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Protective effect of *Zingiber zerumbet Smith* extract on thermotolerance

Hisakazu Kobayashi[1,2], Shuji Ueda[1], Yun Sangsoon[1], Akiho Kushiya[1], Ryosuke Tsumagari[1], Minoru Yamanoue[1] and Yasuhito Shirai[1]*

[1]Department of Applied Chemistry in Bioscience, Graduate School of Agricultural Science, Kobe University, Kobe, Japan. [2]Fuso Chemical Co. Ltd., Osaka, Japan.

***Corresponding author:** Yasuhito Shirai, Ph.D., Department of Applied Chemistry in Bioscience, Graduate School of Agricultural Science, Kobe University, Kobe, Japan.

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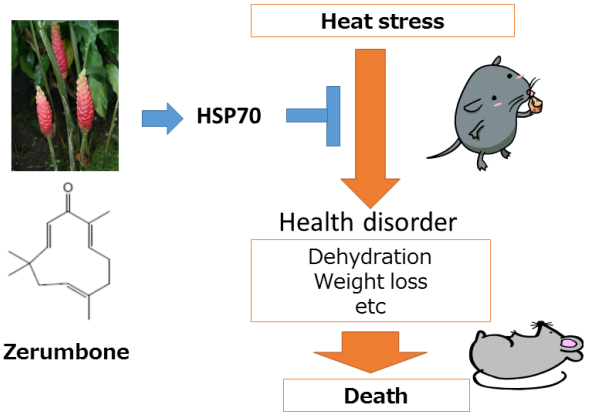
ABSTRACT

***Zingiber zerumbet* extract prevented the heat stress-induced weight loss via HSP70**



Background: Global warming causes severe heat conditions. Heat stress contributes to higher morbidity of heatstroke in human and mortality in livestock. To protect them from heat stress, thermotolerance mechanisms were widely studied, and some studies suggest relationship between heat shock proteins (HSPs) and thermotolerance. HSPs were not induced by only heat shock but also some stimulations including bioactive compounds from plants. *Zingiber zerumbet* is a perennial herb found in many tropical countries, including Thailand. The rhizome of *Zingiber zerumbet* contains zerumbone that is a bioactive compound to induce HSPs expression in animal cells.

Objective: To prevent higher morbidity of heatstroke in human and mortality in livestock by the heat stress, we investigated the effect of zerumbone, the extract of *Zingiber zerumbet Smith*, on thermotolerance, using a cell line and mice.

Methods: The murine liver hepatoma cell line, Hepa1c1c7 cells, were incubated in medium supplemented with extract from rhizome of *Zingiber zerumbet Simith* containing zerumbone, and then the expression of heat shock proteins (HSP) 40, 70 and 90 were investigated by western blotting. Furthermore, we established the evaluation system of thermotolerance using mice, and studied the effect of the extract on the growth rate of mice under the heat shock treatment. Briefly, 4 weeks old C57BL6 mice were fed that with the extract (or vehicle) for a week before the first heat shock treatment (38 °C for an hour). Before and after five days heat treatment, body weights were measured. The protein expressions of heat shock proteins in liver were measured by western blotting using HSPs antibodies.

Results: The extract of *Zingiber zerumbet* rhizome, equivalent to 50 μM zerumbone, significantly increased the expression of heat shock proteins (HSP40, HSP70, HSP90). The growth rate of the mice under the heat treatment were lower than control. The feeding with the extract containing 25 ppm zerumbone have significantly attenuated the decline of the growth rate led by the heat treatment, whereas there was little effect on mouse growth rate grown under normal conditions. The protein expression of HSP70 in the liver of zerumbone-fed mice was upregulated compared with control mice, equivalent to heat treatment without zerumbone. On the other hand, both treatments of zerumbone and heat resulted in highest HSP70 expression among four groups.

Conclusion: Our study demonstrated that oral administration of the extract of *Zingiber zerumbet Smith* led to the attenuation of decline of growth rate induced by heat treatment. HSP70 expression in murine liver was enhanced by either feeding the extract or heat treatment. More interestingly, HSP70 expression was further enhanced by both treatments of zerumbone and heat. These results suggested that zerumbone may contribute to thermotolerance via, at least, HSP70 expression.

