Hepatoprotective effect of germanium-containing *Spirulina* in rats with D-galactosamine- and lipopolysaccharide-induced hepatitis

Orie Yoshinari[1,2]*, Yoshiaki Shiojima[2] and Kiharu Igarashi[1]

[1]*Department of Bioresource Engineering, Faculty of Agriculture, Yamagata University, Yamagata 997-8555, Japan*
[2]*Ryusendo Company Limited, Tokyo 171-0021, Japan*

Abstract

In the present study, the protective effects of dietary *Spirulina* (SP) and germanium-containing *Spirulina* (GeSP) were compared in rats with liver injury induced by an intraperitoneal injection of D-galactosamine and lipopolysaccharide (GalN/LPS). Wistar rats were fed one of the following diets: the basal diet (GalN/LPS-CON group; *n* 6), the basal diet supplemented with 5% SP or GeSP (GalN/LPS-SP and GalN/LPS-GeSP group, respectively; *n* 7 each). After administering these diets for 7 d, each rat was intraperitoneally injected with GalN/LPS. Increases in plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were suppressed in the GalN/LPS-GeSP group (GalN/LPS-CON *v.* GalN/LPS-GeSP: ALT 1052 (SEM 187) *v.* 509 (SEM 88) IU/l and AST 2183 (SEM 368) *v.* 1170 (SEM 196) IU/l) following the injection of GalN/LPS. Plasma levels of interferon-γ (IFN-γ) and TNF-α in GeSP-fed rats were significantly lower when compared with those in the GalN/LPS-CON group (GalN/LPS-CON *v.* GalN/LPS-GeSP: IFN-γ 142·8 (SEM 17·5) *v.* 66·8 (SEM 9·7) pg/ml and TNF-α 72·3 (SEM 15·4) *v.* 31·2 (SEM 6·8) pg/ml). However, the decrease in these levels observed in the GalN/LPS-SP group was not as prominent as those observed in the GalN/LPS-GeSP group. Furthermore, the increase in liver catalase (CAT) and glutathione peroxidase (GPx) activities, as well as the level of oxidised glutathione (GSSG), was more suppressed in GeSP-fed rats (GalN/LPS-CON *v.* GalN/LPS-GeSP: CAT 457 (SEM 47) *v.* 262 (SEM 54) U/mg liver protein; GPx 1·30 (SEM 0·11) *v.* 0·53 (SEM 0·09) U/mg liver protein; GSSG 2·18 (SEM 0·33) *v.* 1·31 (SEM 0·24) mmol/kg liver) after the injection of GalN/LPS. These changes were more pronounced in the GalN/LPS-GeSP group than in the GalN/LPS-SP group. These results suggest that GeSP could afford a significant protective effect in the alleviation of GalN/LPS-induced hepatic damage. In addition, the results indicate that GeSP is more effective than SP.

Key words: Germanium-containing *Spirulina*: D-Galactosamine and lipopolysaccharide: Liver injury: Antioxidant enzymes

Hepatic failure induced by the injection of D-galactosamine and lipopolysaccharide (GalN/LPS) has been considered as an inflammatory response, involving the accumulation of mononuclear cells in the liver and an increase in plasma alanine transaminase and aspartate aminotransferase activities. This phenomenon is observed in patients with acute hepatic failure[1]. GalN/LPS-induced liver injury is also known to cause cytokine release (TNF-α is the main mediator) that contributes to increased oxidative stress and the formation of reactive oxygen species, followed by hepatocyte death[2,3].

The blue-green alga *Spirulina* (*Spirulina platensis*, SP) is used as a health food source because it contains large amounts of vitamins, minerals and amino acids. Its consumption by humans and rodents is believed to be efficacious in improving diabetes[4], osteopenia[5] and immunity[6]. Furthermore, it has been reported that SP and its component phycocyanin mitigate D-galactosamine-induced liver injury in rodents[7,8]. Recently, studies have reported that not only SP but also Fe- and Se-rich SP are beneficial to human health[9–11].

