Sedative-Hypnotic and Receptor Binding Studies of Fermented Marine Organisms

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Abstract

This study was performed to investigate the sedative-hypnotic activity of γ-aminobutyric acid (GABA)-enriched fermented marine organisms (FMO), including sea tangle (FST) and oyster (FO) by Lactobacillus brevis BJ20 (L. brevis BJ20). FST and FO were tested for their binding activity of the GABA_A-benzodiazepine and 5-HT_2C receptors, which are well-known molecular targets for sleep aids. We also measured the sleep latency and sleep duration during pentobarbital-induced sleep in mice after oral administration of FST and FO. In GABA_A and 5-HT_2C receptor binding assays, FST displayed an effective concentration-dependent binding affinity to GABA_A receptor, similar to the binding affinity to 5-HT_2C receptor. FO exhibited higher affinity to 5-HT_2C receptor, compared with the GABA_A receptor. The oral administration of FST and FO produced a dose-dependent decrease in sleep latency and increase in sleep duration in pentobarbital-induced hypnosis. The data demonstrate that FST and FO possess sedative-hypnotic activity possibly by modulating GABA_A and 5-HT_2C receptors. We propose that FST and FO might be effective agents for treatment of insomnia.

Key Words: Fermented marine organisms, GABA_A receptor, 5-HT_2C receptor, Pentobarbital-induced sleep, Sedative-hypnotic activity

INTRODUCTION

Insomnia is one of most prevalent sleep disorders and approximately more than a third of adults populations suffer from chronic insomnia and poor sleep. These estimates are even higher among adults with coexisting medical or psychiatric illness (Doghramji, 2006). Traditionally, sedative-hypnotic drugs such as γ-aminobutyric acid (GABA)-A-benzodiazepine (BZD) receptor or serotonin (5-HT) receptor agents, have been prescribed to treat sleep disturbances, but certain drugs in this class have limited benefits due to side effects such as impairments of cognitive function and memory, changes in appetite, diarrhea, daytime drowsiness, headache or unusual dreams (Attele et al., 2000; Roth et al., 2001; Borja and Daniel, 2006). Therefore, natural sleep aids with sedative-hypnotic effects are being increasingly sought after by the general population as an alternative to prescription drugs to improve sleep quality and avoids side effects. Many studies on the sedative-hypnotic efficacy of terrestrial plants have been performed. Several marine plants among them are known to have biological activities, but the use of these products has been restricted as a potential source of natural sleep drugs.

The molecular targets of medicinal plants producing sedative-hypnotic activity have more recently focused on regulation of GABAergic and serotonergic neurotransmission in the central nervous system (CNS), which is involved in stress and sleep regulation (Attele et al., 2000; Abourashed et al., 2004). GABA, the major inhibitory neurotransmitter in the CNS, functions to maintain a balance between neuronal excitation and inhibition (Johnston, 2005). GABAergic neurotransmission plays a key role in sleep regulation, and the BZD binding site on the GABA receptor is a target for most sedative-hypnotic agents (Johnston, 2005). BZD agents potentiate the ability of GABA to cause membrane hyperpolarization by allowing a Cl-influx. As a result, the inhibition of neurotransmission is achieved, and subsequently these agents produce sedative-
Preparation of fermented marine organisms

Fermented marine organisms were prepared as described by Kang et al. (2012). For the production of GABA-enriched preparations, FMO were added to water at a ratio of 1:15 (w/v) followed by 3% yeast extract and 1% glucose, each based on the amount of yeast extract (control), sea tangle and oyster, to aid in the fermentation process. After autoclaving at 121°C for 30 min, each mixture received 5% w/v of a culture of *L. brevis* (Korean Collection for Type Culture accession number 11377BP). The suspension was thoroughly mixed and incubated at 37°C.

