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# Hepatoprotective effect of *Arazyme* on $CCl_4$-induced acute hepatic injury in SMP30 knock-out mice

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**Abstract**

Arazyme is a novel protease produced by the *HY*-3 strain of *Aranicola proteolyticus*, which is a Gram-negative aerobic bacterium that has been isolated from the intestine of the spider *Nephila clavata*. This study focused on the hepatoprotective effect of Arazyme on carbon tetrachloride ($CCl_4$)-induced acute hepatic injury in senescence marker protein 30 (SMP30) knock-out (KO) mice and SMP30 wild-type (WT) mice. WT mice and SMP30 KO mice were divided into eight groups as follows: (i) two negative control groups (G1, G5) which were treated with a single intraperitoneal (i.p.) olive oil injection. (ii) Two positive control groups (G2, G6) which received a single i.p. $CCl_4$ (0.4 mL/kg) injection. (iii) Two vitamin C-treated groups (G3, G7) which received a single oral administration of vitamin C (100 mg/kg) and were injected with a single i.p. $CCl_4$ (0.4 mL/kg). (iv) Two Arazyme-treated groups (G4, G8) which received a single oral administration of Arazyme (500 mg/kg) and were injected with a single i.p. $CCl_4$ (0.4 mL/kg). Through present study, we could find that Arazyme-treated groups showed decreased degree of liver injury, increased expression of SMP30, decreased expression of phospho-Smad3 (p-Smad3), elevated expression of antioxidant proteins including sorbitol dehydrogenase, dihydropteridine reductase (DHPR), dehydrofolate reductase (DHFR), NADH dehydrogenase, glutathione *S*-transferase kappa 1 (GSTK1) and phospholipid hydroperoxide glutathione peroxidase (PHGPx) compared with non-Arazyme-treated groups. Therefore, it is concluded that Arazyme plays a significant role in protecting injured hepatocytes by increasing the expression of SMP30, inhibiting the transforming growth factor-β (TGF-β)/Smad pathway and elevating the expression of antioxidant proteins.


*Keywords:* Arazyme; SMP30; Hepatoprotective effect; Proteome; Antioxidant protein

## 1. Introduction

Liver injury can be induced by various factors, and hepatotoxins such as $CCl_4$, ethanol and acetaminophen which are metabolized by cytochrome P450 2E1 (CYP2E1) (Sun et al., 2001). Unstable free radicals and reactive oxygen species (ROS) are generated by this metabolic pathway (Kuzu et al., 2007) and these free radicals and ROS induce liver cell apoptosis and necrosis. The ROS and free radicals induce the up-regulation of tumor necrosis factor-alpha (TNF-α), interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) in necrotic hepatocytes. These increased cytokines accelerate the progression of liver injury from the acute stage to chronic liver disease.

Many previous studies have reported that senescence marker protein-30 (SMP30), an important 34-kDa aging marker

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molecule originally identified from rat livers, is notable for its androgen-independent decrease in the livers of aging rats (Fujita et al., 1992). SMP30 maintains calcium homeostasis (Fujita, 1999) and has gluconolactonase (GNL) activity, which is involved in L-ascorbic acid (vitamin C) biosynthesis (Kondo et al., 2006). As a result of the free radical scavenger ability of vitamin C, it is able to work as a powerful antioxidant for acute or chronic tissue injuries and organ injuries (Buettner, 1993; Buettner and Jurkiewicz, 1996). Therefore, SMP30 prevents the apoptosis and necrosis of hepatocytes by maintaining calcium homeostasis and biosynthesis of vitamin C as a powerful antioxidant in hepatocytes. However, SMP30 shows an androgen-independent decrease with age and the expression of SMP30 also decreases due to liver injury caused by lipopolysaccharide (LPS)-induced ROS, administration of $CCl_4$, and 3,3′,5-triiodo-L-thyronine (T3) treatment (Sar et al., 2007). The decrease of SMP30 expression in aging and in injury to organs such as the liver facilitates cell apoptosis and necrosis (Ishigami et al., 2002).