It has been reported that some metals such as zinc and gallium reduce liver damage[12,13], and germanium is also considered to exhibit this effect. Germanium is present in all living plant and animal matter in micro-trace quantities. Although inorganic germanium (germanium dioxide) has been shown to have toxic effects[14], organic germanium has therapeutic attributes including immune enhancement[15] and antioxidative effects[16,17]. Organic germanium is well known to be effective in protecting against liver injury in mice through reducing the production of interferon-γ (IFN-γ) or TNF-α[18,19]. Thus, germanium-containing *Spirulina* (GeSP) could be expected to show stronger hepatoprotective activity than SP due to the synergistic effects of both

germanium and SP. Therefore, in the present study, we produced GeSP and examined its effect.

The safety of GeSP, which is cultured in the presence of germanium dioxide, has been confirmed in animals[20]. There were almost no differences between the GeSP-fed group and the control groups in the growth, weight and histological findings of the organs (e.g. brain, heart, liver and kidney).

In the present study, the protective effects of dietary SP and GeSP were compared in rats with GalN/LPS-induced liver injury by measuring parameters related to liver injury.

Materials and methods

Spirulina germanium fortification

SP was grown in a basal medium (pH 10·5) supplied with aeration. All chemicals were purchased from Wako Pure Chemical Industries. The basal medium contained Na_2CO_3 (12·6 g/l), $NaHCO_3$ (10·0 g/l), Na_2SO_4 (3·0 g/l), $NaNO_3$ (2·2 g/l), KCl (0·5 g/l), K_2HPO_4 (0·5 g/l), $MgCl_2.6H_2O$ (0·5 g/l), EDTA (0·07 g/l), $CaCl_2.2H_2O$ (0·05 g/l), $FeSO_4.7H_2O$ (5·0 mg/l), H_3BO_3 (286 μg/l), $MnCl_2.4H_2O$ (184 μg/l), $ZnSO_4.7H_2O$ (220 μg/l), $CuSO_4.5H_2O$ (19·7 μg/l), $KCr(SO_4)_2.12H_2O$ (48·0 μg/l), MoO_3 (37·5 μg/l), NH_4VO_3 (23·0 μg/l), $NiSO_4.6H_2O$ (22·5 μg/l), $Co(NO_3)_2.6H_2O$ (24·5 μg/l), $Ti(SO_4)_2$ (4·0 μg/l) and $Na_2WO_2.2H_2O$ (1·8 μg/l), and was adjusted to pH 10·5. GeSP was grown in this medium in the presence of germanium dioxide. The pH was adjusted to 12 by adding 5 % NaOH. At the end of the growth period, biomass was recovered and filtered through a 20 μm membrane, thoroughly washed with tap water and dried.

Animal care

The care and use of the rats followed the institutional guidelines of Yamagata University. Male Wistar rats (6 weeks old) were purchased from Japan SLC, Inc. The rats were housed individually in stainless-steel cages with a 12 h light–12 h dark cycle at 22 ± 2°C and 40–60 % humidity. Diet and water were given *ad libitum*. After acclimatisation for 5 d, the rats were divided into five groups based on the body weight of the rats. Statistics were used to ensure that there were no significant differences between the groups. Of these five groups, two were fed on a basal diet (CON and GalN/LPS-CON; *n* 6) and three were fed on a basal diet supplemented with 5 % SP or GeSP (GalN/LPS-SP and GalN/LPS-GeSP; *n* 7) (Table 1). As SP and GeSP contained about 60 % protein, the quantity of casein was adjusted in such a way that the protein level was equal among all the diets. The diet that included GeSP contained 30 parts per million germanium. The concentration of germanium was determined by flame atomic absorption spectrometry.

After administering these diets for 7 d, rats assigned to the GalN/LPS groups were intraperitoneally injected with GalN/LPS (800 mg GalN + 30 μg LPS/kg body weight, GalN (D-galactosamine hydrochloride) and LPS (from *Escherichia coli*)). Rats in the other groups were injected with saline. At 22 h after the injection of GalN/LPS, rats from each group were anaesthetised and blood was collected by cardiac puncture.