The GABA content in the culture broth was measured as follows. A 1 ml sample was taken and diluted 20-fold using 0.02N HCl. The dilution solution (2 ml) was filtered through a 0.2 μm membrane filter (dissmic-25cs, Toyo Roshi Kaisha, Tokyo, Japan). The GABA and glutamate in the filtered sample (20 μl) were measured using Amino acid analyser (AAA L-8900, Hitachi High-Technologied Co., Tokyo, Japan) with a flow rate of 0.3 ml/min.

**Animals**

The male Spraque-Dawley rats, weighting 180-200 g, were used for the receptor binding assay. The male ICR mice were used, weighting 18-22 g each, for the pentobarbital-induced sleep test. All animals were purchased from Orient Animal Corp (Kyungki-do, Republic of Korea), and were group-housed under a reversed 12:12 h light-dark cycle (light on from 08:00-20:00 h). The animals had free access to food and water at a room temperature that was controlled from 20-25°C. All animals were handled daily for at least 7 days prior to the experiment. All animal care and testing conditions were in accordance with the IACUC (Institutional Animal Care and Use Committee) in College of Korean Medicine, the Kyung Hee University.

**[3H]-flumazenil binding assay**

The GABAA-BZD receptor affinity test was carried out as described by Cho et al. (2010). Briefly, the cerebral cortex of each rat was homogenized in 20 volumes of Tris-citrate buffer (50 mM, pH 7.1) and centrifuged at 27,000×g for 15 min. The pellets were washed three times with 20 volumes of Tris-citrate buffer using the same centrifugation conditions. The final washed pellet was resuspended in 20 volumes of Tris-HCl buffer (50 mM, pH 7.4) and incubated at 37°C for 30 min to remove endogenous GABA, followed by centrifugation at 27,000×g for 10 min. The pellet was resuspended in Tris-citrate buffer (500 ml binding buffer per gram of original tissue) and used for the binding assay. Membrane suspension (180 μl) was added to 10 μl of the test solution (0.001-20 mg/ml) and 10 μl of 1 nM (final concentration) of [3H]-flumazenil (Ro 15-1788; Perkin Elmer Life and Analytical Sciences Waltham, MA, USA) in a 96-well plate, mixed, and incubated at 4°C for 40 min. The binding reaction was terminated by filtration onto Whatman GF/C glass fiber filter with ice cold Tris-HCl buffer. The filters were dried for 30 min and suspended in Wallac Microbeta plate scintillation fluid. The amount of radioactivity on the filters was determined using a Wallac 1450 Microbeta liquid scintillation counter (Perkin Elmer Life and Analytical Sciences). Specific binding was calculated as total binding minus non-specific binding, which were determined using binding buffer and clonazepam (1mM, final concentration), respectively.

**[3H]-mesulergine binding assay**

5-HT2C receptor binding assay was performed expressing human 5-HT2C receptor membrane (Perkin Elmer Life and Analytical Sciences). Membrane diluted in 50 mM Tris-HCl (pH 7, 4 mM CaCl2, 0.1% ascorbic acid) at a concentration of 4 μg/ml. Membrane suspension (180 μl) was added to 10 μl of a test solution (0.001-20 mg/ml) and 10 μl of 1 nM (final concentration) of [3H]-mesulergine (Perkin Elmer Life and Analytical Sciences, Waltham, MA, USA) in 96-well plate, mixed, and incubated at 27°C for 60 min. The binding reaction was termi-
nated by filtration onto Whatman GF/C glass fiber filter with ice cold Tris-HCl buffer (50 mM, pH 7.4). The filters were dried for 30 min and suspended in Wallac Microbeta plate scintillation fluid. The amount of radioactivity on the filters was determined as described above. Specific binding was calculated as described above, except using final concentrations of 100 μM mainserin.

**Pentobarbital-induced sleep test in mice**

The pentobarbital-induced sleep test was carried out according to the modified method described by Ma et al. (2009).

