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Hydrolysate Preparation with High Content of 5-Hydroxytryptophan from Liquid Egg Protein and Its Sleep-Potentiating Activity

Jung Il Kwon¹, Yooheon Park², Sung Hee Han*, and Hyung Joo Suh*

BK21Plus, College of Health Science, Korea University, Seoul 02841, Korea

¹Human Milk R&D Team, Maeil Dairies Co. Ltd.

²Research Institute of Biotechnology, Dongguk University, Goyang 10326, Korea



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*Corresponding authors

Sung Hee Han

BK21Plus, College of Health Science,
Korea University, Seoul 02841, Korea
Tel: +82-2-3290-5639
Fax: +82-2-921-7207
E-mail: sungheeh3@gmail.com

Hyung Joo Suh

BK21Plus, College of Health Science,
Korea University, Seoul 02841, Korea
Tel: +82-2-3290-5639
Fax: +82-2-921-7207
E-mail: suh1960@korea.ac.kr

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Abstract

Alcalase hydrolysis of liquid egg white was used to produce 5-hydroxytryptophan (HTP) under various conditions and investigate the sleep-potentiating activity of liquid egg white hydrolysate (LEH) on pentobarbital-induced sleep. Alcalase hydrolysis yielded the highest content of 5-HTP (13.50 µg/mL), while neutrase hydrolysis showed the lowest 5-HTP content (5.23 µg/mL). The liquid egg white to water ratio (1:1) was optimal for the production of 5-HTP with high amino-nitrogen (A-N) content and degree of hydrolysis. The 5-HTP, amino-nitrogen, and degree of hydrolysis increased until 24 h of hydrolysis and slightly increased thereafter during hydrolysis with 2% and 5% enzyme addition. 5-HTP administration at doses of 6 and 9 mg/kg significantly increased sleep duration and decreased sleep latency time compared to that in the control ($p < 0.05$). LEH (150 mg/mouse), which was equivalent to 5-HTP at 6 mg/kg, significantly decreased sleep latency time and increased sleep duration time compared to that in the control ($p < 0.05$). Oral administration of LEH showed sleep-potentiating effects because of 5-HTP. The sleep-potentiating activity of LEH may have occurred through 5-HTP in our pentobarbital-induced sleep model. LEH may be a valuable alternative to sleep enhancement and may be used as a sleep-potentiating agent.

Keywords 5-hydroxytryptophan, liquid egg white, alcalase, hydrolysate, sleep-potentiating activity

Introduction

5-Hydroxytryptophan (5-HTP) is synthesized from the amino acid tryptophan. The body absorbs tryptophan, converts it to 5-HTP, and then generates serotonin, both centrally nervous and peripherally nervous system (Goldman-Rakic and Brown, 1981). Both tryptophan and 5-HTP can penetrate the blood-brain barrier.

5-HTP is a natural amino acid and biosynthetic intermediate of the neurotransmitter serotonin. The body absorbs tryptophan, converts it to 5-HTP, and then converts 5-HTP to serotonin (Haleem, 2017). 5-HTP functions as a neurotransmitter in dopamine and noradrenergic neurons (Haleem, 2017) and can alleviate a state of awakening (Ursin, 1976); thus, 5-HTP has a sleep-enhancing effect (Ursin, 2002). Insomnia induced by *p*-chlorophenylalanine was restored by 5-HTP, which can restore sleep by accelerating serotonin synthesis (Zhao *et al.*, 2004). This *p*-chloro-

rophenylalanine insomnia model demonstrates the importance of 5-HTP and serotonin in sleep regulation (Smith and Kennedy, 2003). However, the clinical use of hypnotics often has many side effects, such as drug dependence, tolerance, rebound insomnia, and other side effects. Over the past several decades, there has been an increased interest in the use of complementary and alternative medicine for insomnia to avoid these side effects (Sarris and Byrne, 2011). Natural sleep alternative supplements are increasingly used worldwide because of their safety and efficacy (Gyllenhaal *et al.*, 2000). Various natural supplements, including *Valeriana officinalis*, *Humulus lupulus*, and 5-HTP, are readily available (Yang *et al.*, 2017). In addition, many researchers are interested in the sleep-promoting effects of natural supplements and their ingredients.