Table 1. Composition of the diets (%)

Groups...	CON	GeSP	GalN/LPS CON	GalN/LPS SP	GalN/LPS GeSP
n	6	6	7	7	7
Casein	20	17	20	17	17
α-Maize starch:sucrose (2:1)	65·5	62·5	65·5	62·5	62·5
Maize oil	5	5	5	5	5
Cellulose	5	5	5	5	5
Vitamin mixture*	3·5	3·5	3·5	3·5	3·5
Mineral mixture†	1	1	1	1	1
SP				5	
GeSP‡		5			5

GalN/LPS, D-galactosamine and lipopolysaccharide; CON, group fed on basal diet; GeSP, germanium-containing *Spirulina*; SP, *Spirulina*.
* AIN-93G-MX, which contained 25 g bitartrate/100 g, were obtained from Oriental Yeast Company Limited.
† AIN-93-VX, which contained 25 g bitartrate/100 g, were obtained from Oriental Yeast Company Limited.
‡ The diet that included GeSP contained 30 parts per million germanium.

Plasma was prepared by centrifuging the heparinised whole blood at 1000 **g** for 20 min. A portion of the liver was excised, quickly frozen in liquid N_2 and stored at −80°C until use.

Measurement of plasma enzyme activities and cytokine levels

The activities of alanine transaminase, aspartate aminotransferase and lactate dehydrogenase were measured enzymatically with a commercial kit, i.e. the Transaminase CII test and LDH-UV test, respectively (Wako Pure Chemicals).

IFN-γ, IL-10 and TNF-α levels were also measured by using commercial ELISA kits, i.e. the Amersham Interferon Gamma Rat Biotrak ELISA System, the Amersham Interleukin-10 Rat Biotrak ELISA System and the Amersham Tumor necrosis factor Alpha Rat Biotrak ELISA System, respectively (GE Healthcare).

Measurement of liver antioxidant enzyme activities

Liver samples (0·6 g) were homogenised with a 5-fold volume of potassium phosphate buffer (0·1 M, pH 7·4, containing 1 mM-EDTA) and a 2-fold volume of KCl (2·3 %), followed by centrifugation at 10 000 **g** for 20 min at 4°C. The supernatants obtained were used for the measurement of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activities.

Superoxide dismutase activity was measured by using the xanthine/xanthine oxidase system[21]. Catalase activity was determined by using the method of Chance & Meahly[22], i.e. measuring the decrease in absorbance at 240 nm due to the decomposition of H_2O_2. Glutathione peroxidase activity was determined from the decrease in the NADPH level due to the reaction[23,24]. Glutathione reductase activity was also measured by monitoring the decrease in the absorbance of NADPH at 340 nm by using oxidised glutathione as a substrate[25]. Protein concentrations were determined by using the method of Lowry *et al.*[26], using bovine serum albumin as the standard.

Measurement of the levels of glutathione, thiobarbituric acid-reactive substances, α-tocopherol and ascorbic acid

To measure the levels of the reduced form of glutathione and the oxidised form of glutathione (GSSG), homogenates were prepared by homogenising liver samples with a 10-fold volume of 0·036 M-perchloric acid. The supernatant obtained by centrifuging the homogenates at 13 500 g for 5 min at 4°C was filtered through a 0·2 μm filter. Reduced form of glutathione and GSSG contents in the filtrate were determined by ion-pairing reverse-phase HPLC coupled to a coulometric detector according to the method of Harvey *et al.*[27].

The concentration of TBARS was measured according to the method of Uchiyama & Mihara[28], using a homogenate prepared by mixing 0·5 g of the frozen liver sample with a 9-fold volume of a cold solution of 1·15 % KCl.

Liver α-tocopherol and ascorbic acid levels were determined by using HPLC, according to the method of Sugimoto *et al.*[29].

