Int. J. Indust. Entomol. Vol. 2, No. 1, 2001, pp. 15~18

International Journal of *Industrial Entomology*

Effect of Silk Fibroin on the Protection of Alcoholic Hepatotoxicity in the Liver of Alcohol Preference Mouse

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(Received 5 December 2000; Accepted 20 January 2001)

Silk fibroin (SF) derived from the domestic silk worm, *Bombyx mori*, is the natural protein and widely used as bio-functional materials as well as apparels. We studied the liver protective effect of SF from alcohol-induced hepatotoxicity in the alcohol preference mouse. To increase more absorption of SF in experimental animals, molecular weight of SF was lowered by 2N of HCl aqueous solution at 100°C for 48 hrs. SF was added to liquid diet with alcohol and fed to the alcohol preference mice for 4 weeks. To assess the liver function, the concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and cholesterol present in either blood or liver tissue were measured. As compared with non-SF treated group, the SF-treated showed significantly low concentrations of ALT, AST, cholesterol and triacylglycerol values, respectively. Histopathological examination revealed that the extent of hepatocyte injury in the SF-treated group was reduced when it was compared with non SF-treated group. These results suggest that SF may have liver protective effects against alcohol-induced hepatotoxicity.

Key words : Silk fibroin, Alcohol, Hepatotoxicity, Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Cholesterol, Triacylglycerol.

Introduction

Silk fibroin (SF) acquired from the domestic silk worm, *Bombyx mori* is a natural protein with high molecular weight (about 370 KDa) and it possesses a property of high crystallinity (Nahm and Shin, 1998). It was known that the repeat unit of crystalline region of SF is (-Gly-Ala-Gly-Ala-Gly-X-)n, where X=Ala or Ser (Bhat and Nadiger, 1980; Sakabe *et al.*, 1989). Recent studies on applications of SF showed that it can be used in biomaterial fields, such as cosmetics (Komatsu, 1999), biosensors to which enzyme is immobilized (Yu *et al.*, 1995; Demura and Asakura, 1989), dressing matrix for wound (Minoura *et al.*, 1995; Gotoh *et al.*, 1998) and drug release matrix (Hanawa *et al.*, 1995; Min *et al.*, 1998; Kang *et al.*, 2000). SF has also been studied health supplemental diet. Many evidences indicate that SF decreases the concentration of cholesterol in blood (Chen *et al.*, 1993; Sugiyama *et al.*, 1985), accelerates alcohol metabolism (Leu *et al.*, 1993), promotes insulin secretion (Leu *et al.*, 1993), and controls the concentration of blood glucose (Leu *et al.*, 1993).

It has been already known that some amino acid such as glycine (Yin *et al.*, 1998) and alanine (Leu *et al.*, 1993) could recover the liver injury induced by alcohol. We hypothesized that SF might have liver protective effect against alcohol-induced hepatotoxicity because SF had been mostly composed of glycine (42%) and alanine (32%) (Nahm and Shin, 1998). In this report, we tested effects of low molecular weight of SF (LMSF), which can be effectively digested and absorbed, against alcohol-induced hepatotoxicity in alcohol preference mice by assessing biochemical and histopathological evaluation of the liver function.

Materials and Methods

Preparation of low-molecular weight of SF (LMSF)

SF was prepared by degumming raw cocoon with Na_2CO_3 to remove sericin and other impurities. Pure SF was treated

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with 2 N of HCl aqueous solution at 100°C for 48 hrs and neutralized using NaOH. The salts produced were removed through ion exchange chromatography.

Animals and diet

Alcohol preference mice (C57BL/6J, 6 weeks, body weight: 20-22 g) were obtained from Center for Experimental Animal at Hallym University in Korea. Mice were housed individually in stainless steel wire cage at 20-22°C with a 12 hrs light-dark cycle (light for 07:00 A.M.-19:00 P.M.). All mice were divided into three groups, Control group (n=6), Al-AF group (n=6) (where AF represents hydrochloric acid treated fibroin), and Alcohol group (n=12). Control diet (Lieber and DeCarli Liquid Rat Diets #710027) and alcohol liquid diet (#710260) were obtained from Dyets (Pennsylvania, USA). The alcohol liquid diet is calorifically balanced to the control diet by the addition of 7% alcohol to give the final calorific content of 4.2 kJ^{-1}. The food energy components of the control diet were: 47% carbohydrate, 18% protein, 35% fat.