All experiments were performed between 1:00 and 5:00 pm and mice were fasted for 24 h prior to the experiment. For oral administration, FO or FST or diazepam (DZP, 2 mg/kg), as positive control were suspended in 0.5% (w/v) carboxymethylcellulose (CMC)-saline. FO or FST (200 and 400 mg/kg) or diazepam (DZP, 2 mg/kg) were administered orally to animals, 30 min before pentobarbital injection. Following the intraperitoneal injection (i.p.) of pentobarbital (hypnotic dosage, 40 mg/kg), each mouse was observed for measurement of sleep latency and sleeping time. The mice lost the righting reflex over 3min were considered to be asleep. The sleep latency was recorded from the pentobarbital injection to the sleep onset and sleeping time was defined as the difference of time between loss and recovery of the righting reflex.

**Statistical analyses**

In the binding assay, displacement binding curves were fitted to a one-site competition binding model. The displacement (%) of radio-ligand binding was determined as $\left[1 - \frac{\text{DPM}_{\text{sample}} - \text{DPM}_{\text{NSB}}}{\text{DPM}_{\text{TB}} - \text{DPM}_{\text{NSB}}} \right] \times 100$. In the pentobarbital-induced sleep experiments, all data were analyzed using a one-way analysis of variance (ANOVA) for multiple comparisons where necessary as means ± S.E.M. Statistical differences among the groups were further analyzed using the Tukey’s post hoc method. The level of significance was set to $p<0.05$.

**Fig. 1.** Changes in GABA and glutamic acid in FO (A) and FST (B) solution during fermentation with *L. brevis*.

**Table 1.** Free amino acid contents of fermented sea tangle solutions with *L. brevis*

| Amino acids                  | Fermented sea tangle solution | Fermented oyster solution |
|------------------------------|-------------------------------|---------------------------|
|                              | With *L. brevis* (0 days later) mg/l sample | With *L. brevis* (5 days later) mg/l sample | With *L. brevis* (0 days later) mg/l sample | With *L. brevis* (5 days later) mg/l sample |
| Phosphoserine                | 24.6                          | 33.0                      | 9.88                        | 147.06                        |
| Taurine                      | 14.4                          | 26.3                      | 3785.20                     | 3785.93                        |
| Phosphoethanolamine          | 8.09                          | 7.65                      | 5.59                        | 0.00                          |
| Aspartic acid                | 1834                          | 1285                      | 1612.93                     | 2048.35                        |
| Threonine                    | 1.43                          | 4.37                      | 14.66                       | 81.14                          |
| Serine                       | 32.9                          | 1.67                      | 27.73                       | 181.18                        |
| Glutamic acid                | 2651                          | 0.00                      | 8200.50                     | 0.00                          |
| Glycine                      | 7.86                          | 30.2                      | 9.66                        | 688.07                        |
| Alanine                      | 198                           | 814                       | 0.00                        | 0.00                          |
| DL-2-Aminobutric acid        | 5.31                          | 33.3                      | 0.00                        | 10.66                         |
| Valine                       | 4.79                          | 44.8                      | 12.77                       | 4.21                          |
| Phenylalanine                | 0.00                          | 26.7                      | 5.21                        | 76.07                         |
| β-Alanine                    | 2.26                          | 10.1                      | 1.04                        | 133.54                        |
| Aminoisobutyric acid         | 1.15                          | 4.55                      |                             |                               |
| β-Aminobutyric acid (GABA)   | 0.00                          | 2465                      | 0.00                        | 4845.43                       |
| Aminooethanol                | 14.9                          | 14.2                      | 8.58                        | 15.65                         |
| Ornithine                    | 2.84                          | 2.80                      | 4.57                        | 1.38                          |
| Lysine                       | 0.00                          | 9.62                      | 5.45                        | 51.96                         |
| Histidine                    | 0.00                          | 0.00                      | 0.98                        | 99.38                         |
| Arginine                     | 2.40                          | 12.4                      | 6.44                        | 5.28                          |
| Proline                      | 116                           | 85.3                      | 88.57                       | 0.00                          |
RESULTS

