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Toxicological assessment of enzyme-treated asparagus extract in rat acute and subchronic oral toxicity studies and genotoxicity tests

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ABSTRACT

The safety of enzyme-treated asparagus extract (ETAS) developed as a novel anti-stress functional material was assessed in acute and subchronic studies and genotoxicity assays. In the acute oral dose toxicity study, all rats survived during the test period and ETAS did not influence clinical appearance, body weight gain and necropsy findings at a dosage of 2000 mg/kg body weight. Thus, the 50% lethal dose (LD_{50}) of ETAS was determined to be greater than 2000 mg/kg. The 90-day subchronic study (500, 1000 and 2000 mg/kg body weight, delivered by gavage) in rats reported no significant adverse effects in food consumption, body weight, mortality, urinalysis, hematology, biochemistry, necropsy, organ weight and histopathology. In the micronucleus test of mice, the incidence of micronuclei in ETAS-administered groups (500, 1000 and 2000 mg/kg/day, injected twice) was equivalent to that of the negative control group, while the positive control group receiving mitomycin C showed a high incidence. The potential of ETAS to induce gene mutation was tested using four *Salmonella typhimurium* strains and *Escherichia coli* WP2*uvrA*. The test sample was not mutagenic to the test strains. These results support the safety of ETAS as food and dietary supplement.

1. Introduction

In the modern age, people are subjected to numerous stresses that contribute to various physical and psychological disorders such as insomnia, digestive disorders and fatigue. Severe stress can result in more serious problems. In Japan, more than one million persons are laden with clinical depression, and over 30,000 people commit suicide each year. Stress is a complex issue and different types of treatments are known to suppress stresses. However, since current treatment models focus on expressed symptoms in hopes that the underlying physical and psychological issues resolve, new approaches are clearly needed for fundamental remission of stress. Results from previous studies suggest some metabolic pathways that might help fight stress-related disorders (Han et al., 2012; Murrow and Debnath, 2013; Zarate et al., 2013). Central to these pathways is a family of molecules known as heat shock proteins (HSPs) (Richter et al., 2010; Murshid et al., 2013), which have a number of subgroups classified according to their molecular weights (e.g., HSP10, HSP27, HSP40, HSP60, HSP70, HSP90, HSP110, etc.). Among the HSP subgroups, HSP70 is one of the most prominent and well-studied members. The protein is constitutively expressed in a variety of organs like the digestive tract, liver and kidney (Tsukimi and Okabe, 2001; Flohé et al., 1998; Beck et al., 2000), and is involved in apoptosis-suppressive and anti-inflammatory activities that are cytoprotective (Hirata et al., 2009; Nishida et al., 2010). Several substances have been found to up-regulate HSP70 expression so far. Paeoniflorin, which is a main ingredient derived from peony (*Paeonia lactiflora*), was previously discovered as a natural product to induce HSP70 via enhancement of phosphorylation and DNA-binding property of heat shock factor 1 (HSF-1) (Yan et al., 2004). Teprenone (geranylgeranylacetone; GGA), an acyclic polyisoprenoid developed and clinically used in Japan, binds to C-terminal of HSP70 directly contacting with HSF-1 and brings about the dissociation of the HSP70 from HSF-1 to produce free HSF-1 involved in HSP70 expressions (Otaka et al., 2007). Accordingly, the compound protects human gastric mucosa resulting from induction of HSP70 (Yanaka et al., 2007).

In the search to identify botanical sources that could support HSP70 induction activity, various plants grown in Hokkaido were screened and the most powerful HSP70 induction was noted from asparagus (*Asparagus officinalis* L.). On the basis of this observation, an enzyme-treatment process was developed to produce a functional food material termed enzyme-treated asparagus extract (ETAS). We have elucidated that the extract contains 5-hydroxymethyl-2-furfural (HMF) (Fig. 1, compound **1**) and its novel derivative named asfural (Fig. 1, compound **2**), which are some of active ingredients to enhance HSP70 expression (Ito et al., 2013). Asfural

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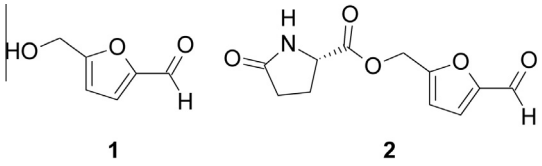

Fig. 1. Chemical structures of 5-hydroxymethyl-2-furfural (**1**) and asfural (**2**).

is considered to be a condensation reaction product of HMF and pyroglutamic acid derived from glutamine contained in asparagus. Their chemical structures are extremely different from those of paeoniflorin and teprenone. Although the underlying mechanism of HMF and asfural on HSP70-inducing effect remains to be clarified, it may be distinguished from the mechanism of action via HSF-1, which is a potential target for paeoniflorin and teprenone. Asparagus is a major vegetable that has also been used in traditional medicine since ancient times and is known to have diuretic, anti-fatigue and metabolism-enhancement effects (Ye et al., 1994; Hasani-Ranjbar et al., 2009; Negi et al., 2010; Guarrera and Savo, 2013). Recent studies demonstrated that asparagus extract regulates blood glucose levels in diabetic rats and has oxidant defense activity (Hafizur et al., 2012; Tiveron et al., 2012). In our study, HMF and asfural as well as ETAS up-regulated HSP70 mRNA in a human promyelocytic leukemia cell line, HL-60 cells, at the concentration showing no cytotoxicity (Ito et al., 2013). The latest study reported that ETAS possesses neuroprotective effect and attenuates cognitive impairment in senescence-accelerated mice (Sakurai et al., 2013).