Many recent studies have shown that ROS are pivotal factors in the pathogenesis of liver diseases, including viral hepatitis, alcoholic hepatitis, ischemic liver injury and drug toxicity (Albano, 2002; Pessayre, 1995). Many antioxidants have been proposed for use in the prevention of acute toxic liver injury, but most antioxidants do not show sufficient protective effects in acute liver injury (Wu et al., 2007). Many studies have reported that several traditional herbal medicines have a protective effect against ROS-induced injury. However, substances such as those extract from insects have not been studied sufficiently to determine their protective effects against liver injuries. Arazyme is a novel 51.5-kDa metalloprotease that is secreted into the culture medium by the microorganism *Aranicola proteolyticus* at present called as *Serratia proteamaculans*, an aerobic Gram-negative symbiotic bacterium isolated from the intestine of the spider *Nephila clavata* (Bersanettia et al., 2005; Kwak et al., 2007). This study is focused on examining the effects of the oral administration of Arazyme produced by the *HY*-3 strain of *A. proteolyticus*, in comparison with vitamin C, on acute $CCl_4$-induced hepatic injury in SMP30 knock-out (KO) mice and wild-type (WT) mice.

## 2. Materials and methods

### 2.1. Animals and experimental design

12-Week-old, male, specific pathogen free, SMP30 KO mice ($n = 20$) and WT mice ($n = 20$) weighing 23–25 g were used in this study. The WT mice were purchased from Japan SLC, Inc. and maintained for 1 week before experimental use. The SMP30 KO mice composed of 10 male mice and 20 female mice were obtained from Tokyo Metropolitan Institute of Gerontology and housed in a room at $22 \pm 3$ °C, relative humidity $50 \pm 10\%$, a 12-h light–dark cycle and were given food and water ad libitum. The SMP30 KO mice were bred and first generation male SMP30 KO mice were used in this study. Genomic DNA of the SMP30 KO mice was purified from mouse-tail tissue using a combination of several procedures according to a literature (Henneberger et al., 2000). Genotypes were determined by PCR using the primer TA4(5′-CAAGTAACTCTAGGTATGGAC-3′), TS3(5′-CTAGCCATGGTGGATGAAGAT-3′) and NEO(5′-TCGTGCTT-TACGGTATCGCCGCTCCCGATT-3′) to detect the normal SMP30 allele. Animal experiments were performed in accordance with the NIH guidelines for the care and use of laboratory animals. Each of the WT mice and SMP30 KO mice were divided into four groups. At 24 h after the $CCl_4$ injection, all animals were sacrificed. These groups and experimental design are schematically shown in Fig. 1. The dose of $CCl_4$ was determined according to a literature (Huang et al., 1995) and the determination of vitamin C dose was made according to a previous study (Kondo et al., 2006).

| Group | Animal type | Number | Treatment |
|---|---|---|---|
| G1 | Wild mice | n=5 | Olive oil |
| G2 | Wild mice | n=5 | $CCl_4$ (i.p.) |
| G3 | Wild mice + Vit C (p.o.) | n=5 | $CCl_4$ (i.p.) |
| G4 | Wild mice + Arazyme (p.o.) | n=5 | $CCl_4$ (i.p.) |
| G5 | SMP30 KO mice | n=5 | Olive oil |
| G6 | SMP30 KO mice | n=5 | $CCl_4$ (i.p.) |
| G7 | SMP30 KO mice + Vit C (p.o.) | n=5 | $CCl_4$ (i.p.) |
| G8 | SMP30 KO mice + Arazyme (p.o.) | n=5 | $CCl_4$ (i.p.) |

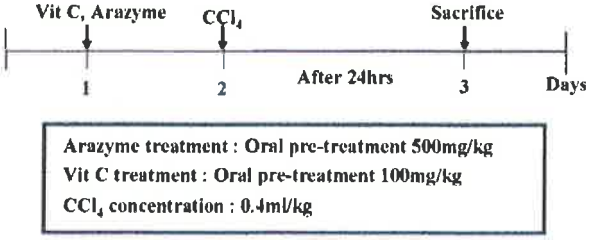


Fig. 1. Experimental design as described in Section 2. Group 1 (G1) and group 5 (G5) were treated with a single i.p. olive oil injection. Group 2 (G2) and group 6 (G6) received a single i.p. $CCl_4$ (0.4 mL/kg) injection. Group 3 (G3) and group 7 (G7) received a single oral administration of vitamin C (100 mg/kg) and were injected with a single i.p. $CCl_4$ (0.4 mL/kg). Group 4 (G4) and group (G8) received a single oral administration of Arazyme (500 mg/kg) and were injected with a single i.p. $CCl_4$ (0.4 mL/kg). At 24 h after the $CCl_4$ injection, all animals were sacrificed. i.p., intraperitoneal. p.o., per orally.

### 2.2. Histopathology

The sections of liver were cut to 4 μm in thickness and stained with hematoxylin and eosin (H&E). To grade liver damage, Grades 0–3 were used. Grade 0: no morphologic evidence of injury. Grade 1: scattered pericentral necrosis seen uniformly, but only within a few hepatocytes circumference in zone 3 (pericentral region of hepatic lobule). Grade 2: pericentral necrosis seen uniformly, frequently reaching to zone 2 (intermediate region of hepatic lobule). Grade 3: widespread hepatocellular necrosis, extending beyond a five hepatocytes circumference around the central vein, frequently reaching to zone 1 (periportal region of hepatic lobule).

