Arch Pharm Res Vol 25, No 5, 643-646, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Phytol, SSADH Inhibitory Diterpenoid of *Lactuca sativa*

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(Received May 30, 2002)

The succinic semialdehyde dehydrogenase (SSADH) inhibitory component was isolated from the EtOAc fraction of *Lactuca sativa* through repeated column chromatography; then, it was identified as phytol, a diterpenoid, based on the interpretation of several spectral data. Incubation of SSADH with the phytol results in a time-dependent loss of enzymatic activity, suggesting that enzyme modification is irreversible. The inactivation followed pseudo-first-order kinetics with the second-rate order constant of 6.15×10^{-2} $mM^{-1}min^{-1}$. Complete protection from inactivation was afforded by the coenzyme NAD^+, whereas substrate succinic semialdehyde failed to prevent the inactivation of the enzyme; therefore, it seems likely that phytol covalently binds at or near the active site of the enzyme. It is postulated that the phytol is able to elevate the neurotransmitter GABA levels in central nervous system through its inhibitory action on one of the GABA degradative enzymes, SSADH.

Key words: *Lactuca sativa,* Anticonvulsant, SSADH inhibition, Phytol

INTRODUCTION

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system, where it is released at up to 40% of all synapses (Olsen & Avoli, 1997). The metabolism of GABA in brain is carried out by three enzymes. The Glutamate decarboxylase (GAD; EC 4.1.1.15) catalyzes the conversion of glutamate to GABA. Subsequently, GABA degradation is achieved in a two-step reaction, GABA is transaminated by GABA-transaminase (GABA-T; EC 2.6.1.19) to succinic semialdehyde (SSA), which is then converted to succinate by the enzyme succinic semialdehyde dehydrogenase (SSADH; EC 1.2.1.24) Succinate thus enters the tricarboxylic acid cycle.

Impairment of the GABA function is widely recognized to provoke seizures, whereas facilitation of GABA function has an anticonvulsant effect (Löscher, 1999). Thus, enhancement of the brain GABA level by activating GAD or inhibiting GABA degradation is the basic action mechanism of drugs used in the treatment of convulsive disorders. An antiepileptic drug Vigabatrin, an ethyl analogue of GABA, exerts its pharmacological effects through the inhibition of GABA degradation process (Jung *et al.*, 1977).

Lactuca sativa (Chrysanthemum) is a biannual herb widely cultured in Korea, not only as a vegetable source but for medical use. The aerial parts and seed are known to have sleeping-inducing effect or remedy effect for several urination troubles (Soka, 1985). Some flavonoid glycosides of quercetin or luteolin together with lactucins (Khalil *et al.*, 1991), the latter of which were known to be sleeping-inducing components, were isolated from this plant; however, the principal component of the plant manifesting antispasmodic activity has not yet been reported so far.

In this study, the isolation of an anticonvulsant compound from the aerial parts of *Lactuca sativa* and the evaluation of its inhibitory effects on SSADH were carried out.

MATERIALS AND METHODS

Materials

The *Lactuca sativa* was purchased at Suwon Agriculture-Fishery Market. NAD^+, SSA were purchased from Sigma Chemical Co. (St. Louis, U.S.A). Gastrodin was isolated from the rhizome of *Gastrodia elata* Blume (Baek *et al.*, 1999).

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Instruments

^{1}H-(400 MHz) and ^{13}C-NMR (100 MHz) spectra were measured with Unity-INOVA (Varian, U.S.A.) spectrometer. EI mass spectra were taken on a JEOL JMS-AX505WA spectrometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. IR spectra were taken with a Perkin-Elmer 599B spectrometer. Absorption spec-troscopic measurements were carried out in Kontron UVIKON Model 930 double beam spectrophotometer.

Enzyme purification and assay

The purification of bovine brain SSADH was performed by a method previously described (Lee *et al.*, 1995). For precise kinetic data, the formation of NADH was measured by following the increase in absorbance at 340 nm at which NADH is known to have a molar absorption coefficient of $6.22 \times 10^3 M^{-1}cm^{-1}$. All assays were performed in duplicate and the initial velocity data was correlated with a standard assay mixture containing 0.1 mM succinic semialdehyde and 5 mM NAD^+ in 0.1 M sodium pyrophosphate (pH 8.4) at 25°C. Protein concentration was determined by the Bradford method with bovine serum albumin as a standard (Bradford, 1976).