Statistical analyses

The normality of the animal distribution was tested on the body weight of the rats using a one-way ANOVA with Tukey's test. Results were analysed using Student's t test if two groups (CON and GeSP groups) were compared or by a two-way ANOVA followed by Tukey's test if three groups (among the GalN/LPS groups) were tested. If variances were inhomogeneous in Student's t test, results were analysed using the Welsh test. Data are expressed as means with their standard errors. A P value of less than or equal to 0·05 was considered as significant.

Results and discussion

The protective effects of SP and GeSP against GalN/LPS-induced liver injury in rats are given in Table 2. No differences were observed in body-weight gain and food intake among the groups, regardless of the treatment with GalN/LPS. Plasma alanine transaminase, aspartate aminotransferase and lactate dehydrogenase activities were significantly lower in the GalN/LPS-GeSP group than in the GalN/LPS-CON group. Although the alanine transaminase activity in the GalN/LPS-SP group had a tendency to decrease ($P<0·1$), the aspartate aminotransferase activity was not different from that in the GalN/LPS-CON group. Lactate dehydrogenase activities in the GalN/LPS-SP and GalN/LPS-GeSP groups were significantly lower than that in the GalN/LPS-CON group; however, there was no difference between the GalN/LPS-SP and GalN/LPS-GeSP groups. Although a histological evaluation of the liver was not performed in the present study, previous studies have shown that there were no histopathological differences in the liver of rats fed SP or GeSP when compared with rats fed a normal diet[20,30]. With regard to parameters related to liver injury, the present results suggest that the liver of rats in the GalN/LPS-SP and GalN/LPS-GeSP groups was not as damaged as the liver of rats in the GalN/LPS-CON group. In particular, the results for aminotransferase activities indicate that liver damage in rats fed the GalN/LPS-GeSP diet was milder than that in rats fed the GalN/LPS-SP diet.

Feeding of GeSP significantly inhibited the GalN/LPS-mediated increase in plasma IFN-γ and TNF-α levels. The levels of these cytokines tended to be lower in the GalN/LPS-SP group than in the GalN/LPS-CON group ($P<0·1$). Moreover, plasma levels of IL-10 in the GalN/LPS-SP and GalN/LPS-GeSP groups were significantly elevated compared with those in the GalN/LPS-CON group. The animals injected with GalN/LPS have been reported to develop severe liver injury, which is dependent on the induction of TNF-α and IFN-γ[31,32], whereas IL-10 protects from liver injury through reducing the mRNA expression of IFN-γ and TNF-α[33]. These results support the possibility that the administration of SP and GeSP protects against liver damage induced by the injection of GalN/LPS; furthermore, the results show that GeSP was more effective in alleviating liver damage than SP.

GalN/LPS-induced liver injury is also known to cause increased formation of reactive oxygen species, followed by hepatocyte death[2,3]. Therefore, we analysed the activities of liver antioxidant enzymes and glutathione levels. Superoxide dismutase and catalase activities did not differ between the CON and GeSP groups that received no GalN/LPS treatment. The increased catalase and glutathione peroxidase activities observed following the injection of GalN/LPS were decreased in the group fed on GeSP. Although GSSG levels did not differ between the two groups that were not injected with GalN/LPS, the GSSG level in the GalN/LPS-GeSP group was significantly lower than that in the GalN/LPS-CON group. It has been reported that GSSG levels are increased by oxidative stress[29]. These results may suggest that SP, especially GeSP, ameliorates oxidative stress induced by the injection of GalN/LPS. However, GeSP tended to reduce the activities of antioxidant enzymes (glutathione peroxidase and glutathione reductase) in the absence of GalN/LPS treatment. At present, the mechanism by which GeSP influences these enzymes is unclear. Goodman[16] suggested that organic germanium (bis(carboxylgermanium) sesquioxide) might function as an electron donor. Thus, GeSP itself may also act as an antioxidant in the body. A study has reported that high amounts of phycocyanobilin present in *Spirulina* inhibit the activity of NADPH oxidase[34]. Moreover, various studies have suggested that germanium compounds may have protective effects against liver injury, as well as antioxidant effects[17,19,20]. Thus, GeSP is believed to exert synergistic effects of both *Spirulina* and germanium.