When the nutritional value and safety of protein foods or new protein components are established, their functional and biological properties are also examined (Richardson, 1977). The biological activity of enzyme hydrolysates has been widely investigated. The functional, nutritional, and biological properties of proteins can be modified by enzymatic hydrolysis (Yu *et al.*, 2007), which can produce various small peptides and free amino acids (Quist *et al.*, 2009). The biological activity of protein hydrolysates is related to a variety of factors, including protein sources and enzymes (Keoghane *et al.*, 1985).

Therefore, enzymatic hydrolysis of egg protein to produce value-added hydrolysates is very attractive for improving insomnia. The goal of this study was to develop a material containing a high content of 5-HTP using liquid egg white for possible applications of this neurotransmitter in sleep disorder therapy. To increase the 5-HTP content in liquid egg white, the liquid egg white hydrolysate (LEH) obtained from enzymatic hydrolysis was subjected to various treatments. We established enzymatic hydrolysis conditions to increase 5-HTP content based on evaluation of the 5-HTP and amino-nitrogen contents and degree of hydrolysis (DH) of the hydrolysate. The sleep-promoting effect of LEH with a high content of 5-HTP was also evaluated in a pentobarbital-induced sleep model.

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Materials and Methods

Chemicals and enzymes

L-Leucine, 2,4,6-trinitrobenzenesulphonic acid (TNBS) and ficin were purchased from Sigma-Aldrich (USA). Alcalase, neutrase, flavourzyme, and protamex were purchased from Novozymes (Denmark). Collupulin was purchased from DSM Corp. (Netherlands). Table 1 shows the characteristics of the enzymes used to prepare the hydrolysate.

Preparation of liquid egg white hydrolysate (LEH)

Egg white solution (30 mL of liquid egg white in 70 mL of distilled water) was pre-incubated at 45°C for 20 min before enzyme hydrolysis. Hydrolysis was carried out at pH 6.0 for 12 h, and the enzyme reaction conditions are shown in Table 1. After hydrolysis, the enzyme was inactivated by heat treatment at 90°C for 15 min. The hydrolysate was centrifuged at 2,800×g for 20 min in a refrigerated centrifuge (Beckman model J2-21, Beckman Coulter, Inc., USA) and the supernatant was lyophilized (TFD, Ilshin, Korea).

Quantification of amino-nitrogen (A-N) and degree of hydrolysis (DH)

A-N content was measured as described by Benjakul and Morrissey (1997). After adding 2.0 mL of 0.2125 M phosphate buffer (pH 8.2) and 1.0 mL of 0.01% TNBS solution to the diluted hydrolysate (125 µL), the solution was mixed thoroughly and incubated in a dark room for 30 min in a 50°C water bath (Model W350, Memmert, Germany). The reaction was terminated by the addition of 2.0 mL of 0.1 M sodium sulfite. After cooling the mixture at 22±0.5°C for 15 min, the absorbance was measured at 420 nm and the A-N content was expressed as L-leucine. The DH was calculated as follows (Benjakul and

Table 1. Enzyme characteristics

Enzyme	Source	Optimum conditions		Type
		Temperature (°C)	pH	
Alcalase	<i>Bacillus sp.</i>	50-60	8.0-9.0	Endo
Neutrase	<i>B. amyloliquefaciens</i>	45	6.0-7.0	Endo
Protamex	<i>Bacillus sp.</i>	35-60	5.5-7.5	Complex
Flavourzyme	<i>Aspergillus sp.</i>	45-50	5.0-7.0	Complex
Collupulin	<i>Caruca papaya</i>	50-70	5.0-7.5	Endo
Ficin	<i>Ficus carica</i>	45-55	5.0-6.0	Endo

Morrissey, 1997):

$$DH = [(L_t - L_o)/(L_{max} - L_o)] \times 100$$

where L_t is the amount of A-N released at time t , L_o is the A-N content in the original liquid egg white, And L_{max} is the total A-N content in the original liquid egg white obtained after acid hydrolysis at 100°C for 24 h with 6 M HCl.

Pentobarbital-induced sleep test

Animal experiments were conducted with the approval of Korea University Animal Care Committee (KUACUC-20160824-1). Animals used in the experiment (4-wk-old ICR mice) were obtained from Central Lab Animal, Inc. (Korea). Rodents freely consumed food and water, and the animal breeding room was maintained at 24°C, 50-60% atmospheric humidity, and a 12-h light/dark cycle. Mice were tested for pentobarbital-induced sleep after 5 d of adaptation.