Chronic ethanol treatments

At the beginning of the experiment, mice in two groups (Alcohol group and Al-AF group) received 1% ethanol for 1 day, then 3% ethanol for 3 days, followed by 6% ethanol for 4 days. Finally, 10% alcohol was given for 4 weeks to carry out the experiments. LMSF was added to alcohol liquid diet and then fed Al-AF for 4 weeks. Mice in Control group received only control liquid diet. All diets were changed daily to give fresh diet to mice. Diet intakes of the experimental mice were monitored daily and body weights were checked weekly. After 4 weeks, all the animals were killed for histopathological examination of the liver.

Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma

Blood was withdrawn from suborbital sinus of the experimental animals with heparinized tubes before experiment and after 2 or 4 weeks. Plasma was separated within 30 minutes by centrifuging. The activities of ALT and AST in plasma were measured by ultraviolet rate method with Hitachi 750 auto-analyzer according to the counsel of JSCC (Japanese Society for Clinical Chemistry)

Determination of lipid accumulation in the liver

One gram of liver was homogenized in 2 ml of 0.9% sodium chloride solution and then 9ml of CM solution (chloroform : methanol=2 : 1) was added to the solution. After vigorous mixing, 6 ml of chloroform layer was taken to measure lipid amount in the liver tissue. With enzymatic colorimetric method, total cholesterol (TC) and triacylglycerol (TG) accumulated in the liver were measured with spectrophotometer (Hitachi photometer 4020, Boehringer Mannhem) at 505 nm and 550 nm, respectively.

Histopathological investigation

The liver tissue was fixed in 4% neutral-buffered formaldehyde, dehydrated and embedded in paraffin. Four micrometers paraffin sections were cut and stained with hematoxylin-eosin to assess steatosis, inflammation and necrosis.

Statistical analysis

Means of the values of ALT, AST, TC and TG between two groups were compared by use of Students *t*-test from EXCEL program. Test of significance of the estimates between two groups was carried out at $p<0.01$ and $p<0.05$ according to student *t*-test.

Results

Activities of ALT and AST in plasma

When hepatocytes are broken by the external stimulations such as alcohol or virus, ALT and AST are released and flow into blood. As the liver is injured, the levels of ALT and AST in blood are known to increased (Lindros and Jarvelainen, 1998). Fig. 1 and 2 show the concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma, which are often used as standard markers for the liver function. Before feeding alcohol, the values of alcohol group and Al-AF did not show any difference compared with control group. However, after 2 weeks, the values of ALT and AST of alcohol

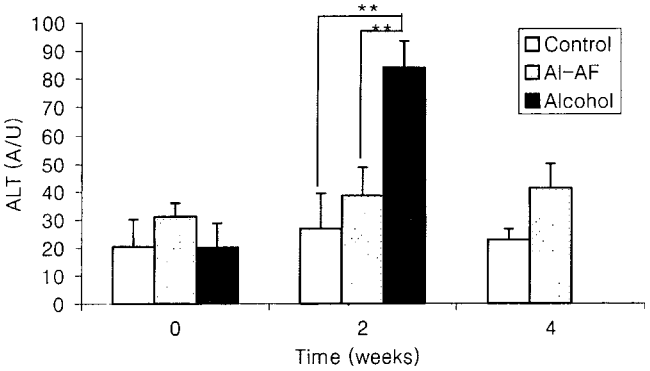

Fig. 1. Change of the concentration of alanine aminotransferase in plasma as conducting time. Control diet was fed Control group (n=6). Ten percentage of alcohol and alcohol diet with low molecules of silk fibroin (LMSF) treated with hydrochloric acid was fed Al-AF group (n=6). Alcohol and alcohol liquid diet without any LMSF were fed alcohol group (n=12). **significant at $p < 0.01$ according to student *t*-test.