Isolation of SSADH inhibitory component, phytol, from *Lactuca sativa*

The aerial parts of *Lactuca sativa* (20 kg, raw materials) was cut into pieces and extracted with 100% MeOH (72 L) and 80% aqueous MeOH solution (25 L) at room tem-perature. The filtration of the extracted solution and the evaporation under reduced pressure yielded methanolic extracts. The extracts were dissolved in water (1 L) and extracted with EtOAc (2 L) and *n*-BuOH (1.6 L), successively, to afford a EtOAc fraction (56 g) and *n*-BuOH fraction (35.7 g), respectively.

The EtOAc fraction (56 g) was subjected to silica gel column chromatography (750 g), which was eluted stepwise-gradiently with $CHCl_3$-MeOH (10:1→7:1→5:1→3:1) to be divided into eight subfractions (LSE-1~LSE-8). The second fraction (LSE-2, 16.6 g) was successively applied to silica gel column chromatography (250 g, *n*-hexane:EtOAc = 5:1→3:1→$CHCl_3$-MeOH=10:1) to yield eleven subfractions (LSE-2-1~LSE-2-11). The 100 mg of the fourth fraction (LSE-2-4, 4.2 g) was applied to ODS (50 g) column chromatography (acetone-MeOH-H_2O=3:7:1→9:3:1) to afford a purified compound **1** (81 mg).

Compound 1 (phytol) : colorless oil; $[\alpha]_D$ = +0.2° (c=1.2, $CHCl_3$); EI/MS *m/z*: 296 (M^+), 278, 263, 196, 182, 126, 123, 71, 57; IR ($CHCl_3$), 3334, 2954, 2923, 2868, 1669 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$, δ), 5.40 (1H, dq, *J*=6.8, 1.4 Hz, H-2), 4.14 (2H, d, *J*=6.8 Hz, H-1), 1.99 (2H, t, *J*=7.0 Hz, H-4), 1.66 (3H, br. s, H-20), 1.00-1.66 (methine&me-thylene), 0.87 (6H, d, *J*=6.3 Hz, H-16, 17), 0.85 (3H, d, *J*=6.1 Hz, H-18), 0.84(3H, d, *J*=6.6 Hz, H-19); ^{13}C-NMR (100MHz, $CDCl_3$, δc) 140.14 (C-3), 123.11 (C-2), 59.34 (C-1), 39.85 (C-4), 39.34 (C-5), 37.40 (C-9), 37.34 (C-6), 37.26 (C-8), 36.65 (C-10), 32.76 (C-11), 32.66 (C-7), 27.95 (C-15), 25.12 (C-12), 24.77 (C-13), 24.45 (C-14), 22.69 (C-19), 22.59 (C-20), 19.72 (C-18), 19.68 (C-16), 16.13 (C-17).

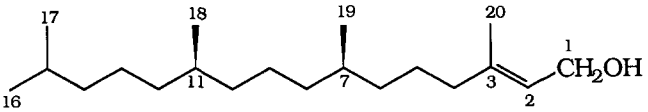

Fig. 1. Chemical structures of phytol isolated from *Latuca sativa*

Reaction of SSADH with phytol

SSADH was preincubated with sample in 0.05M 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 the addition of phytol.

RESULTS AND DISCUSSION

Isolation of phytol from *Lactuca sativa.*

Since abnormally 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-T, SSADH or SSAR) would be useful in attempts to elevate GABA levels in certain pathological conditions.

The MeOH extracts of *Lactuca sativa* showed inhibitory effect on a SSADH. Among EtOAc, *n*-BuOH and water soluble fractions obtained from MeOH extracts of *Lactuca sativa* through solvent fractionation, the EtOAc fraction exhibited the inhibitory activity (Table I). Therefore, activity-guided fractionation of EtOAc fraction through silica gel and ODS column chromatography was carried out to finally afford a SSADH inhibitory component (Table I), compound 1, by the yield of 0.017 %.