Furthermore, liver α-tocopherol, ascorbic acid and TBARS levels were measured to determine the status of oxidative stress in the liver. There were no differences in the levels of α-tocopherol and ascorbic acid in the CON and GeSP groups that were not injected with GalN/LPS. However, their levels decreased in rats injected with GalN/LPS and the levels improved in those fed with GeSP, but not with SP. These (α-tocopherol and ascorbic acid) might not be consumed, because Gesp itself functions as an antioxidant in the body. The level of TBARS in the GalN/LPS-GeSP group had the tendency to decline compared with that in the GalN/LPS-CON group ($P=0·102$), but there was no significant difference between the groups. These results suggest that the high oxidative status of the rat liver following the injection of

Table 2. Effects of dietary *Spirulina* (SP) and germanium-containing *Spirulina* (GeSP) on D-galactosamine- and lipopolysaccharide-induced hepatitis in rats

(Mean values with their standard errors)

	CON (*n* 6)		GeSP (*n* 7)		GalN/LPS CON (*n* 6)		SP (*n* 7)		GeSP (*n* 7)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Initial body weight (g)	88·9	2·1	88·9	1·6	88·8	1·6	88·8	1·3	88·8	1·9
Body-weight gain (g/8 d)	32·7	1·1	34·5	1·0	28·5	1·5	28·5	1·2	30·0	1·9
Food intake (g/8 d)	95·8	0·5	96·9	0·3	91·7	1·8	92·2	1·6	93·1	1·2
Plasma ALT (IU/l)	42·5	5·2	44·5	4·3	1052	187	614†	86	509*	88
Plasma AST (IU/l)	65·9	5·0	50·5	2·7	2183	368	1380	187	1170*	196
Plasma LDH (μmol/min per litre)	324	27	371	30	40 006	10 259	9273**	1541	5430**	1249
Plasma IFN-γ (pg/ml)	82·3	13·9	92·2	16·2	142·8	17·5	101·1†	18·9	66·8**	9·7
Plasma IL-10 (pg/ml)	33·1	13·2	41·8	9·1	80·4	10·3	109·9*	14·9	148·9**	5·3
Plasma TNF-α (pg/ml)	14·4	1·0	16·7	2·6	72·3	15·4	45·1†	8·7	31·2**	6·8
Liver SOD (U/mg protein)	3·25	1·04	1·74	0·28	4·42	1·11	2·69	0·40	1·94†	0·37
Liver CAT (U/mg protein)	254	30	247	38	457	47	373	63	262*	54
Liver GPx (U/mg protein)	0·90	0·25	0·46††	0·05	1·30	0·11	0·88†	0·13	0·53*	0·09
Liver GSSG-R (U/μg protein)	38·4	4·1	16·3††	4·1	65·89	29·71	22·62	3·25	9·72†	2·47
Liver GSH (mmol/kg liver)	3·07	0·30	3·58	0·17	2·75	0·25	2·92	0·29	2·71	0·27
Liver GSSG (mmol/kg liver)	0·97	0·24	1·03	0·16	2·18	0·33	1·53†	0·09	1·31*	0·24
Liver α-tocopherol (μg/g liver)	26·9	1·8	27·1	2·1	22·8	1·9	27·4	2·1	32·7*	3·5
Liver ascorbic acid (μg/g liver)	222	13	219	13	144	13	184	12	224**	21
Liver TBARS (nmol/g liver)	96·4	4·7	88·3	7·4	110·1	6·6	100·3	3·2	96·8	3·9

GalN/LPS, D-galactosamine and lipopolysaccharide; CON, group fed on basal diet; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; IFN-γ, interferon-γ; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GSSG-R, glutathione reductase; GSH, reduced form of glutathione; GSSG, oxidised form of glutathione; TBARS, thiobarbituric acid-reactive substances.

Mean values were significantly different from those of the GalN/LPS-CON group: † $P < 0·1$, * $P < 0·05$, ** $P < 0·01$.

†† Mean values were significantly different from those of the CON group ($P < 0·1$).