The changes of GABA and glutamate in fermented marine organisms during fermentation by L. brevis

Fig. 1 shows the changes of GABA and glutamic acid in the sea tangle and oyster solution during fermentation with L. brevis. The glutamic acid contents in sea tangle and oyster solution were 2,651 and 8,200 mg/L, respectively before fermentation. However, its concentrations were dramatically decreased during fermentation. GABA was not detected in the intact sea tangle and oyster solution. However, GABA dramatically increased during fermentation with L. brevis. The concentrations were 2,465 and 4,845.43 mg/L after 5 days of fermentation, respectively. These results indicate that the glutamic acid in the sea tangle and oyster solution was completely converted to GABA by L. brevis.

The free amino acid contents of the fermented sea tangle and oyster solution with L. brevis were shown in Table 1. After fermentation of sea tangle, most of the glutamic acid was converted to GABA, and some levels of amino acids such as aspartic acid, serine, and glycine were decreased. The contents of alanine, valine, glycine, phenylalaline and lysine dramatically were increased after fermentation of sea tangle. Furthermore, glutamic acid of oyster has been converted to GABA and phosphoserine, aspartic acid, threonine, serine and glycine after fermentation.

Binding affinity of fermented marine organisms to GABA_A and 5-HT_2C receptor

Table 2 and 3 presents the % displacement of [3H] flumazenil (GABA_A receptor agonist) and [3H] mesulergine (5-HT_2C specific agonist) binding with unlabeled FMO obtained with seven concentrations. FST showed moderately dose-dependent activity to GABA_A receptor, which displaced over 50% of [3H] flumazenil binding at a concentration of 20 mg/ml. In addition, FO had a weak bonding activity for GABA_A receptor. The IC50 values of FO and FST were 2.25 ± 1.1 and 1.05 ± 1.15 mg/ml, respectively as seen in Fig. 2. In the 5-HT_2C receptor binding assay, FO displayed over 60% of [3H] mesulergine binding at a concentration of 20 mg/ml. FST showed moderately dose-dependent activity in 5-HT_2C binding assay similar to its affinity of GABA_A recep-

| Table 2. | [3H] flumazenil binding of fermented marine organisms to the GABA_A-BZD receptor |
|----------|---------------------------------------------------------------------------------------------------|
| Species  | Displacement (%) of [3H] flumazenil binding at different plant extract concentration (mg/ml) |
|          | 0.001 | 0.01 | 0.1 | 1 | 2.5 | 5 | 10 | 20 |
| Control  | 5.6 ± 1.4 | 4.1 ± 2.6 | 1.6 ± 2.2 | 5.3 ± 0.8 | -1.9 ± 4.3 | 5.0 ± 1.0 | 2.8 ± 3.9 | -2.2 ± 5.0 |
| FO       | -12.9 ± 2.2 | -11.7 ± 0.9 | -12.6 ± 0.5 | -10.9 ± 1.4 | 6.3 ± 2.4 | 18.4 ± 1.2 | 31.5 ± 2.3 | 33.9 ± 0.9 |
| FST      | -10.7 ± 3.3 | -4.7 ± 2.0 | 0.3 ± 2.2 | 4.5 ± 2.3 | 24.1 ± 1.1 | 41.3 ± 2.4 | 45.9 ± 1.8 | 51.4 ± 3.3 |