Compound **1**, colorless oil, showed the absorbance bands due to the hydroxyl (3334 cm^{-1}) and olefine (1669 cm^{-1}) in the IR spectrum ($CHCl_3$) and molecular ion peak (M^+) at *m/z* 296. In the ^{1}H-NMR spectrum (400 MHz, $CDCl_3$), an olefinic methine (δ5.40, tq), an oxygenated methylene (δ4.14, d), and an allyl methylene (δ1.99) signals were observed. Also in the high magnet field region, an allyl singlet methyl (δ1.66) and four doublet methyls (δ0.84, 0.85, 0.87 (x2)) including several methine or methylene signals were observed indicating compound

Table I. The Effect of fractions obtained from *Lactuca sativa* on SSADH activity

Fr.*	LSM	LSE	LSB	LSW							
Activity**	51	42	92	108							
Fr.	LSE1	LSE2	LSE3	LSE4	LSE5	LSE6	LSE7	LSE8			Gastrodin***
Activity	69	38	58	112	135	97	89	113			31
Fr.	LSE2-1	LSE2-2	LSE2-3	LSE2-4	LSE2-5	LSE2-6	LSE2-7	LSE2-8	LSE2-9	LSE2-10	LSE2-11
Activity	104	87	66	27	46	89	88	102	92	84	91

*LSM : MeOH extracts, LSE : EtOAc fr., LSB : BuOH fr., LSW : water fr.
SSADH (10uM) in 0.1M sodium pyrophosphate (pH 8.4) at 25°C was inactivated with 40 μg of each fractions and 10 μg of gastrodin*. Remaining activity (%) was determined after 15 min of incubation.

1 to be an aliphatic alcohol with one double bond. In the ^{13}C-NMR spectrum (100MHz, $CDCl_3$) twenty signals consisting of one olefinic quaternary(δc 140.14) and methine (δc 123.11), one oxygenated methylene (δc 59.34), five methyls (δc 22.69, 22.59, 19.72, 19.68, 16.13), nine methylene and three methine signals were observed. These results led to the conclusion for compound **1** to be a non-cyclic aliphatic diterpenoid. Compound **1** was finally identified as phytol through the comparison of several physical and spectral data with those of literatures (Singh, 1991). Phytol, an aliphatic acyclic diterpenoid, is usually found as a component part of chlorophyll. The very rare plants have been reported to contain the phytol itself so far.

In this study, lactucins, known as the principal component manifesting sleeping-inducing effect of *Lactuca sativa*, were not detected in the fractions exhibiting SSADH inhibitory activity. Therefore, though the inhibitory activity of phytol (**1**), which was first isolated from *Lactuca sativa*, on SSADH was relatively low in comparison to gastrodin (Table I) from the rhizome of *Gastrodia elata* (Baek *et al.*, 1999), it may be usable as an effective anticonvulsant and antiepileptic therapeutic drug.

Time-dependent inactivation of SSADH by phytol

Incubation of SSADH with varying concentrations of phytol resulted in a time-dependent loss of enzyme activity. Plots of the logarithm of remaining activity versus time at different sample concentrations, indicated in each case pseudo first-order kinetics (Fig. 2). A double reciprocal plot yielded a straight line that did not pass through the origin (inset, Fig. 2), suggesting that a reversible SSADH-phytol complex was present before the formation of an irreversible complex. Such an inhibition scheme can be illustrated in the following equation:

$$E + I \underset{}{\overset{K_d}{\rightleftharpoons}} EI \xrightarrow{K_{inact}} E\text{-}I' \qquad \text{(Eq. 1)}$$

where E is the enzyme, I is the inhibitor, k_d is the dissociation constant of the reversible enzyme-inhibitor complex EI, and K_{inact} is the rate constant of the formation of the irreversibly inactivated complex E-I. Inhibition following typical saturation kinetics has been analyzed by Kitz and Wilson (1962). The apparent first-order rate constant for activity loss by phytol, k_{app}, is given in the folmaining activity) against pre-incubation time.

$$K_{app} = \frac{[I] \cdot K_{inact}}{[I] + K_d} \qquad \text{(Eq. 2)}$$

The values of k_d and K_{inact} determined from a linear plot of $1/k_{app}$ against 1/[I] were 1.63 mM and $1.67 \times 10^{-3} s^{-1}$, respectively (Fig. 2, inset). The second-order rate constant for the overall inhibition, k_i, as calculated from the equation $k_i = k_{inact}/k_d$, was $6.15 \times 10^{-2} mM^{-1} min^{-1}$ (Table II).