Asparagus consumption is commonly worldwide without any major reported side effects, but in order to assess the safety of the unique ETAS, several toxicity studies were conducted. In this paper, we report the results from rat acute and subchronic toxicity studies of orally administered ETAS and from genotoxicity studies including a micronucleus assay in mice and a bacterial reverse mutation assay (Ames test).

2. Materials and methods

2.1. Materials

Enzyme-treated asparagus extract (ETAS) provided by Amino Up Chemical Co., Ltd. (Sapporo, Japan) was produced from asparagus (*A. officinalis* L.) grown in Hokkaido. ETAS was manufactured according to a method described previously (Ito et al., 2013). In brief, raw asparagus was extracted with hot water at 121 °C for 45 min, and treated with sucrase C (Mitsubishi-Kagaku Foods Corporation, Tokyo, Japan) and macerozyme A (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) for 24 h. After enzyme inactivation and centrifugation, the supernatant was concentrated *in vacuo*. The obtained ETAS solution was mixed with dextrin (Pindex; Matsutani Chemical Industry Co., Ltd., Hyogo, Japan) that was used as filler, and spray dried following sterilization to achieve production of the ETAS powder. We used two lots of the ETAS powder in this investigation, which were lot No. 111021 containing 43.4 wt% of ETAS and 57.6 wt% of dextrin for acute oral dose toxicity study, micronucleus test and Ames test, and lot No. HSP1209S containing 50.4 wt% of ETAS and 49.6 wt% of dextrin for subchronic 90-day oral dose toxicity study. Their components were shown in Table 1, and all dosages and concentrations for the current studies were corresponding to those of ETAS but not the ETAS powder.

2.2. Acute oral dose toxicity study in rats

This study was conducted at Biotoxtech Co., Ltd. (Chungcheongbuk-do, Korea) in accordance with Good Laboratory Practice (GLP) standard and OECD425 guidelines, and approved by the Institutional Animal Care and Use Committees of Biotoxtech Co., Ltd. Female Crl:CD(SD) rats at the age of 7 weeks were purchased from Orientbio, Inc. (Seongnam, Korea). Following quarantine and acclimation, five healthy rats (8 weeks old) were selected randomly. The first dose level of ETAS was started at 175 mg/kg body weight (one rat). Based on the survival of the rat for at least 48 h after previous dosing, next dose levels were determined with dose progression of factor 3.2. Actually, 2nd step (550 mg/kg, one rat) and 3rd, 4th and 5th steps (2000 mg/kg, total three rats) were selected and evaluated. The doses were calculated by AOT425StatPgm (Version 1.0, 2001) according to the "OECD Guideline for Testing of Chemicals 425, Acute Oral Toxicity-Up-and-Down Procedure." The starting dose level was selected as 175 mg/kg because there was no available toxicity information on the test substance. The limit dose level was selected as 2000 mg/kg in sigma 0.5 according to AOT425StatPgm. Animals were maintained in a temperature- and humidity-controlled room at 20–24 °C and 40–67%, respectively, under a 12-h light–dark cycle (lights on 07:00 to 19:00), fed a standard pelleted rodent chow (Teklad Certified Irradiated Global 18% Protein Rodent Diet 2918C; Harlan Laboratories, Inc., IN, USA), and given water *ad libitum*.

All animals were observed for their general condition and clinical signs for 30 min and at 1, 2, 4 and 6 h after dosing on day 0, and once daily thereafter for 14 days (days 1–14). Body weight was recorded on days 0, 1, 3, 7 and 14. On day 14, all animals were anesthetized with CO_2 and exsanguinated from the abdominal aorta. Complete gross postmortem examinations were performed on all animals. The 50% lethal dose (LD_{50}) value was calculated by the statistical program AOT425StatPgm.

Table 1
Component of ETAS powder used in the current studies.

Component	Lot No.	
	111021	HSP1209S
Moisture (wt%)	3.3	3.1
Protein (wt%)	7.0	10.9
Total fat (wt%)	0.2	0.8
Ash (wt%)	2.7	4.0
Available carbohydrate and dietary fiber (wt%)	86.8	81.2
Calorie (kcal/100 g)	377	376

2.3. Subchronic 90-day oral dose toxicity study in rats

2.3.1. Study design

This study was conducted at Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan) according to GLP standard and OECD408 guidelines, and approved by the Ethics Committee for Animal Experiments of Safety Research Institute for Chemical Compounds Co., Ltd. Male and female Crl:CD(SD) rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Following quarantine and acclimatization, 40 male and 40 female rats (5 weeks old) were selected and randomly allocated to four groups (10 rats/group) by gender. Animals were maintained in a temperature- and humidity-controlled room at 19–23 °C and 31–49%, respectively, under a 12-h light–dark cycle (lights on 08:00 to 20:00), fed a standard pelleted rodent chow (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan), and given water *ad libitum*. Four groups of male and female rats received ETAS at a dosage of 0, 500, 1000 or 2000 mg/kg body weight by oral gavage once daily for 90 consecutive days. The highest dose was set at 2000 mg/kg, because the LD_{50} value was considered to be greater than 2000 mg/kg, and no test substance-related effects were evident in clinical signs, body weight data and necropsy findings in the acute oral dose toxicity study.