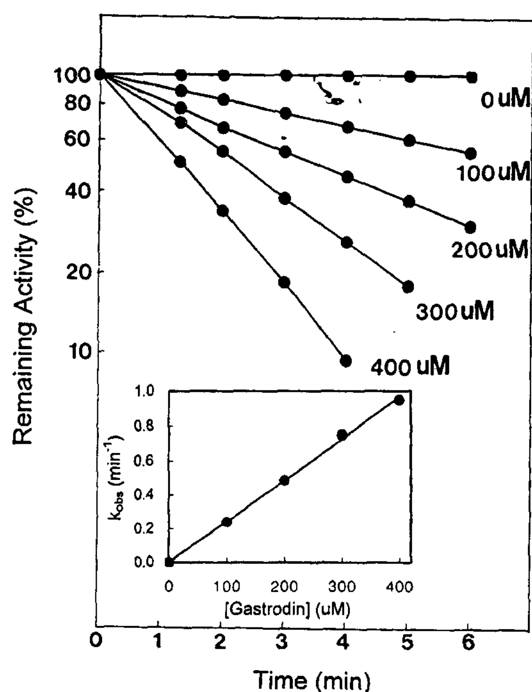

Fig. 2. Determination of the rate constant (Kobs) for the inactivation of succinic semialdehyde dehydrogenase at different concentration of gastrodin. The enzyme (5 μM) was incubated with 100 μM, 200 μM, 300 μM, and 400 μM gastrodin in 0.05 M potassium phosphate buffer, pH 8.0 at 25°C, respectively. Aliquots withdrawn from the incubation mixtures were tested for enzyme activity. The inset shows the dependence of the observed rate constant (Kobs) on gastrodin concentration.

studies were carried out in the presence of substrate or coenzyme NAD^+ to define the sites of gastrodin modification. The reaction of succinic semialdehyde dehydrogenase with gastrodin was completely blocked by incubation of the enzyme with coenzyme NAD^+ but not with substrate (Table I).

Spectroscopic properties

The modified enzyme by gastrodin was not reactivated by dialysis against 0.05 M phosphate buffer(pH 8.0). The absorption spectrum of the dialyzed inactive enzyme exhibited an increase in absorption bands at 215 nm and 280 nm which demonstrated presence

Table 1. Inactivation of succinic semialdehyde dehydrogenase by gastrodin at pH 8.0

Reaction mixture	Remaining Activity (%)
Enzyme (10 μM)	100
Enzyme (10 μM)+gastrodin (400 μM)	15
Enzyme (10 μM)+SSA (20 μM)+ gastrodin (400 μM)	24
Enzyme (10 μM)+NAD+(500 μM)+ gastrodin (400 μM)	98

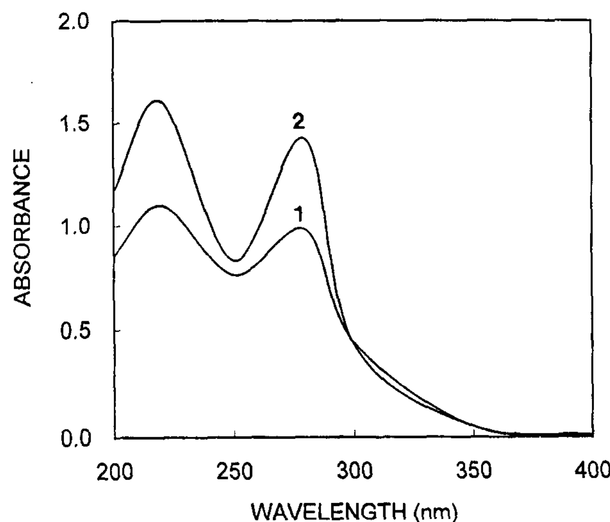

Fig. 3. Absorption spectra of native and modified succinic semialdehyde dehydrogenase with gastrodin. The enzyme (10 μM) was incubated without (**1**) or with 400 μM gastrodin (**2**) in 0.05 M potassium phosphate buffer, pH 8.0, 25°C. The reaction mixture was dialyzed against the same buffer.

