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Antioxidative Effect of Enzymatic Hydrolysate of Horn and Hoof in Rat

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The antioxidative effects of enzymatic hydrolysate of horn and hoof (EHHH) from cow and buffalo, keratin-containing livestock waste, were studied using rat with carbon tetrachloride (CT)-induced liver injury. There was no effect on rat growth such as body weight gain, kidney or liver weight, when rats were fed on a diet containing EHHH. When liver injury was induced in rats using CT, the value of rat liver thiobarbituric acid-reactive substances (TBARS) increased. However, EHHH effectively reduced the value of the liver TBARS. Furthermore, EHHH restored the liver mitochondria catalase activity, which was reduced by CT.

Keywords: horn and hoof, enzymatic hydrolysate, carbon tetrachloride induced-liver injury, antioxidant activity

Many studies have been performed on the enzymatic hydrolysates of proteins. The physiological functions present in peptides were found to be produced from proteins by enzymatic hydrolysis. Studies on the physiological functions of peptides reported HIV-I protease inhibiting activity (Lee & Maruyama, 1998), angiotensin I-converting enzyme inhibiting activity (Maruyama & Suzuki, 1982; Maruyama *et al*., 1985; Yokokawa *et al*., 1992) and antioxidative effects (Hua-Ming *et al*., 1995; Mosammat *et al*., 1999).

Large quantities of livestock waste such as horn, hoof, bone and feather are produced during food processing and meat processing. These wastes contain large amounts of the proteins keratin and collagen, and can be utilized as protein sources for new food materials. Keratin is present at high levels in animal horns and hoofs and in feathers. Since proteins in these wastes are hard proteins, their treatment is very difficult. Thus, only a part of this livestock waste is used as feed and foaming agents for fire extinguishers (Hoshino, 1976). Kida *et al*. (1995) developed an apparatus and set of conditions for enzymatic hydrolysis to effectively utilize the protein of horn and hoof, and were able to produce an enzymatic hydrolysate. Morimura *et al*. (2002) developed an efficient extraction process for collagen from pig skin and fish bone, and studied the physiological functions of the produced peptide. Furthermore, Ohba *et al*. (2003) found that enzymatic hydrolysates of collagen and keratin derived from livestock waste and fish waste have physiological functions of antioxidative activity and ACE inhibition activity.

The administration of carbon tetrachloride (CT) is known to cause liver injury in rats. The CT is metabolized within the liver cells, then chlorine radicals are generated, and these radicals cause liver injury (Koga *et al*., 2001). Miyazawa *et al*. (1990) reported that thiobarbituric acid-reactive substance (TBARS)-values of the liver were increased by the administration of CT.

In the present study, we investigated the antioxidative effects of EHHH using rats with CT-induced liver injury.

Materials and Methods

Materials and chemicals A mixture of horn and hoof from cow and buffalo provided by Nishida Co. (Kumamoto) was used. CT and olive oil were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). All other chemicals were purchased from Nacalai Tesque Co. (Kyoto).

Preparation of enzymatic hydrolysate Enzymatic hydrolysate of horn and hoof (EHHH) was prepared according to the method of Kida *et al*. (1995). The enzyme used was sabinase 16. OL (Novozymes Japan, Ltd., Chiba) derived from *Bacillus subtilis*. A mixture of horn and hoof was pretreated under a pressure of 7 kgf/cm^2 at 170 with steam for 1.5 h, and then powdered by crusher (R-8, Nihon Rikagaku Kikai, Co. Ltd., Tokyo). This powder was hydrolyzed by the enzyme at 60˚C and pH 8.3 for 1 h. After enzymatic hydrolysis, the reaction mixture was separated continuously by centrifugation (10,000×*g*), and then the supernatant was concentrated using ultrafiltration (molecular weight cut-off Ca. 20,000). This concentrate was lyophilized and called UF-concentrate. Then, the permeate of ultrafiltration was concentrated using a reverse osmosis-membrane and the concentrate was lyophilized and called RO-concentrate. In a preliminary experiment *in vitro*, the antioxidative activity of RO-concentrate was stronger than that of UF-concentrate. Further experiments used the RO-concentrate of EHHH.

Animal experiments Five-week-old male Wistar rats (body weight 120–130 g) were obtained from Kyodo Co. (Kumamoto). In the preliminary breeding, all the rats were fed a commercial standard diet (CLEA Rodent Diet CE-2, CLEA JAPAN Inc. Tokyo) for 5 days before use in the experiment. The

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Table 1. Effects of enzymatic hydrolysate of hoon and hoof on the body weight, liver and kidney of rats.