| Table 3. | [3H]-mesulergine binding of fermented marine organisms to the 5-HT_2C receptor |
|----------|---------------------------------------------------------------------------------------------------|
| Group    | Displacement (%) of [3H] mesulergine binding at different plant extract concentration (mg/ml) |
|          | 0.001 | 0.01 | 0.1 | 1 | 2.5 | 5 | 10 | 20 |
| Control  | 11.8 ± 4.1 | 0.5 ± 7.4 | -1.3 ± 7.1 | 15.2 ± 6.8 | 5.3 ± 10.2 | 17.3 ± 1.2 | -6.3 ± 2.5 | 3.9 ± 2.7 |
| FO       | -0.8 ± 7.4 | 4.2 ± 9.9 | 15.5 ± 2.8 | 17.9 ± 3.2 | 45.1 ± 3.9 | 58.3 ± 3.4 | 61.9 ± 0.8 | 62.2 ± 0.6 |
| FST      | 20.5 ± 3.9 | 22.3 ± 1.7 | 24.4 ± 1.4 | 29.6 ± 2.5 | 51.9 ± 4.9 | 53.0 ± 1.5 | 58.2 ± 2.1 | 59.1 ± 1.3 |

Fig. 2. Dose-response curves and IC50 values of FO (A) and FST (B) in the GABA_A-BZD receptor binding assay. Each data point is expressed as mean ± SD (n=3).
tor, which displaced over 50% of [3H]mesulergine binding at a concentration of 20 mg/ml. The IC50 values for FO and FST were determined to be 1.34 ± 1.11 and 1.56 ± 0.26, respectively as seen in Fig. 3.

Effects of fermented marine organisms on pentobarbital-induced sleep in mice

The sedative-hypnotic effects of FMO were tested by measuring the sleep onset and sleep duration time pentobarbital-induced sleep after oral administration of FMO in mice. ANOVA analysis of pentobarbital test revealed that sleep onset and sleep duration time were significantly different across all groups (F5,59=15.394, p<0.001; F5,59=5.459, p<0.01, respectively). The oral administration of FST and FO produced a dose-dependent decrease in sleep latency and increase in sleep duration in mice with hypnotic dose (40 mg/kg) of pentobarbital as seen in Fig. 4. The most pronounced hypnotic activity of FO was observed at 400 mg/kg in the sleep onset time (p<0.05) (Fig. 4A) and sleep duration time (p<0.01) (Fig. 4B), compared to the control group. FST significantly decreased sleep onset time (p<0.05) (Fig. 4A) and increased sleep duration time (p<0.05) (Fig. 4B) at a dose of 400 mg/ml compared with the control mice. However, FST and FO at a low dose (200 mg/kg, p.o) had no significant hypnotic effects on the sleep onset time. As a positive control, DZP (2 mg/kg, p.o) significantly potentiated the pentobarbital-induced sleep behaviors, compared with control group (p<0.001 for sleep onset time, p<0.05 for sleep duration time).

DISCUSSION

The results of the current study demonstrate the sedative-hypnotic activity of gamma-amino butyric acid (GABA)-enriched fermented marine organisms (FMO), including sea tangle (FST) and oyster (FO) by Lactobacillus brevis BJ20 (L. brevis). It was shown that glutamic acid of marine organisms was converted to GABA after fermentation by L. brevis. In GABAA and 5-HT2C receptor binding assays, FST displayed an effective concentration-dependent binding affinity to GABAA receptor, similar to the binding affinity to 5-HT2C receptor. FO exhibited higher affinity to 5-HT2C receptor, compared with the GABAA receptor. The oral administration of FST and FO produced a dose-dependent decrease in sleep latency and increase in sleep duration in pentobarbital-induced hypnosis. Our data suggest that FST and FO possess sedative-hypnotic activity possibly by modulating GABAA and 5-HT2C receptors.

In the 5-HT2C receptor binding assay, FO showed a moderate concentration-dependent binding activity with an IC50 value of 1.34 mg/ml, whereas FO had a weak binding affinity to GABA-A receptor with an IC50 value of 2.25 mg/ml. In addition, FST had similar binding activity to GABA-A and 5-HT2C receptors.
ceptor with an IC50 value of 1.05 and 1.56 mg/ml, respectively. This result indicates that FMO contains at least two natural ligands which bind to the GABA_A-BZD and 5-HT2C receptors and is more sensitive to GABA_A receptor than 5-HT2C receptor. The results are in agreement with the reports that FMO contains active compounds such as GABA sedative-hypnotic effects mediated by potentiation of GABA receptors (Johnston, 2005).