### 2.3. Immunohistochemistry

Sections of liver were deparaffinized in xylene and toluene, rehydrated in a graded alcohol series, incubated in a solution of 3% hydrogen peroxide in methanol for 30 min and microwaved at 750 W for 10 min in 10 mmol/L citrate buffer, pH 6.0. Tissue sections were washed with PBS. After being blocked with rabbit serum (Vector Laboratories, Burlingame, CA, USA) for 1 h, the sections were immunostained with a primary antibody; the primary antibodies used were a polyclonal rabbit anti-Smad3 antibody at a dilution of 1:100 (Santa Cruz Biotechnology, Inc., CA, USA), a monoclonal rabbit anti-phospho-Smad3 (p-Smad3) antibody at a dilution of 1:200 (Cell Signaling Technology, Inc.), a polyclonal rabbit anti-p-Smad2/3 antibody at a dilution of 1:800 (Santa Cruz Biotechnology, Inc., CA, USA), a polyclonal anti-SMP30 antibody at a dilution of 1:5000 and a polyclonal rabbit antibody to CYP2E1 (Chemicon International, Inc., USA) at a dilution of 1:800. The antigen–antibody complex was visualized by an avidin–biotin peroxidase complex solution using an ABC kit (Vector Laboratories, Burlingame, CA, USA). The sections were rinsed in distilled water and counterstained with Mayers hematoxylin. For a negative control, the primary antibody was replaced by phosphate-buffered saline.

### 2.4. Immunoblot analysis

Snap-frozen liver tissues were homogenized in RIPA buffer containing 0.1 mmol/L $Na_3VO_4$ and protease inhibitor cocktail tablets (Roche, Mannheim, Germany). The lysate was centrifuged at 4000 rpm for 10 min at 4 °C to remove solid tissue and debris and then the supernatant was centrifuged at 14,000 rpm for 20 min at 4 °C to obtain soluble cytosolic protein. The protein concentration was measured by using the Bradford method (Bradford, 2007). The proteins were boiled for 10 min with 1× electrophoresis sample buffers. Eighty micrograms of the samples per lane were separated by 10–12% SDS–polyacrylamide gel electrophoresis. After blocking the membrane with a 3% bovine serum albumin in Tris-buffered saline (TBS), SMP30 was detected by using a rat polyclonal anti-SMP30 antibody. Smad3 was detected by using a polyclonal rabbit anti-Smad3 antibody. p-Smad3 was detected by using a polyclonal rabbit anti-p-Smad3 antibody. To confirm an equal quantity of protein loading, β-actin was detected by using a polyclonal goat anti-β-actin antibody at a dilution of 1:500. After washing the membrane in TBS, blots were incubated with a horseradish peroxidase (HRP)-conjugated anti-rat, rabbit and goat IgG. A specific binding was detected by using the Super Signal West Dura Extended Duration Substrate (PIERCE, Rockford, IL, USA) and exposing the blots to Medical X-ray Film (Kodak, Tokyo, Japan). Reproducibility of each band was confirmed through triple-replicate experiments. The images of protein bands were scanned, and band intensities were quantified using ImageJ software (National Institutes of Health, USA).

### 2.5. Liver tissue preparation for proteomic analysis

For 2D PAGE sample preparation, the frozen liver tissue (200 mg) was homogenized in 3 mL of a sample buffer (40 mmol/L Tris, 8 mol/L urea, 4% CHAPS (w/v), 1 mmol/L EDTA, and 10 mmol/L dithioerythritol) containing a protease inhibitor cocktail tablet (Roche, Mannheim, German). To obtain a soluble fraction after 8000 rpm for 10 min, the supernatant, the total homogenate, was centrifuged at 10,000 rpm for 10 min at 4 °C. Trichloroacetic acid (TCA) 50% was added to the supernatant to a final concentration of 12.5%. The protein precipitate was collected, then mixed with 100% ethanol and finally, it was left at −70 °C for 20 min. The samples were centrifuged at 12,000 rpm for 15 min at 4 °C and the protein precipitate was washed with 70% ethanol and dried. A dry pellet was dissolved in the sample buffer and the soluble fraction was collected after centrifugation at 15,000 rpm for 40 min at 15 °C.