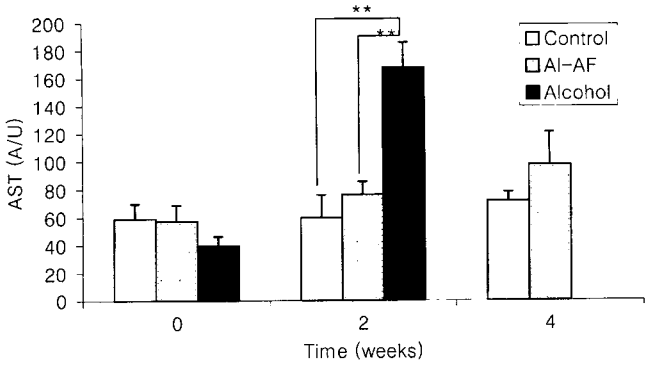

Fig. 2. Change of the concentration of aspartate aminotransferase in plasma as conducting time. Control diet was fed control group (n=6). Ten percentage of alcohol and alcohol diet with low molecules of silk fibroin (LMSF) treated with hydrochloric acid was fed Al-AF group (n=6). Alcohol and alcohol liquid diet without any LMSF were fed alcohol group (n=12). **significant at $p < 0.01$ according to student *t*-test.

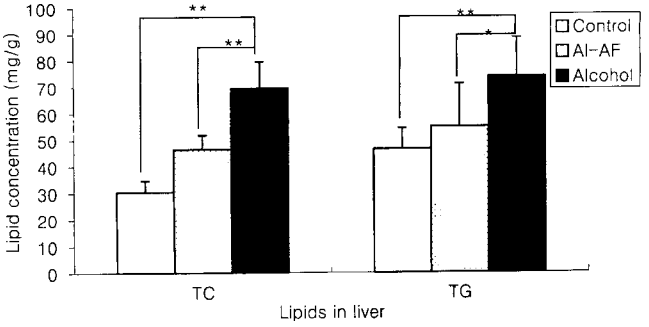

Fig. 3. Values of total cholesterol (TC) and triacylglycerol (TG) in the liver after 4 weeks except alcohol group (after 2 weeks). Control diet was fed control group (n=6). 10% of alcohol and alcohol diet with low molecules of silk fibroin (LMSF) treated with hydrochloric acid was fed Al-AF group (n=6). Alcohol and alcohol liquid diet without any LMSF were fed alcohol group (n=12). **significant at $p < 0.01$ and *significant at $p<0.05$ according to student *t*-test.

group increased significantly, whereas those of AL-AF group remained in similar ranges as to control group. After 4 weeks, the values of Al-AF group remained at the similar concentrations compared to those of control group. The values of alcohol group after 4 weeks were not able to measure because all the animals in alcohol group died before experiment was completed.