Before the pentobarbital-induced sleep experiment, mice were fasted for 24 h and all samples were dissolved in physiological saline. Sleep latency and duration were assessed following oral administration of 5-HTP (3, 6, and 9 mg/kg) or LEH (The amount of 5-HTP equivalent to 6 mg/kg). The mice were injected with pentobarbital (42 mg/kg) 40 min after sample administration. Mice were individually housed, and sleep latency and duration were measured as described by Yang *et al.* (2013). Mice that did not sleep for 15 min after pentobarbital-injection were excluded from the experiment.

Statistical analysis

All data were analyzed by using the Statistical Package for Social Sciences version 12.0 (SPSS Inc., USA). The results are presented as the mean±standard deviation. Differences between groups were evaluated by one-way analysis of variance and Duncan's multiple range tests. Statistic values of $p < 0.05$ were considered significant.

Results and Discussion

Enzymatic hydrolysis of liquid egg white

The aim of this study was to optimize the conditions to increase 5-HTP content by hydrolyzing liquid egg white. Enzymatic hydrolysis was monitored by measuring 5-HTP and A-N contents and DH. Alcalase hydrolysis yielded the highest content of 5-HTP (13.50 µg/mL), while neutrase

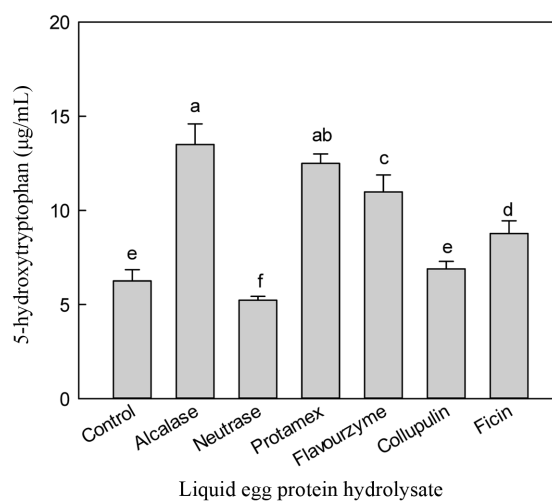


Fig. 1. The 5-hydroxytryptophan content of LEH prepared with various proteases. Thirty milliliters of egg white liquid was mixed with 70 mL of deionized water. The solution was pre-incubated at 45°C for 20 min prior to enzymatic hydrolysis with various proteases. Hydrolysis was conducted at pH 6.0 for 12 h. Error bars represent the standard deviation of triplicate measurements. Different letters indicate significant differences with the same protease ($p < 0.05$).

treatment showed the lowest 5-HTP content (5.23 µg/mL) (Fig. 1). While non-enzymatic hydrolysis (control) showed a low level of 5-HTP (6.25 µg/mL), treatment with protamex, flavourzyme, collupulin, and ficin gave 5-HTP contents from 6.89 to 12.5 µg/mL.

Alcalase hydrolysis yielded the highest A-N (15.27 mg/mL) content, while the control showed the lowest A-N (1.53 mg/mL) content (Fig. 2). Hydrolysis using neutrase, collupulin, papain, and ficin resulted in A-N contents of 1.72-7.86 mg/mL. Unlike the results for liquid egg white hydrolysis, the neutrase hydrolysate of egg white powder showed the highest A-N content (Cho *et al.*, 2014). During the production of egg white powder, egg white proteins undergo thermal, physical, interfacial, and chemical treatments that can cause functional damage (Mine, 1995). Lechevalier *et al.* (2005) reported that heat treatment affected the tertiary structure of proteins, such as protein folding or aggregation. Therefore, liquid egg white and powder showed differences in the specificity of proteolytic cleavage.