### 2.6. Two-dimensional gel electrophoresis

The first dimensional isoelectric focusing (IEF) experiment was carried out on commercial 18 cm, pH 3–10 immobilized pH gradient (IPG) strips at 25 °C with a maximum current setting of 50 μA/strip in an IPGphor electrophoretic unit (Amersham Biosciences, Uppsala, Sweden). The strips were rehydrated overnight in 340 μL of a Destreak Rehydration solution containing a 0.5% IPG buffer. After rehydration, 800 μg of the protein sample was loaded onto the anodic end of an IPG strip. The IEF was performed at 25 °C according to the following steps: 50 V for 7 h, 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 2 h and 8000 V for 80,000 V h. After focusing process, the strips were equilibrated twice for 15 min. The first equilibration solution contained 30% glycerol, 6 mol/L urea, 2% sodium dodecyl sulfate (SDS), 50 mmol/L Tris–HCl, pH 8.8, 65 mmol/L dithiothreitol (DTT), and a trace of bromophenol blue. The second equilibration was performed with the same equilibration solution, except that DTT was replaced by 260 mmol/L iodoacetamide. Separation for the second dimension was performed on 1.0-mm thick 8–12% polyacrylamide gels at 15 °C, at a constant current of 60 V per gel.

### 2.7. Image analysis and data processing

The images of the protein spots were scanned, and spot intensities were obtained using ImageMaster 2D Platinum software (Amersham Biosciences, Uppsala, Sweden). After global normalization, reproducible spots were selected from among the triple-replicate experiments using a spot intensity-dependent standard deviation. Statistical analysis of the spot intensities was performed at $P < 0.05$ by a non-parametric Mann–Whitney $U$-test using SPSS for Windows (Release 12.0.1, SPSS Inc., USA). The mean of the relative intensities (% of volume) of the remaining spots was used in the following fold-change calculation.

### 2.8. Matrix-assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF/MS)

Briefly, the spots were destained with 30% methanol. Two hundred millimolars per liter ammonium bicarbonate was added to the gel pieces and micromixed for 30 min. The gel pieces were shrunk by dehydration in 100% acetonitrile, which was then removed before the pieces were dried. An enzymatic digestion was performed by adding 20 μL of 0.0125 μg/μL sequence grade modified trypsin (Promega, Madison, USA) in 50 mmol/L ammonium bicarbonate and 5 mmol/L calcium chloride, and incubated at 37 °C for 16 h. The extracted digestion mixtures were dried and then the residue was suspended in 0.1% trifluoroacetic acid (TFA). One microliter of the extracted sample was dispensed onto the MALDI sample plate with 1 μL of matrix solution (40 mg/mL α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA and 50% acetonitrile) and dried under ambient conditions. Database searches were carried out with the MS-Fit (http://prospector.ucsf.edu). Proteomic analysis was performed as described by our previous study (Yun et al., 2004).

### 2.9. Statistical analysis

All data taken from each group were expressed as mean ± S.D. and analyzed for statistical significance using a non-parametric Mann–Whitney $U$-test using SPSS for Windows (Release 12.0.1, SPSS Inc., USA). The value of statistical significance was set at $P < 0.05$ or $P < 0.01$.