	Group No.			
	1	2	3	4
Body weight (g)[a)]	187.6± 8.27	191.0± 6.20	191.3± 6.14	182.4± 5.10
Body weight (g)[b)]	169.4± 8.57	172.3± 5.01	174.2± 3.74	164.7± 4.87
Liver (g)	6.1± 0.60*	6.1± 0.42*	10.8± 0.78	10.8± 0.87
Kidney (g)				
Right	0.9± 0.07	0.9± 0.14	1.0± 0.05	1.0± 0.19
Left	0.8± 0.10	0.9± 0.09	1.0± 0.08	1.0± 0.13

[a)]Before administration of CCl_4.
[b)]Before dissection.
Group 1 and 3 were fed a standard diet. Group 2 and 4 were fed a standard diet containing EHHH. Group 1 and 2 were administrated olive oil. Group 3 and 4 were administrated a mixture of CT and olive oil. Results are expressed as means±SD (n=5). Asterisks were indicating significant differences from group 3 (p<0.001)

rats were kept under the conditions of a 12-h light/ dark cycle (08:00–20:00 light) at 22˚C. After the preliminary feeding, the rats were classified into four groups (5 rats/group). Groups 1 and 3 were fed commercial standard diet, while Groups 2 and 4 were fed commercial standard diet containing 3% enzymatic hydrolysate-RO concentrate of horn and hoof. Water was provided freely during the experiment. On day 6 of feeding, Groups 1 and 2 were given olive oil (0.6 ml/100 g body weight) through a stomach tube, while groups 3 and 4 were given a mixture of CT and olive oil (0.2 ml-CT/0.6 ml-olive oil/100 g body weight). However, all the rats were fasted from 12 h before to 24 h after the administration of CT. Twenty-four hours after the CT treatment, the rats were sacrificed under anesthesia with ether, and the liver and kidney were excised and immediately weighed, and stored at −80˚C until needed for measuring the catalase activity and TBARS. All measured values were analyzed statistically.

Measurement of liver TBARS The liver TBARS were determined by the method of Uchiyama and Mihara (1978). The rat liver was homogenized with cold 1.15% KCl solution to make a 10% homogenate. Next, phosphate solution (1%, 0.5 ml) and TBA solution (0.6%, 1.0 ml) were added to the 10% rat liver homogenate (0.5 ml) and the mixture was reacted in a boiling water bath for 45 min. After cooling, *n*-butanol (4.0 ml) was added to the reaction mixture and mixed vigorously. The butanol phase was separated by centrifugation at 2000×g for 15 min and the absorbance was measured at 535 nm and 520 nm using a spectrophotometer. The difference in absorbance was used as the TBA value. The protein contents of the sample were determined by the method of Lowry-Folin (Folin *et al*., 1927; Lowry *et al*., 1951). The TBARS value is shown as nmol of malondialdehyde per mg of protein.

Measurement of liver mitochondria catalase activity To determine the catalase activity, a rat liver mitochondria fraction was prepared by the method of Del Boccio *et al*. (1990). The right liver lobe (0.5 g) was homogenized in 2.5 ml of an ice-cold 0.1 M phosphate buffer (pH 7.4) containing 1.0 mM EDTA. The homogenate was mixed with 2.3% KCl solution (5.0 ml), and then the mixture was centrifuged at 600×g for 3 min at 4˚C to remove the fibrous materials. The supernatant was recentrifuged at 1400×g for 10 min at 4˚C to remove the cellular debris. The supernatant was subsequently sonicated at 30 W for 2 min at 4˚C, and then the suspension was centrifuged at 10,000×g for 30 min at 4˚C to obtain a precipitate of the mitochondrial fraction. This precipitate was suspended in 0.1 M phosphate buffer (pH 7.4) and the catalase activity was determined by the method of Tomita and Sano (1983) with modifications. Hydrogen peroxide

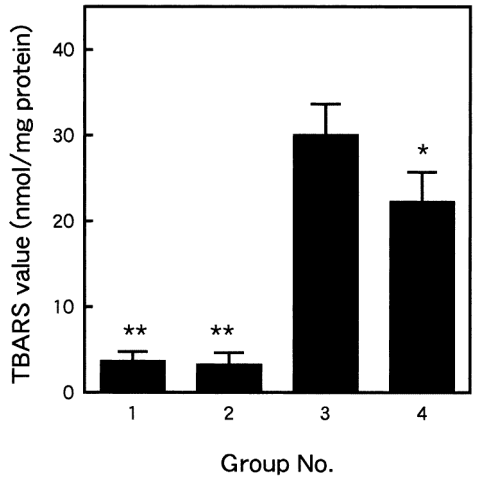

Fig. 1. Effect of enzymatic hydrolysate of EHHH on the liver TBARS in CT-treated rats. Group No. is shown in Table l. Results were expressed as means±SD. (n=5). Asterisks indicate significant differences from Group 3. (*), p<0.05; (**), p<0.001.