DH is the most widely used indicator of proteolytic degradation in hydrolysis processes. Therefore, DH was measured to estimate the degradation extent of liquid egg white. DH values of hydrolysates were 0-43.2% after 24 h hydrolysis (Fig. 2). Alcalase showed the highest DH value

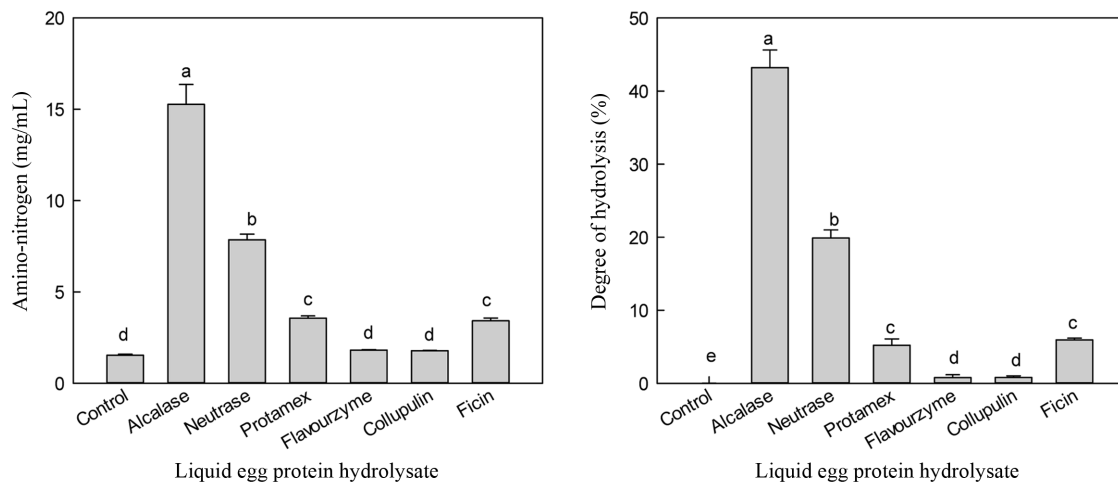


Fig. 2. Amino-nitrogen and degree of hydrolysis (DH) of egg white liquid prepared by various proteases at 1:50 (w/w) enzyme/substrate and 1:2 (V/V) liquid egg/water. Hydrolysis was carried out at 50°C for 24 h. $DH = [(L_t - L_o)/(L_{max} - L_o)] \times 100$, where L_t is the amount of α -amino acid released at time t , L_o is the amount of α -amino acid in the original egg white liquid, and L_{max} is the total α -amino acid in the original egg white liquid obtained after acid hydrolysis (6 M HCl at 100°C for 24 h). Error bars represent the standard deviation of triplicate measurements. Different letters indicate significant differences with the same protease ($p < 0.05$).

(43.2%), which was more efficient in hydrolyzing liquid egg white hydrolysis compared to the other enzymes tested.

Alcalase showed a high degree of DH, which may be because alcalase contains an endo-protease that enhances the hydrolysis of liquid egg white. For enzymes showing a high DH, to the likelihood of obtaining egg white proteins composed of amino acids or short peptides is also high. Therefore, alcalase is ideal for commercial application because of its low cost and high recovery of A-N. Based on our results, alcalase is suitable for the production of LEH containing high 5-HTP content.

Changes in 5-HTP, A-N, and DH of LEH with various ratios of egg white liquid to water

Enzyme specificity, enzymatic hydrolysis conditions, and substrate concentration used in proteolysis greatly affect the molecular weight and amino acid sequence of the hydrolysates and their biological activities (van der Ven *et al.*, 2002). Taking into account the economic considerations in protein recovery from liquid egg white, various substrate concentrations were compared to determine the most suitable concentration for substrate hydrolysis.

Fig. 3 shows the changes in 5-HTP according at various substrate concentrations. A substrate to water ratio of 1:2 showed higher production of 5-HTP compared to other ratios such as 2:1. However, there were no significant differences in 5-HTP content between the 1:2 and 1:1 ratios of egg-white and water.

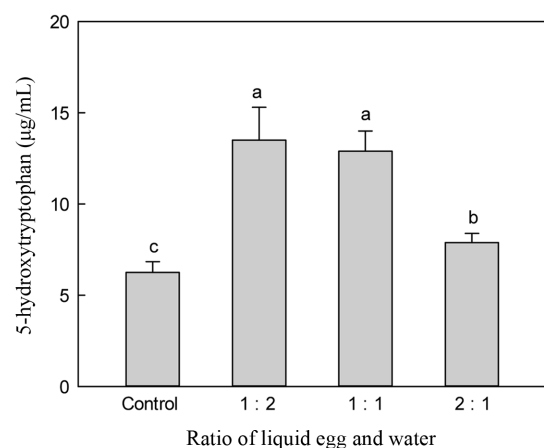


Fig. 3. 5-hydroxytryptophan content of ELH with various ratios of egg liquid to water. Hydrolysis was carried out at 50°C for 24 h. Error bars represent the standard deviation of triplicate measurements. Different letters indicate significant differences with the same protease ($p < 0.05$).