Time-dependency of the inhibitory process could be used to show that the type of inhibition is irreversible. To

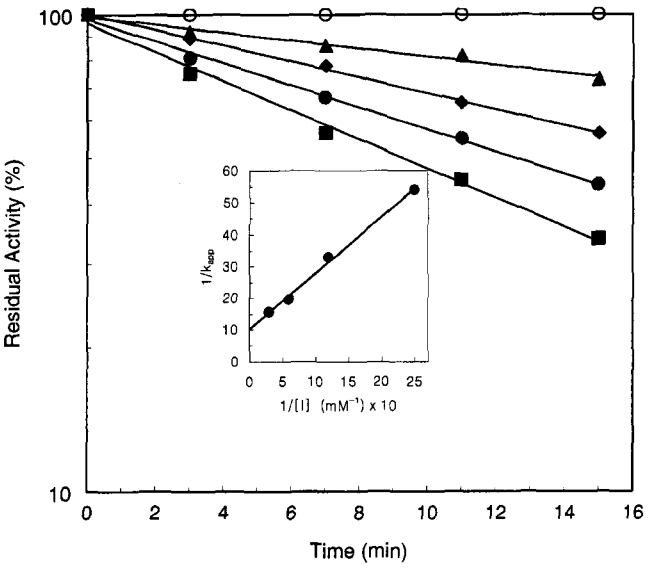

Fig. 2. *Time-dependent Inactivation of SSADH by phytol.*
SSADH (10 μM) in 50 mM sodium pyrophosphate buffer, pH 8.4 at 25°C, was incubated with no (○), 0.4 (▲), 0.8 (□), 1.6 (●), and 3.2mM (■) of phytol. At given time intervals, aliquots withdrawn from the incubation mixture were tested for residual enzyme activity. All values are the means of three enzyme samples. The line is the best fit by regression analysis performed by the software Microsoft Excel. Inset, double-reciprocal plot of $1/k_{app}$ *versus* 1/[I] allowing the determination of k_d and k_{inact}.

Table II. Kinetic parameters for irreversible inhibition of SSADH by phytol.

k_d	K_{inact}	k_i
mM	sec^{-1}	$mM^{-1}min^{-1}$
1.63	1.67×10^{-3}	6.15×10^{-2}

k_d is the dissociation constant for the initial reversible complex, and K_{inact} is the pseudo first-order rate constant for the conversion of the reversible complex to the irreversibly inhibited enzyme. The values for k_d and K_{inact} were determined by nonlinear regression of Equation 2 to the data presented in Fig. 1. The bimolecular reaction constant k_i was determined as K_{inact} / k_d.

Table III. Protection of SSADH by substrates during inactivation with phytol

Reaction mixture	Remaining Activity (%)
SSADH	100
SSADH + phytol	32
SSADH / SSA + phytol	38
SSADH / NAD^+ + phytol	98

SSADH (10uM) in 0.1M sodium pyrophosphate (pH 8.4) at 25°C was inactivated with 3.2 mM of phytol. Remaining activity was determined after 15 min of incubation. Protections were achieved by pre-incubating 5 mM NAD^+ and 1 mM of succinic semialdehyde for 20 min before the start of the inactivation by phytol.

further evaluate the irreversibility of the interaction between the enzyme and inhibitor, the ability to recover catalytic activity by dilution was examined. The inhibitory effect by a reversible inhibitor would be reduced in parallel to the degree of dilution, whereas inhibition by an irreversible inactivator would remain unchanged. SSADH was pre-incubated for 30min at 25°C with 20 μg of phytol and then diluted by up to 20-fold prior to the measurement of enzyme activity. The inhibitory effect was not significantly changed by dilution (data not shown). Taken together, these observations indicate that phytol acts as an irreversible inactivator of SSADH.

In order to assess that the inactivation of the sample is active-site directed, the ability of substrates to protect the enzyme active site from inactivation was tested. The residual activity was dramatically increased when the enzyme was pre-incubated with coenzyme NAD+, whereas only little or no protecting effect was observed with substrate SSA (Table III). This result clearly shows that the binding site of phytol is more likely to be located at or near the coenzyme binding site of the enzyme, rather than at the substrate SSA binding site.

ACKNOWLEDGEMENTS

This study was supported by the Brain Neuroscience Research Grant (M1-0108-00-0019) from the Ministry of Science and Technology and in part from the Korea Science and Engineering Foundation (2000-1-21300-001-2), and a research grant from Hallym University (2000).

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