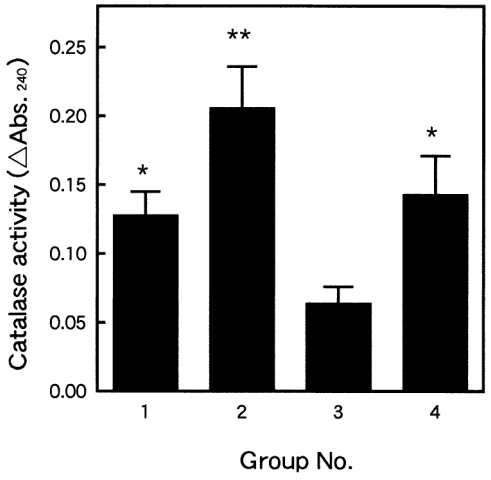

Fig. 2. Effect of enzymatic hydrolysate EHHH on the liver mitochondria catalase in CT-treated rats. Group No. is shown in Table l. Results were expressed as means±SD. (n=5). Asterisks indicate significant differences from Group 3. (*), p<0.05; (**), p<0.001.

(30 mM, 1.0 ml) was added to the suspension of the mitochondrial fraction (2.0 ml) and mixed immediately. The decrease in the absorbance of the reaction mixture at 240 nm after 60 s was measured and taken as the catalase activity. The blank used was 0.1 M phosphate buffer (pH 7.4) instead of the hydrogen peroxide.

Statistical analyses Each value is shown as mean±standard deviation (SD). All date were evaluated by two-way ANOVA and Student's *t*-test to define the significance of differences ($p<0.05$) between means.

Results and Discussion

Effects of the EHHH on growth of rats As shown in Table 1, the liver and kidney weights of rats fed a diet containing EHHH (Group 2) were no different from those of standard rats (Group 1). However, when CT was given to rats, the liver weight increased significantly (Group 3, 4). We consider that the CT is metabolized within the liver cells, then chlorine radicals are generated, and these oxidative stress cause hypertrophy of the liver. There was no difference on body weight gain, however food intake in the rats fed a standard diet and diet containing EHHH (data not shown). Thus, we considered that EHHH had no effect on the growth of rat growth.

Effect of the EHHH on rat liver TBARS Suda *et al.* (1997) have reported that the liver TBARS value increased after administration of CT. Thus, we examined the effects of EHHH on rat liver TBARS. As shown in Fig.1, when rats were fed a diet containing EHHH (Group 2), there was no difference in the liver TBARS values compared with those of standard rats (Group 1). When CT was given to rats, the liver TBARS values increased significantly (Group 3), whereas the increases in these values were controlled by feeding with EHHH (Group 4). These results indicate the possibility that EHHH can suppress the increase in liver lipid peroxidation due to CT.

Effect of the EHHH on rat liver mitochondria catalase activity In general, catalase is known to decompose hydrogen peroxide *in vivo*, and to suppress lipid peroxidation (Nakano, 1991; Takeda & Samejima, 1991). Thus, we studied the effects of EHHH on the catalase activity in rat liver mitochondria fraction. As shown in Fig. 2, when CT was given to rats, catalase activity in the rat liver mitochondria fraction decreased by about half (Group 3). However, this decrease in activity was restored to the same level as that of standard rats by feeding with the EHHH. Furthermore, the catalase activity of rats fed a diet containing EHHH (Group 2) was enhanced by about 60% compared with that of standard rats (Group 1). Therefore, it was found that EHHH restored the liver mitochondria catalase activity reduced by the administration of CT.

These results demonstrated the antioxidative effects of EHHH against CT-induced oxidative stress *in vivo*. In these experiments, since the rats were fasted before and after the administration of CT, the EHHH component was believed not to have inhibited the uptake of CT. Thus, EHHH, which was ingested in advance in the animals, is considered to have reduced the CT-induced liver injury. In general, keratin contains large amounts of cysteine as a constituent amino acid and cysteine was reported to show high antioxidative activity (Yamashoji *et al.*, 1979). Thus, it is considered that EHHH has antioxidative activity. However, to clarify the antioxidative mechanism of EHHH *in vivo*, further studies are necessary.

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