Fig. 4 shows the changes in A-N content and DH at various substrate concentrations. A substrate to water ratio of 1:1 showed the highest A-N content among other ratios, such as an egg white liquid to water ratio of 1:2 and 2:1. The 1:2 ratio was not significantly different from the A-N content obtained using a 1:1 ratio. Increasing the water addition to liquid egg white increased DH. However, the costs of manufacturing the desired bioactive peptide are an important consideration. To minimize the energy cost

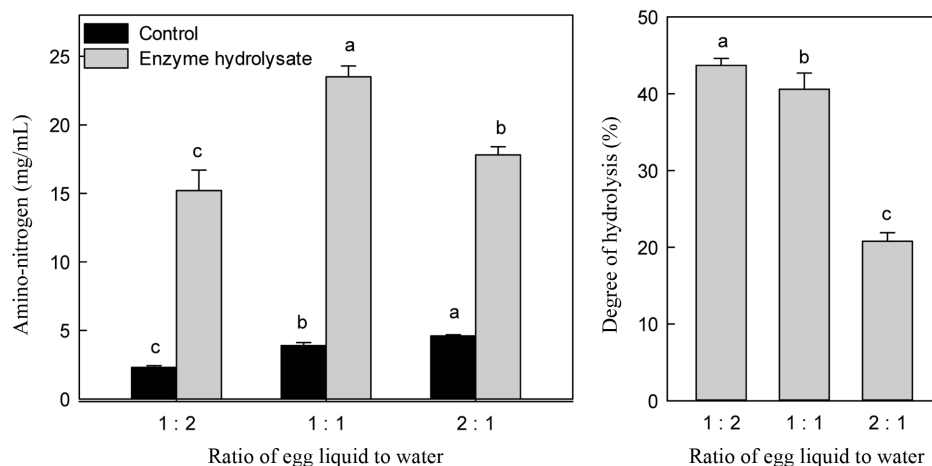


Fig. 4. Amino-nitrogen and degree of hydrolysis (DH) of ELH with various ratios of egg liquid to water. Hydrolysis was carried out at 50°C for 24 h. Error bars represent the standard deviation of triplicate measurements. Different letters indicate significant differences with the same protease ($p < 0.05$).

of water removal to dry hydrolysate and reduce the reaction time, the liquid egg white ratio for alcalase was a substrate to water ratio of 1:1. A high concentration of substrate is very important for decreasing production costs.

Changes of 5-HTP and A-N content, and DH during hydrolysis with 2% and 5% addition of alcalase

5-HTP and A-N contents and DH of alcalase hydrolysate were increased with increasing enzyme addition (Figs. 5 and 6). 5-HTP, A-N, and DH increased until 24 h of hydrolysis and then slightly increased thereafter during hydrolysis with 2% and 5% enzyme addition. Significant changes in 5-HTP, A-N, and DH were observed following enzyme treatment at 2% and 5% addition between 19.19 and 24.84 $\mu\text{g/mL}$, 27.88 and 34.63 mg/mL , and 42.30 and 51.80% at 24 h hydrolysis, respectively. Particularly, 5% alcalase addition did not significantly increase the 5-HTP, DH, and A-N contents after 24 h of hydrolysis (Figs. 5 and 6).

Many researchers have observed similar trends in hydrolysis using various proteases and substrates (Lopez *et al.*, 1998, Qi *et al.*, 1997). The hydrolysis pattern, particularly the decrease in hydrolysis rate, is not fully understood. Alder-Nissen (1986) found that changes in hydrolysis rate are not attributed to substrate exhaustion. Constantinides and Adu-Amankwa (1980) suggested several explanations, such as the depletion of peptide bonds that enzyme intends to hydrolysis, product inhibition, and/or enzyme inactivation.