GalN/LPS was improved by the intake of GeSP. However, the intake period employed in the present study might be too short to have an effect on the lipid peroxide level.

The present study suggests that the intake of GeSP affects the activities of antioxidant enzymes and reduces oxidative stress. Furthermore, it indicates that dietary GeSP exerts a stronger protective effect than SP against liver injury induced by the injection of GalN/LPS. Thus, dietary GeSP could provide beneficial effects to human health.

Acknowledgements

References

1. Bélanger M & Butterworth RF (2005) Acute liver failure: a critical appraisal of available animal models. *Metab Brain Dis* **20**, 409–423.
2. Oberholzer A, Oberholzer C, Bahjat FR, et al. (2001) Genetic determinants of lipopolysaccharide and D-galactosamine-mediated hepatocellular apoptosis and lethality. *J Endotoxin Res* **7**, 375–380.
3. Liu LM, Zhang JX, Luo J, et al. (2008) A role of cell apoptosis in lipopolysaccharide (LPS)-induced nonlethal liver injury in D-galactosamine (D-GalN)-sensitized rats. *Dig Dis Sci* **53**, 1316–1324.
4. Parikh P, Mani U & Iyer U (2001) Role of *Spirulina* in the control of glycemia and lipidemia in type 2 diabetes mellitus. *J Med Food* **4**, 193–199.
5. Ishimi Y, Sugiyama F, Ezaki J, et al. (2006) Effects of *spirulina*, a blue-green alga, on bone metabolism in ovariectomized rats and hindlimb-unloaded mice. *Biosci Biotechnol Biochem* **70**, 363–368.
6. Hirahashi T, Matsumoto M, Hazeki K, et al. (2002) Activation of the human innate immune system by *Spirulina*: augmentation of interferon production and NK cytotoxicity by oral administration of hot water extract of *Spirulina platensis*. *Int Immunopharmacol* **2**, 423–434.
7. Lu J, Ren DF, Wang JZ, et al. (2010) Protection by dietary *Spirulina platensis* against D-galactosamine and acetaminophen-induced liver injuries. *Br J Nutr* **103**, 1573–1576.
8. González R, González A, Remirez D, et al. (2003) Protective effects of phycocyanin on galactosamine-induced hepatitis in rats. *Biotecnol Aplicada* **20**, 107–110.
9. Puyfoulhoux G, Rouanet JM, Besançon P, et al. (2001) Iron availability from iron-fortified *spirulina* by an *in vitro* digestion/Caco-2 cell culture model. *J Agric Food Chem* **49**, 1625–1629.
10. Cases J, Vacchina V, Napolitano A, et al. (2001) Selenium from selenium-rich *Spirulina* is less bioavailable than selenium from sodium selenite and selenomethionine in selenium-deficient rats. *J Nutr* **131**, 2343–2350.
11. Cases J, Wysocka IA, Caporiccio B, et al. (2002) Assessment of selenium bioavailability from high-selenium *spirulina* subfractions in selenium-deficient rats. *J Agric Food Chem* **50**, 3867–3873.
12. Zhou Z, Wang L, Song Z, et al. (2004) Abrogation of nuclear factor-kappaB activation is involved in zinc inhibition of lipopolysaccharide-induced tumor necrosis factor-alpha production and liver injury. *Am J Pathol* **164**, 1547–1556.
13. Krecic-Shepard ME, Shepard DR, Mullet D, et al. (1999) Gallium nitrate suppresses the production of nitric oxide and liver damage in a murine model of LPS-induced septic shock. *Life Sci* **65**, 1359–1371.
14. Lin CH, Chen TJ & Chen SS (2009) Functional changes on ascending auditory pathway in rats caused by germanium dioxide exposure: an electrophysiological study. *Toxicology* **256**, 110–117.
15. Nakamura T, Saito M & Aso H (2012) Effects of a lactobacilli, oligosaccharide and organic germanium intake on the immune responses of mice. *Biosci Biotechnol Biochem* **76**, 375–377.