Keywords: *Zingiber zerumbet*, thermotolerance, heat shock protein

INTRODUCTION

The average temperature in the contiguous United States was 52.7°F (11.5°C) in 2019, 1.5 above the 20th century average [1]. Over the past 10 years, the U.S has also experienced the highest number of very warm nights (minimum temperature above 75°F) on record [1]. Similarly, in Japan, annual number of days with a maximum temperature of 35°C or above ("Mousho-bi" in Japanese, meaning extremely hot day) has increased; the total number of "Mousho-bi" in Tokyo was 1 in the 1980's but it increased to 69 in the 2010's [2]. Those reports clearly show that we are facing "Global warming".

Climate change causes various types of impacts on agriculture, forests/forestry and fisheries, depending on the areas and products. Indeed, it is said that extreme temperatures in the United States in 2018 caused about 150 million U.S. dollars in economic damage. In addition, the global warming-induced severe heat conditions affect not only the mortality in livestock and plants, but also human health. Heat stress is one of the causes of higher morbidity of heatstroke in humans. Heat stroke is a life-threatening diseases that results from exposure to high ambient temperature [3].

"Thermotolerance" is the ability to withstand hot conditions. For example, plants and animals

acquire thermotolerance to cope with lethal high temperatures following acclimatization at sublethal high temperatures. Therefore, thermotolerance mechanisms were widely studied to prevent higher morbidity of heatstroke in human and mortality in livestock due to heat stress. Although there are several mechanisms to induce thermotolerance, many studies have suggested relationship between heat shock proteins (HSPs) and thermotolerance.

HSPs are also induced by some stimulations including bioactive compound from plants, so called, "phytochemicals". One of the phytochemicals is zerumbone from the rhizome of *Zingiber zerumbet*. *Zingiber zerumbet* is a plant in the ginger family, and a perennial herb found in many tropical countries. Originally, zerumbone had been identified as an anti-cancer agent [4] and then was shown to be a bioactive compound to induce HSPs expression in animal cells [5, 6].

These facts suggest that oral administration of zerumbone prevents heat stress. However, there is no in vivo evidence that oral administration of Zerumbone prevents heat stress. Therefore, we investigated the effect of oral admistration of zerumbone, the extract of *Zingiber zerumbet Smith*, on thermotolerance, using a cell line and mice.

METHODS

Materials: Anti-40, 60, 70 and 90 kDa HSP antibodies were obtained from BD Bioscience (San Jose, CA, USA).

Cell culture: Hepa1c1c7 cell was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and grown in Ham's F-12K (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 20% (v/v) fetal bovine serum (Biowest, Nuaillé, France), 50 µg/ml endothelial cell growth supplement from bovine neural tissue (Sigma-Aldrich, St. Louis, MO, USA) and 100 µg/ml heparin sodium under a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO_2 at 37 °C.

Extract of Z. zerumbet* rhizome:* Rhizomes of *Zingiber zerumbet Smith* were dried and powdered. The powder was extracted with ethanol at room temperature. The concentration of zerumbone was measured by HPLC.

In vitro evaluation of the protein Expression of HSPs: The murine liver hepatoma cells, Hepa1c1c7, were incubated in medium supplemented with extract of *Z. zerumbet* rhizome containing 50 µM zerumbone, and then the expressions of HSPs 40, 60, 70 and 90 were investigated by western blotting.

Safety assessment of the Z. zerumbet rhizome extract: C57BL6 mice were fed with the diet that contain the extract equivalent to 25 ppm zerumbone from 4 to 12 weeks-old. After 8 weeks oral administration, blood chemical analysis was performed by Oriental yeast Co. Ltd (Osaka, Japan).

Thermotolerance test: We established the evaluation system of thermotolerance using mice, and studied the effect of the extract on the growth rate of mice under the heat shock treatment. Briefly, 4 week old C57BL6 mice were fed daily the extract containing 25 ppm zerumbon (or vehicle) for a week, (so called "preconditioning"), and then were put under heat stress conditions (38°C for an hour / day) or normal conditions for 5 days. During the 5 days, feeding of the extract was continued. Before and after 5 days of heat treatment, body weights were measured. The protein expressions of HSPs in liver and muscle were measured by western blotting using HSPs antibodies.