The ratio of enzyme to substrates suitable for hydroly-

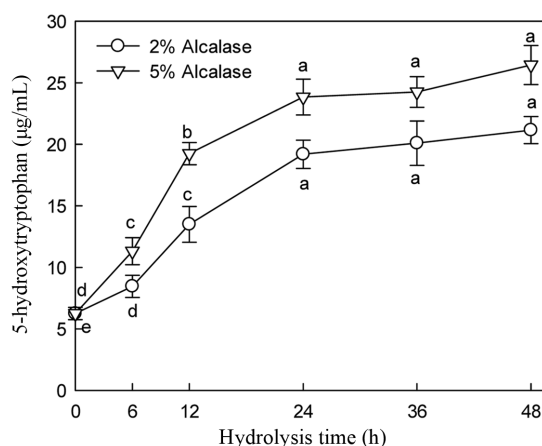


Fig. 5. 5-hydroxytryptophan changes of ELH during hydrolysis with alcalase with different addition amounts of egg white liquid/water [2:1 (V/V)]. Hydrolysis was carried out at 50°C for 48 h. Error bars represent the standard deviation of triplicate measurements. Different letters indicate significant differences with the same protease ($p < 0.05$).

sis was 2-10: 100 (w/w) according to the recommendations of most suppliers (Gibbs *et al.*, 2004). Other studies showed that a range of 1.00-3.00% is an economically feasible enzyme range, with DH increasing as the E/S ratio is increased up to approximately 2.40%, with slight decreases at higher E/S ratios (Wu *et al.*, 2008).

Effect of 5-HTP and LEH on sleep time of pentobarbital-induced sleep in mice

Pentobarbital sodium, a barbiturate compound, is used

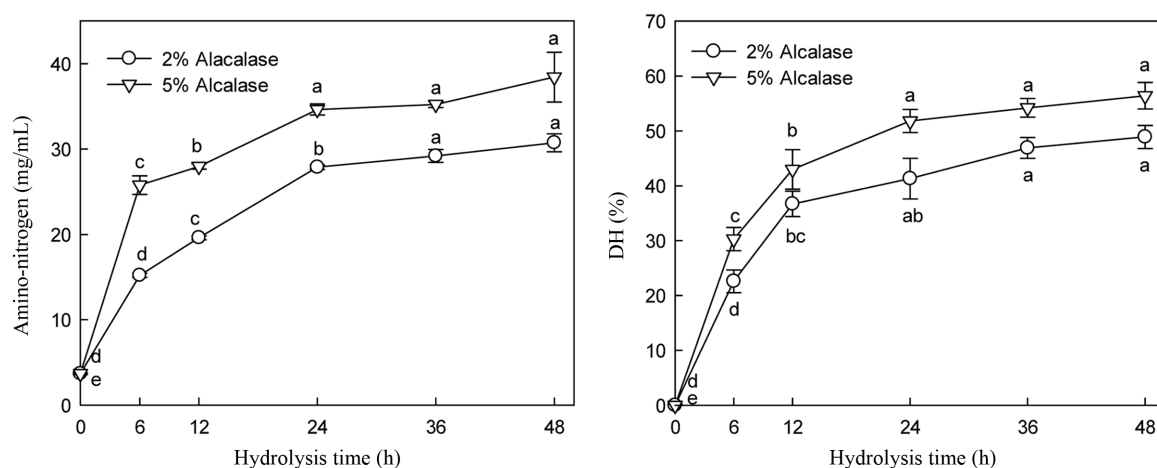


Fig. 6. Amino-nitrogen and degree of hydrolysis (DH) changes in ELH during hydrolysis with alcalase with different addition amounts of egg white liquid/water [2:1 (V/V)]. Hydrolysis was carried out at 50°C for 48 h. Error bars represent the standard deviation of triplicate measurements. Different letters indicate significant differences with the same protease ($p < 0.05$).

to induce sleep in both rodents and humans (Koch-Weser and Greenblatt, 1974). Pentobarbital-induced sleep in mice is the most commonly used method of screening of sedative-hypnotic drugs. Therefore, we used this method to evaluate the sleep-potentiating effects of 5-HTP and LEH.

5-HTP and LEH were administered 40 min before the pentobarbital-induced sleeping test. The 5-HTP and control groups showed differences in the sleep induction effect (Fig. 7). Oral administration of 5-HTP (3, 6, 9 mg/kg) generally increased the sleep duration and decreased the sleep latency time in pentobarbital-induced mice. Particularly, 5-HTP administration of 6 and 9 mg/kg significantly increased sleep duration and decreased sleep latency time compared to that in control mice ($p < 0.05$). However,

5-HTP administration at doses of 6 and 9 mg/kg did not result in significant differences in sleep latency and sleep duration time.