16. Goodman S (1988) Therapeutic effects of organic germanium. *Med Hypotheses* **26**, 207–215.
17. Alil MM, Noaman E, Kamal S, et al. (2007) Role of germanium L-cysteine alpha-tocopherol complex as stimulator of some antioxidant defense systems in gamma-irradiated rats. *Acta Pharm* **57**, 1–12.
18. Yokochi S, Ishiwata Y, Hashimoto H, et al. (1998) Hepatoprotective effect of propagermanium on *Corynebacterium parvum* and lipopolysaccharide-induced liver injury in mice. *Scand J Immunol* **48**, 183–191.
19. Ishiwata Y, Yokochi S, Hashimoto H, et al. (1998) Protection against concanavalin A-induced murine liver injury by the organic germanium compound, propagermanium. *Scand J Immunol* **48**, 605–614.
20. Orimo H, Onda K, Kaseki H, et al. (1984) The chronic intoxication test on germanium contained *Spirulina* product fed to Wistar strain rats. *Jpn J Nutr* **42**, 43–49 (in Japanese).
21. Imanari T, Hirota M, Hayakawa K, et al. (1997) Improved assay method of superoxide dismutase. *Igaku no Ayumi* **101**, 496–497 (in Japanese).
22. Chance B & Meahly AC (1955) *Methods in Enzymology*, vol. 2, pp. 764–775. New York: Academic Press.
23. Paglia DE & Valentine WN (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* **70**, 158–169.
24. Whanger PD, Weswing PH, Schmitz JA, et al. (1977) Effects of selenium and vitamin E on blood selenium levels, tissue glutathione peroxidase activities and white muscle disease in sheep fed purified or hay diets. *J Nutr* **107**, 1298–1307.
25. He P & Yasumoto K (1990) Age-associated changes in glutathione peroxidase and oxidized protein in erythrocytes of senescence-accelerated mice. *Nippon Eiyo Shokuryo Gakkaishi* **43**, 121–125 (in Japanese).
26. Lowry OH, Rosebrough NJ, Farr AL, et al. (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.
27. Harvey PR, Ilson RG & Strasberg SM (1989) The simultaneous determination of oxidized and reduced glutathiones in liver tissue by ion pairing reverse phase high performance liquid chromatography with a coulometric electrochemical detector. *Clin Chim Acta* **180**, 203–212.
28. Uchiyama M & Mihara M (1978) Determination of malonaldehyde precursor in tissue by thiobarbituric acid test. *Anal Biochem* **86**, 271–278.
29. Sugimoto E, Igarash K & Takenaka A (2003) Preventive effect of dietary nasunin on galactosamine-induced liver injury in rats. *Food Sci Technol Res* **9**, 94–99.
30. Yoshino Y, Hirai Y, Takahashi T, et al. (1980) The chronic intoxication test on *Spirulina* product fed to Wistar-strain rats. *Jpn J Nutr* **38**, 221–225 (in Japanese).

31. Josephs MD, Bahjat FR, Fukuzuka K, *et al.* (2000) Lipopoly-saccharide and D-galactosamine-induced hepatic injury is mediated by TNF-alpha and not by Fas ligand. *Am J Physiol Regul Integr Comp Physiol* **278**, R1196–R1201.

32. Kim WH, Hong F, Radaeva S, *et al.* (2003) STAT1 plays an essential role in LPS/D-galactosamine-induced liver apoptosis and injury. *Am J Physiol Gastrointest Liver Physiol* **285**, G761–G768.

33. Nagaki M, Tanaka M, Sugiyama A, *et al.* (1999) Interleukin-10 inhibits hepatic injury and tumor necrosis factor-alpha and interferon-gamma mRNA expression induced by staphylococcal enterotoxin B or lipopolysaccharide in galactosamine-sensitized mice. *J Hepatol* **31**, 815–824.

34. Ravi M, De Lata S, Azharuddin S, *et al.* (2010) The beneficial effects of *Spirulina* focusing on its immunomodulatory and antioxidant properties. *Nutr Diet Suppl* **2**, 73–83.

British Journal of Nutrition