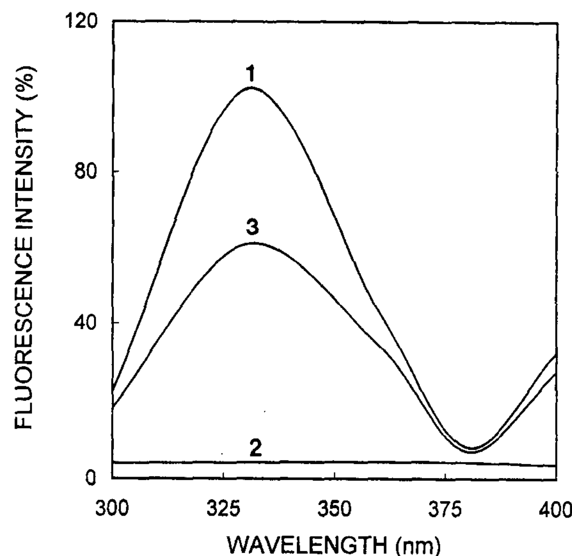

Fig. 4. Fluorescence emission spectra of native and modified succinic semialdehyde dehydrogenase. The enzyme (2 μM) was incubated without (**1**) or with 200 μM gastrodin (**2**) in 0.05 M potassium phosphate buffer, pH 8.0, 25°C. (**3**): gastrodin only (Excitation wavelength: 280 nm).

of the indole chromophore of tryptophan residue. A pronounced effect was observed in the emission spectrum of intrinsic fluorescence, characterized by a large decrease in intensity at 330~340 nm range.

DISCUSSION

Since abnormal low levels of neurotransmitter GABA in brain has been associated with a variety of neurological disorders including epilepsy, seizure and convulsant disorder, a specific inhibitory compound of GABA degradative enzymes (GABA transaminase or succinic semialdehyde dehydrogenase) would be useful in attempts to elevate GABA levels in certain pathological conditions.

Recently, we have screened the effect of MeOH extracts obtained from 40 plants traditionally used as anticonvulsant drugs. Among several plants, the crude extracts of rhizome of *Gastrodia elata* Blume showed inhibitory effect against brain succinic semialdehyde dehydrogenase, not on GABA transaminase. The rhizome of *Gastrodia elata* has been known to have sedative, antispasmodic and anticonvulsant effect (Soka, 1985; Huang 1989, Huh *et al.,* 1995). Therefore we started to isolate the pure active compounds which has inhibitory effect on the GABA degradative enzyme, succinic semialdehyde dehydrogenase, by fractionation and chromatographic procedures. As shown in Fig. 1, we identified pure compounds gastrodin and parishin which have been known as major biological components in *Gastrodia elata.*

Succinic semialdehyde dehydrogenase was irreversibly inactivated by gastrodin, not by parishin, in a pseudo-first order process. Replots of kinetic data yielded a second order rate constant of 1.2×10^3 $M^{-1}min^{-1}$ for the inactivation process at 25°C and pH 8.0.

Interaction of the enzyme with gastrodin was monitored by absorption and emission spectrophotometric methods. Dialyzed inactivated enzyme modified by gastrodin showed an increase in absorption bands at 215 and 280 nm which indicates that gastrodin might react with certain amino acid residues of the active site in the enzyme. The fluorescence intensity was quenched without any change in the emission maximum, indicating that the modification by gastrodin didn't cause an extensive alteration in the structure of succinic semialdehyde dehydrogenase. Complete protection from inactivation was afforded by the coenzyme NAD^+ (Table II). Those lines of results strongly suggest that inactivation occurred due to interaction with tryptophan or cysteine residues located at or near coenzyme NAD^+ binding site. In marked contrast to NAD^+, substrate succinic semialdehyde did not afford any protection against the inactivation by gastrodin.

According to the inhibitory effect of gastrodin against succinic semialdehyde dehydrogenase, we can assume that the inhibition of this enzyme by gastrodin is important in the elevation of neurotransmitter GABA levels in CNS. In summary, we isolated and identified the pure biological compound gastrodin from *Gastrodia elata* and present article is the first report which showed the inhibition of the succinic semialdehyde dehydrogenase by gastrodin. Further studies to elucidate the elevation of GABA levels by this compound in brain and *in vivo* experimental studies may provide insights into approaches for the clinical application of gastrodin as an effective anticonvulsant and antiepileptic therapeutic drug.

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