We also compared the sleep-promoting effect of 5-HTP (6 mg/kg) and LEH (150 mg/mouse), which was equivalent to 5-HTP at 6 mg/kg for sleep latency and sleep duration in mice treated with pentobarbital (42 mg/kg, i.p.). 5-HTP and LEH significantly decreased the sleep latency time compared to that in control mice ($p < 0.05$). There were no significant differences in sleep latency time between 5-HTP and LEH. 5-HTP and LEH also significantly increased the sleep duration time compared to that in control mice ($p < 0.05$). There was a significant difference in sleep duration time between mice treated with 5-HTP and

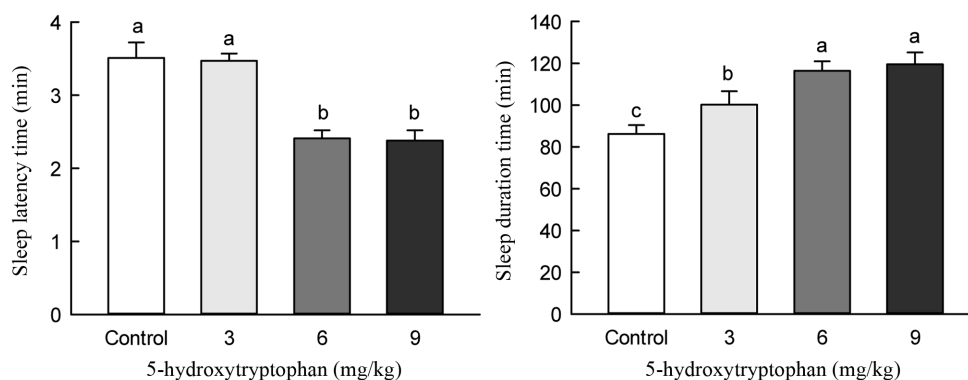


Fig. 7. Effect of 5-hydroxytryptophan (5-HTP) on sleep onset and sleep duration in mice treated with pentobarbital (42 mg/kg, i.p.). Control mice were treated with pentobarbital 40 min after oral administration of 5-HTP and LEH. Each column represents the mean \pm SE of the values obtained from 6 mice. Different letters indicate significant differences ($p < 0.05$) among samples (Duncan's multiple range test).

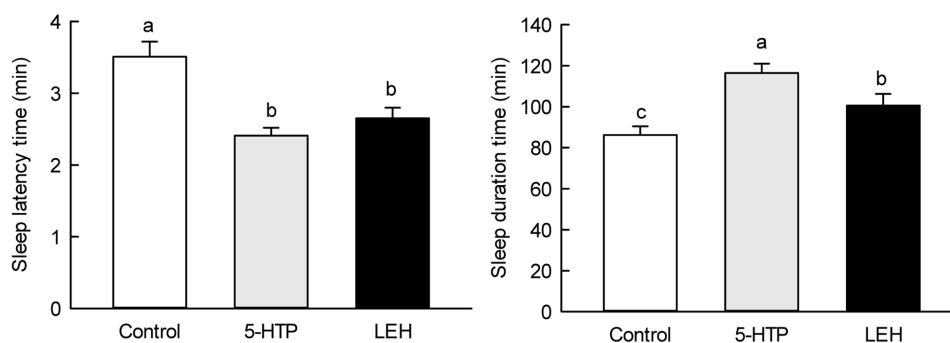


Fig. 8. Effect of 5-hydroxytryptophan (5-HTP) and LEH on sleep onset and sleep duration in mice treated with pentobarbital (42 mg/kg, i.p.). Control mice were treated with pentobarbital 40 min after oral administration of 5-HTP and LEH. LEH was administered at 150 mg/mouse, which was equivalent to 5-HTP at 6 mg/kg. Each column represents the mean \pm SE of values obtained from 6 mice. Different letters indicate significant differences ($p < 0.05$) among samples (Duncan's multiple range test).

LEH ($p < 0.05$). These results demonstrate that oral administration of LEH had sleep-promoting effects. The sleep-promoting effect of LEH was caused by 5-HTP.

The results of this study demonstrate that hydrolysate containing 5-HTP was produced by alcalase from liquid egg white. The hydrolysate is an interesting and valuable substance that can potentiate sleep and can be used as a sleep-potentiating material. Therefore, further studies are required to determine the sleep structure and sleep mechanisms induced by egg hydrolysate for its application as a functional food substance.

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