RESULTS

Safety assessment of Zingiber zerumbet extract: To investigate the side effect of oral administration of *Zingiber zerumbet* extract, we fed 4 week old C57BL6 mice a diet that contained the extract equivalent to 25 ppm zerumbone for 4 weeks and measured their body weights. There was no significant difference in the body weight of test and control mice (Fig. 1A). Also, there was no significant difference in consumption of diet between control and test (data not shown). Next, to check the effect of *Zingiber*

zerumbet extract on kidney, liver and pancreas functions, we measured blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and amylase (AMY) in the respective blood samples. BUN is a marker of kidney function. AST and ALT are for liver, while AMY is for pancreas. Fig. 1B shows there was no significant effect of oral administration of *Zingiber zerumbet* extract on BUN, AST, ALT and AMY. Furthermore, there was no significant difference in the sizes of kidney and liver as well as other organs including spleen, stomach and duodenum (Fig. 1C). Based on the results, we concluded that oral administration of 25 ppm zerumbone exerts no side effect to the mice.

Effect of oral administration of Zingiber zerumbet extract on the loss of body weight by head stress: To investigate protective effect of *Zingiber zerumbet* extract against heat stress-induced body weight loss, we fed mice a diet with or without *Zingiber zerumbet* extract for a week, and then put the mice under heat conditions (38°C for an hour / day) for the following 5 days. As shown in Fig. 2, control mice without heat stress showed about 7.2% body weight gain for the 5 days, while control mice with heat stress showed only a 2 % gain. In contrast, zerumbone-fed mice with heat stress gained 4.2 %; it is significantly higher compared to the control mice with heat stress. Importantly, Zerumbone-fed mice without heat stress showed about 7.0% body weight gain for the 5 days; it was