Although the results of analytical and binding assay indicated the possibility that fermented marine organisms including FST and FO contain natural ligands that bind to the GABA_A-BZD and 5-HT2C receptors, it is difficult to ascertain blood-brain barrier (BBB) penetration of these FMO. Thus, we confirmed the sedative-hypnotic effects of fermented marine organisms through a pentobarbital-induced sleep in mice. The pentobarbital-induced sleep test can be a useful tool for examining the inhibitory effects on the CNS, especially for investigating influences on GABAergic system (Ma et al., 2009, Cho et al., 2010). It has been demonstrated that many hypnotic and anti-anxiety drugs prolong the pentobarbital-induced sleep time (Silva et al., 2007, Ma et al., 2009).

GABA-enriched foods have recently been implicated as effective and safe bioactive substances. Several studies have reported that marine organisms are useful as a functional food source that includes essential bioactive compounds, such as protein, essential fatty acids, vitamins and minerals, neuroactive compounds of catecholamine (Athukorala et al., 2006, Lee et al., 2010). Ginseng or ginseng or ginsenosides isolated from ginseng extract have shown to have high affinity to GABA_A (Kimura et al., 1994) and 5-HT2C receptors (Cho et al., 2010) and also have reported to increase non-rapid eye movement sleep (Lee et al., 2012). Therefore, it has been suggested that GABA-enriched foods may have beneficial effects on sleep behaviors. We tested this hypothesis and found that administration of FST and FO produced a dose-dependent decrease in sleep latency and increase in sleep duration with hypnotic dose of pentobarbital. FO and FST produced a marked hypnotic activity at 400 mg/kg in the sleep onset time and sleep duration time, compared to the control group. The pentobarbital-induced sleep behaviors were significantly potentiated by DZP (2 mg/kg, p.o), which is commonly prescribed to treat psychiatric diseases including anxiety and insomnia. DZP increase action of GABA by binding to the benzodiazepine site on the GABA_A receptor, producing behavioral effects. In the present study, marine organisms are more effective in potentiating sleep behaviors than a positive control DZP. These differential effects may be explained by the fact that DZP only increases effects of GABA by binding to the benzodiazepine site on the GABA_A receptor, whereas FST and FO bind to both GABA_A-BZD and 5-HT2C receptors, and stimulate synergistically 5-HT and GABAergic systems in the brain.

The present study demonstrated that FST and FO have the hypnotic-sedative effects on sleep-related behaviors, showing that fermented marine organisms lead to decrease sleep latency and increase sleep duration. These results suggest potency of fermented marine organisms as a sleep aid and prove that these effects are closely associated GABAergic and serotonergic systems and may be mediated through these transmitter systems. It is certain that these mixed serotonergic and GABAergic mechanisms may be highly implicated in modulating sedative effects of FMO. This suggestion is strengthen by the fact that most of the 5-HT2C receptor-immunoreactive cells are localized in GABAergic neurons in the brain areas (Liu et al., 2007). Thus it is possible that FMO activates the 5-HT2C receptors expressed by GABAergic cells in sleep-related brain areas and may produce decrease in sleep latency and increase in sleep duration.

In summary, our results clearly revealed that most of glutamic acid in sea tangle and oyster solution were converted to GABA during fermentation with L. brevis. The results of the current experiments indicate that GABA-enriched FO and FST display high affinity to GABA_A and 5-HT2C receptors, and produce hypnotic-sedative activity in pentobarbital-treated mice. These results suggest that FO and FST are effective in improving sleep and that serotonergic and GABAergic mechanisms may be highly implicated in modulating sedative effects of FMO. Further investigation is needed of other derivatives and isolation of the active compounds of fermented marine organisms that cause the hypnotic-sedative activity including their pharmacological mechanisms.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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