Lipid accumulations in liver

The accumulated amounts of total cholesterol (TC) and

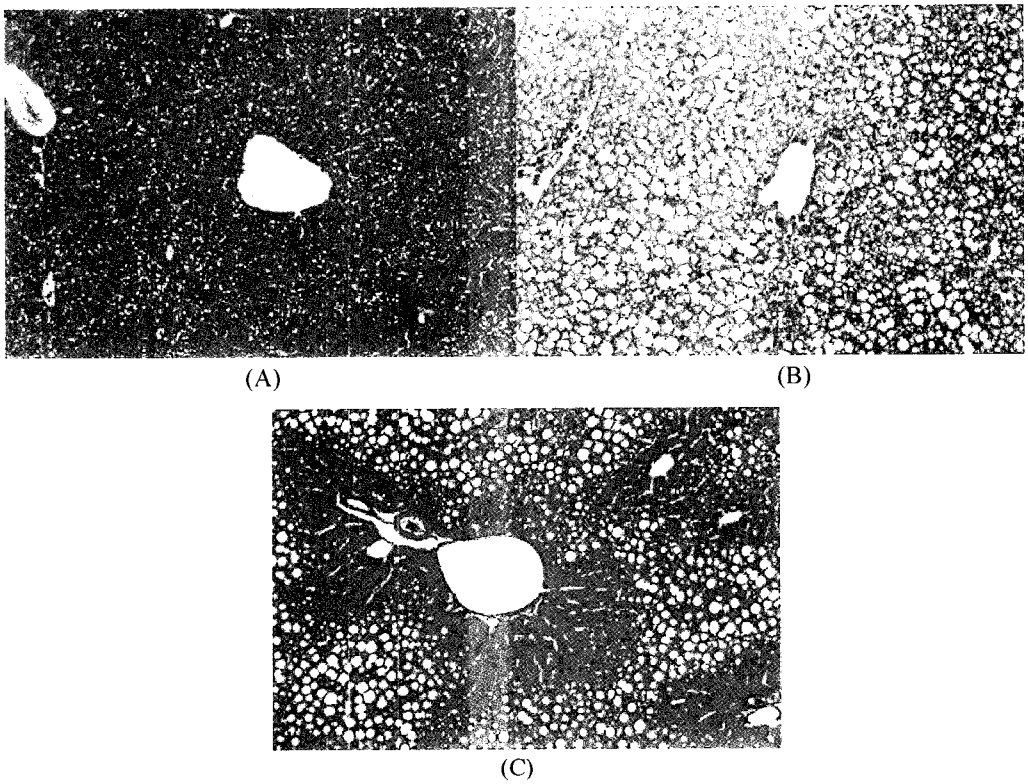

Fig. 4. Histopathology of the liver from experimental animal treated with control diet after 4 weeks (A), alcohol (10%) and alcohol diet without low molecules of silk fibroin (LMSF) after 2 weeks (B), alcohol (10%) and alcohol diet with 5% of LMSF treated with hydrochloric acid (C) after 4 weeks (H. & E.× 100).

triacylglycerol (TG) in the liver were shown in Fig. 3. It is known that tryacylglycerol accumulated in the liver, especially, is major factor of fatty liver. In alcohol group, TC and TG accumulations after 2 weeks were significantly increased compared to those of other groups after 4 weeks.

Histopathological investigation

Fig. 4 is a photomicrograph of the liver tissue from the animals in control group (Fig. 4A) after 4 weeks, alcohol group (Fig. 4B) after 2 weeks and Al-AF group (Fig. 4C) after 4 weeks. In alcohol group, marked lipid accumulation, mild inflammation and necrosis were observed. But, in Al-AF group, lipid accumulation was attenuated to moderate steatosis and mild inflammation and necrosis were observed.

Discussion

It is known that glycine can accelerate recovery from alcohol-induced liver injury because glycine could inhibit the release of TNFa from Kupffer cells by blocking the concentration of Ca^{2+} in Kupffer cells (Yin *et al.*, 1998). Moreover, because alanine can promote alcohol metabolism in the liver, the concentration of alcohol in blood can be reduced rapidly (Leu *et al.*, 1993).

More than 70% of glycine and alanine is present in SF (Nahm and Shin, 1998). But, natural SF cannot be digested in body, for its high molecular weight and high crystallinity. LMSF from hydrochloric acid treatment is digested and absorbed into body more than 90% in rat (Chen *et al.*, 1991) and free amino acids to have functionality against alcoholic injury are produced. So liver function is normalized and the values of ALT, AST in blood are mostly recovered (Fig. 1 and 2). In addition, the accumulation of triacylglycerol in liver is reduced to the level of normal condition (Fig. 3 and 4).

Conclusively, if SF composed of amino acids can be dissolved in water and its molecular weight can be lowered effectively, it can be applicable to health supplemental diet. But until now, we cannot explain exactly the mechanism that SF has a function to protect the liver against alcoholic toxicity. It is necessary to find other factors to be able to interpret the mechanism from further measurements to refer in the next report.

Acknowledgments

This work was supported by grant No. (98-2-15-01-01-2) from the University-Industry Collaborative Research program of the Korea Science and Engineering Foundation.

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