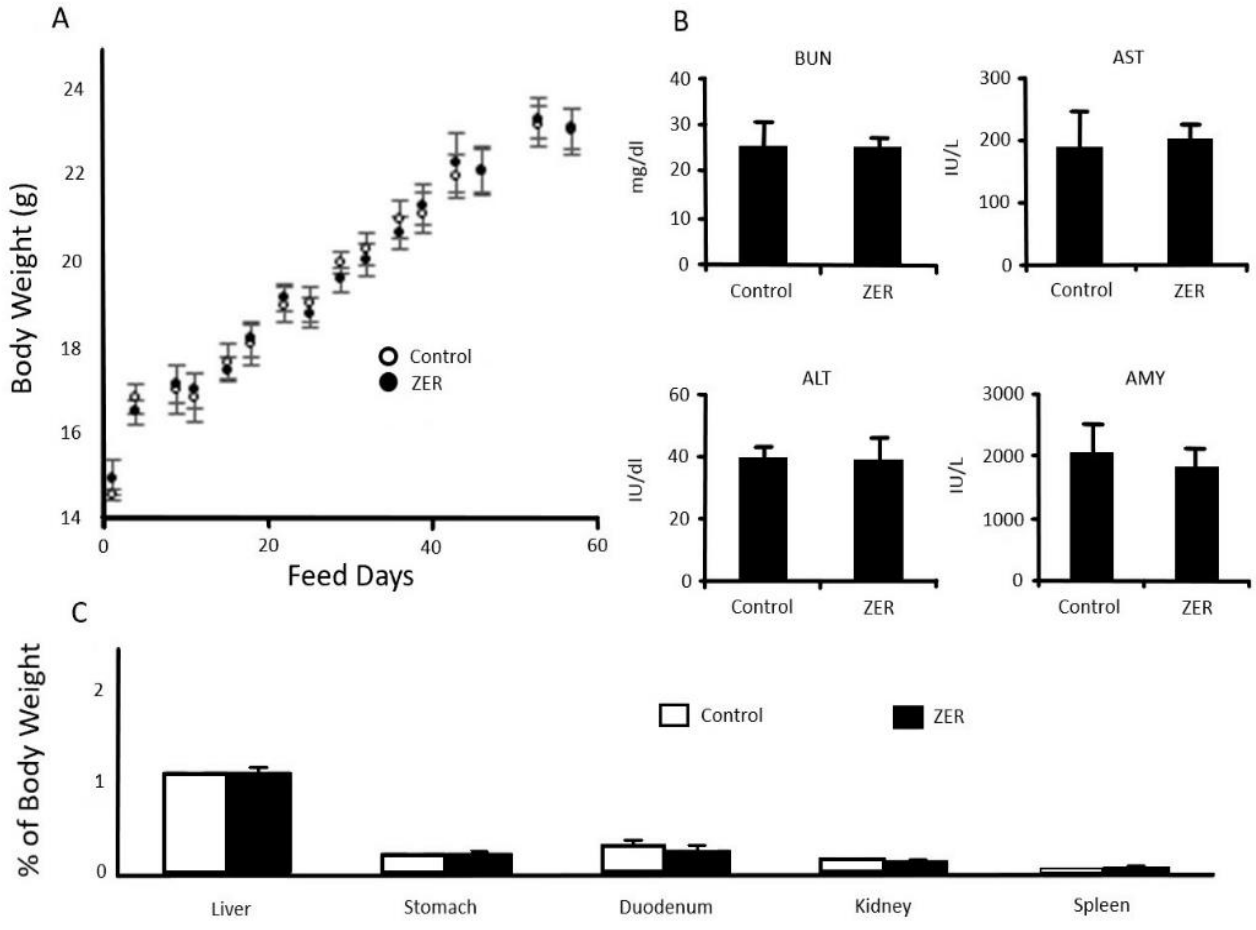


Figure 1: Safety assessment of the Z. Zerumbet extract on (A) body weight, (B) biochemical parameter in blood serum and (C) organs weight.

There is no significant difference between control and test in all datum. At least 5 mice were used in control and test, respectively. ZER represents the mice fed with *Zingiber zerumbet* extract.

similar with control mice without heat stress. These results indicated that pretreatment with *Zingiber zerumbet* extract prevented the weight loss by heat stress.

Effect of oral administration of Zingiber zerumbet extract on the HSP expression: As we mentioned in the introduction, HSPs are key molecules for thermotolerance. Before we checked the amount of HSPs induced by oral administration and/or heat stress *in vivo*, we investigated which type of HSP was induced by *Zingiber zerumbet* extract *in vitro*. As shown in Fig. 3, HSP40, 70 and 90 were significantly induced by 24h incubation with *Zingiber zerumbet* extract. In contrast, HSP60 showed a tendency to be induced but it was not significant. Therefore, we focused on HSP40, 70 and 90. First, we checked the expression of HSP90 in the soleus muscle and liver of control and zerumbone-fed mice with or without heat stress.

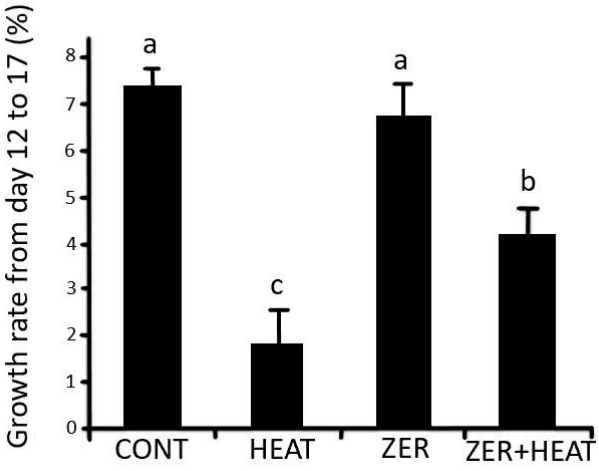


Figure 2. Effect of oral administration of Z. zerumbet extract on body weight loss by heat stress.

Different alphabets show significance analyzed with two-way ANOVA and Scheffe's test among the groups (P <0.05). The error bars show ±SE. n=5 for each condition. ZER represents the mice fed with Zingiber zerumbet extract, and HEAT represents the mice with added heat stress.

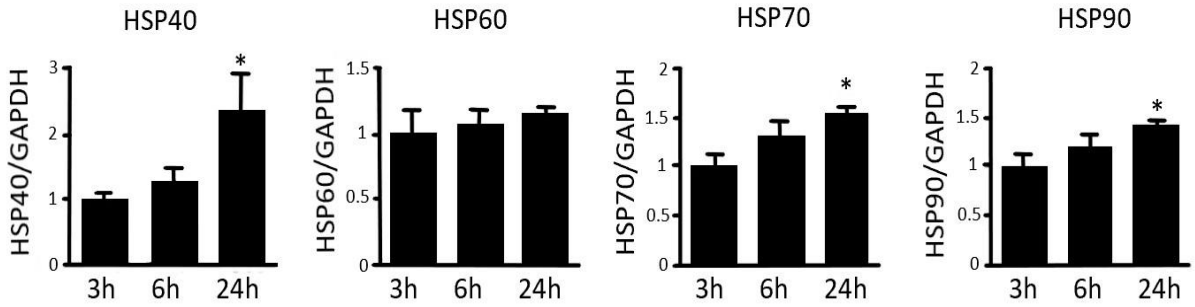


Figure 3. Effect of Z. Zerumbet extract on HSPs expression in Hepa1c1c7 cell line.

Each column show average of the three independent experiments. * shows significant difference compared to control (P <0.01).

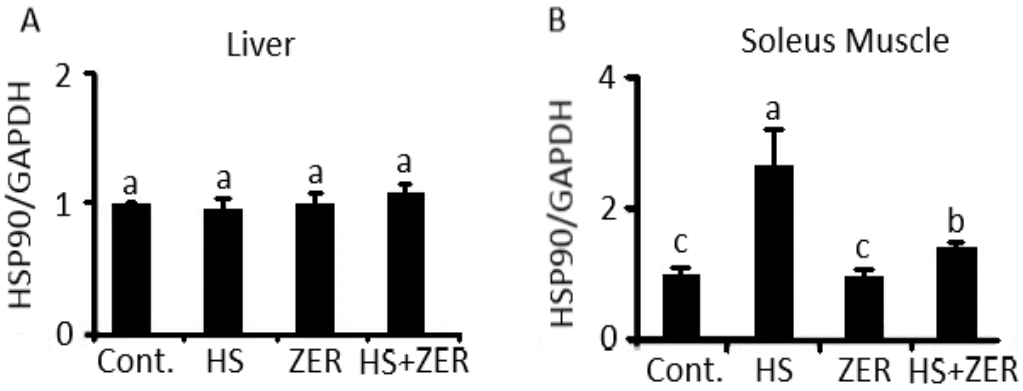


Figure 4. Effect of oral administration of Z. Zerumbet extract on HSP90 expression in liver (A) and Soleus muscle (B).

Expression level of HSP90 was normalized as ratio to that of control and each column shows the average of the results from 5 mice. Different alphabets show significance analyzed with two-way ANOVA and Scheffe's test among the groups ($P < 0.05$). The error bars show ±SE. ZER represents the mice fed with *Zingiber zerumbet* extract, and HS represents mice with heat stress.

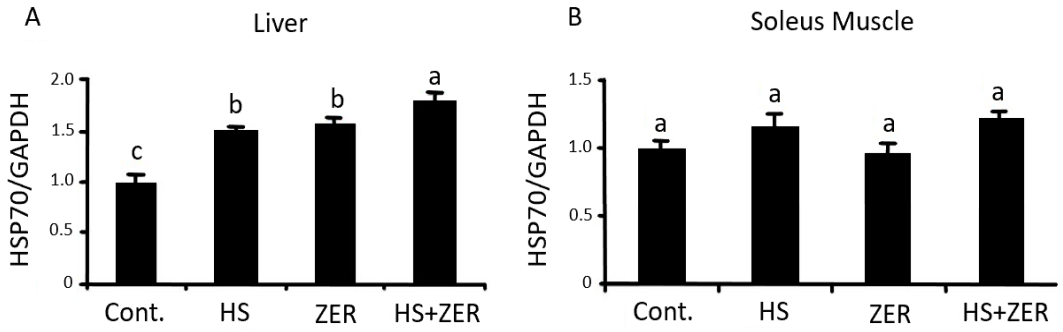


Figure 5. Effect of oral administration of Z. Zerumbet extract on HSP70 expression in in liver (A) and Soleus muscle (B).

Expression level of HSP70 was normalized as a ratio to that of control and each column show average of the results from 5 mice. Different alphabets show significance analyzed with two-way ANOVA and Scheffe's test among the groups ($P < 0.05$). The error bars show ±SE. ZER represents the mice fed with *Zingiber zerumbet* extract, and HS means heat stress.

As shown in Fig. 4A, in the muscle, HSP90 was significantly induced by heat stress but not by oral administration of *Zingiber zerumbet* extract (Fig. 4B). In contrast, there was no significant difference in the expression of HSP90 in the liver (Fig. 4A). Next, we compared amount of HSP70. In contrast to HSP90, HSP 70 was induced by both heat stress and *Zingiber zerumbet* extract in the liver (Fig. 5A). Interestingly, the HSP70 expression was further enhanced by both treatments of Zerumbone and heat (Fig. 5A). On the other hand, there was no significant difference in the expression of HSP70 in the muscle, although it showed a tendency to be induced by heat stress but not by *Zingiber zerumbet* extract (Fig. 5B). HSP40 was not detected in both muscle and liver. These results suggested that induction of HSP70 in the liver may contribute to prevention of weight loss by heat stress.

DISCUSSION

In this study, we showed for the first time that preconditioning by oral administration of zerumbone prevented the weight loss caused by heat stress. Also, we showed that Zerumbone induced HSP40, 70 and 90 in vitro, confirming the previous results [5]. According to Murakami et al, zerumbone is bound to proteins in the cells and the zerumbone-bound proteins are recognized by HSP90, resulting in activation of heat shock factor 1 (HSF1). HSF1 induces expression of several proteins including HSP40 and HSP70 [5,6]. On the other hand, HSP90 was not induced by oral administration of zerumbone, and HSP40 was not detected in our study, suggesting low amount of HSP40 in liver and muscle in vivo. Alternatively, it might be due to our methods employed. In contrast, the *in vivo* induction of HSP70 by zerumbone was detected in the liver but not in the muscle. The difference seemed to be due to distribution of zerumbone. The results suggested that HSP70 in the liver, at least, is a key molecule for the zerumbone-induced thermotolerance. The importance of HSP70 for the thermotolerance is supported by other reports [7,8] and the importance of HSP70 in the liver for cold stress was also reported [9]. However, how HSP70 in the liver contributes to the thermotolerance is still unknown, and we can't rule out the possibilities that other HSPs in some organs are also involved in zerumbone-induced thermotolerance.

Zerumbone is not only one phytochemical to induce HSPs. For example, curucumin induced HSP70 in human leukemia cells [10] and sulforaphane induces HSP27 expression [11]. These facts suggest that not only zerumbone but also other phyotochemicals could prevent heat stress through similar mechanisms to thermotolerance. In other words, oral administration of phytochemicals including zerumbone is an attractive way to protect domestic animals and humans from heat stress. Of course, to utilize phytochemicals for the thermotolerance against heat stress, further experiments would be necessary. But it is a hopeful way to fight against world warming.

In conclusion, our study demonstrated that oral administration of the extract of *Zingiber zerumbet* led to the attenuation of decline of growth rate induced by heat treatment. HSP70 expression in murine liver was enhanced by either feeding the extract or heat treatment. These results suggest that zerumbone may contribute to thermotolerance via, at least, HSP70 expression.

List of Abbreviations: ALT: alanine aminotransferase, AMY: amylase, AST: aspartate aminotransferase, BUN:blood urea nitrogen, HPLC: high performance liquid chromatography, HSF1: heat shock factor 1, HSP: heat shock protein, U.S: United States, ZER: zerumbone.

Competing Interests: There are no conflicts of interest to declare.

Author's Contribution: Yasuhito Shirai designed the experiments and revised the manuscript. Hisakazu Kobayashi performed almost all experiments, analyzed data, and wrote a draft of the manuscript. Yun Sangsoon, Shuji Ueda, Akiho Kushiya and Ryousuke Tsumagari performed some parts of the experiments. Yamanoue Minoru gave a critical advice for the research. All authors read and approved the final version of the manuscript.

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