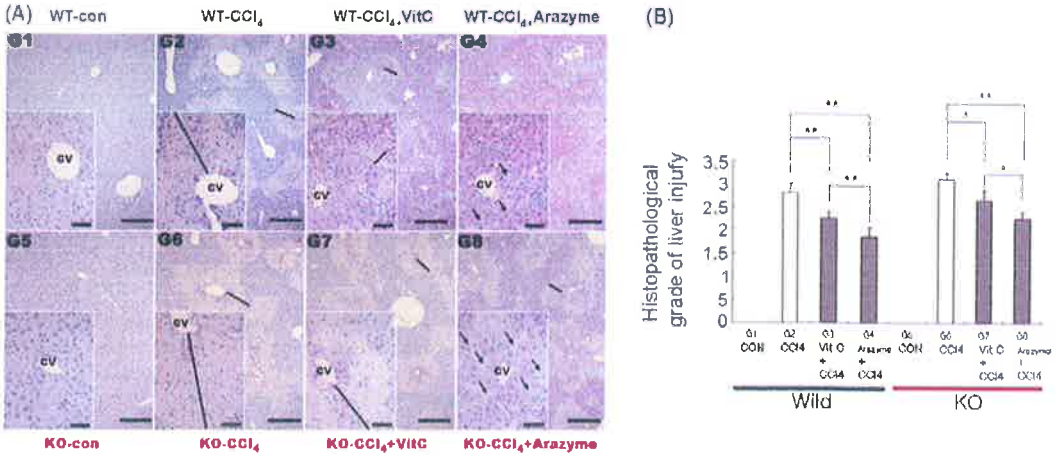


Fig. 2. Histological features of acutely damaged livers of WT and SMP30 KO mice. (A) In contrast to the $CCl_4$-damaged groups (G2, G6), there was a prominent decrease in the degree of centrilobular necrosis and inflammation in the livers of the vitamin C-treated groups (G3, G7) and, especially, the Arazyme-treated groups (G4, G8). In the Arazyme-treated groups (G4, G8), the hepatoprotective effect was more significant than that of vitamin C-treated groups (G3, G7). In the SMP30 KO mice (G6–G8), more severe centrilobular necrotic lesions and more inflammatory cells were observed than in the SMP30 WT mice (G2–G4). H&E stain. Scale bars = 50 μm (high magnification) and 200 μm. CV, central vein. Black line, necrotic area around the central vein. Arrow, damaged hepatocytes. (B) Histopathological grade of liver damage. Data are shown as mean ± S.D. (*$P < 0.05$, **$P < 0.01$).

## 3. Results

### 3.1. Histopathological change in the livers of SMP30 KO mice and WT mice after a single administration of $CCl_4$

The $CCl_4$-treated groups (G2, G6) showed widespread centrilobular necrosis, coagulative necrosis of hepatocytes and infiltration of inflammatory cells with intact periportal zones (Fig. 2A). in contrast to the $CCl_4$-damaged livers (G2, G6), there was a prominent decrease in the degree of centrilobular necrosis and inflammation in the livers of the vitamin C-treated groups (G3, G7) and Arazyme-treated groups (G4, G8). In the livers of the SMP30 KO mice (G6–G8), more severe centrilobular necrosis and more inflammatory cells were observed around the central vein area than were observed in those of SMP30 WT mice (G2–G4). The protective effect of Arazyme on an acutely damaged liver was more significant than the effect of vitamin C, and this effect was more noticeable in the SMP30 WT mice than in the SMP30 KO mice (Fig. 2B).

### 3.2. Decreased expression of SMP30 resulting from a single dose of $CCl_4$ and increased SMP30 expression in the Arazyme-treated group

The results of immunohistochemistry demonstrated that SMP30 was expressed in the cytoplasm and nucleus of the hepatocytes around the central vein of the SMP30 WT mice, but the expression of SMP30 was not detected in the SMP30 KO mice, which confirmed their genotype (Fig. 3A). In the $CCl_4$-treated WT mice (G2), expression of SMP30 was significantly decreased compared with the control WT mice group (G1). However, the Arazyme-treated group (G4) and vitamin C-treated group (G3) showed increased expression of SMP30 in their hepatocytes. The quantity of SMP30 expression was confirmed by immunoblot analysis (Fig. 3C). These results indicate that the decrease in the expression of SMP30 was caused by hepatocyte necrosis by administration of $CCl_4$, and that treatment with Arazyme or vitamin C plays a key role in protecting the liver from acute damage. Consequently, hepatocytes that are protected from $CCl_4$-induced damage by treatment with Arazyme or vitamin C can recover more easily than untreated hepatocytes, due to increased expression of SMP30.

### 3.3. Expression of p-Smad 2/3, p-Smad3 and Smad3 in Arazyme and vitamin C-treated mice

The $CCl_4$-treated group of WT mice (G2) and SMP30 KO mice (G6) showed significantly increased expression of p-Smad3 compared with the control groups (G1, G5). However, the Arazyme-treated groups (G4, G8) and the vitamin C-treated groups (G3, G7) of WT mice and SMP30 KO mice revealed decreased expression of p-Smad3 than the $CCl_4$-treated groups (G2, G6). The level of p-Smad3 expression was greater in the SMP30 KO mice than the WT mice in the immunoblot analysis (Fig. 4C and D). The immunohistochemistry for p-Smad2/3 also corresponded well with the results of the immunoblotting (Fig. 4A and B). The $CCl_4$-treated groups (G2, G6) demonstrated stronger positive expression in the nucleus of the injured hepatocytes around the central vein than did the control mice (G1, G5), the Arazyme-treated groups (G4, G8) and the vitamin C-treated groups (G3, G7). The expression of p-Smad3 and p-Smad2/3 was more strongly positive in the SMP30 KO mice than in the WT mice. However, the quantity of expression of Smad3 showed no significant differences among all the groups of WT and SMP30 KO mice either in the immunoblotting (Fig. 4E and F) and immunohistochemistry (data not shown). These results indicate that Arazyme has hepatoprotective effects mediated by the inhibition of the expression of p-Smad3 and p-Smad2/3,

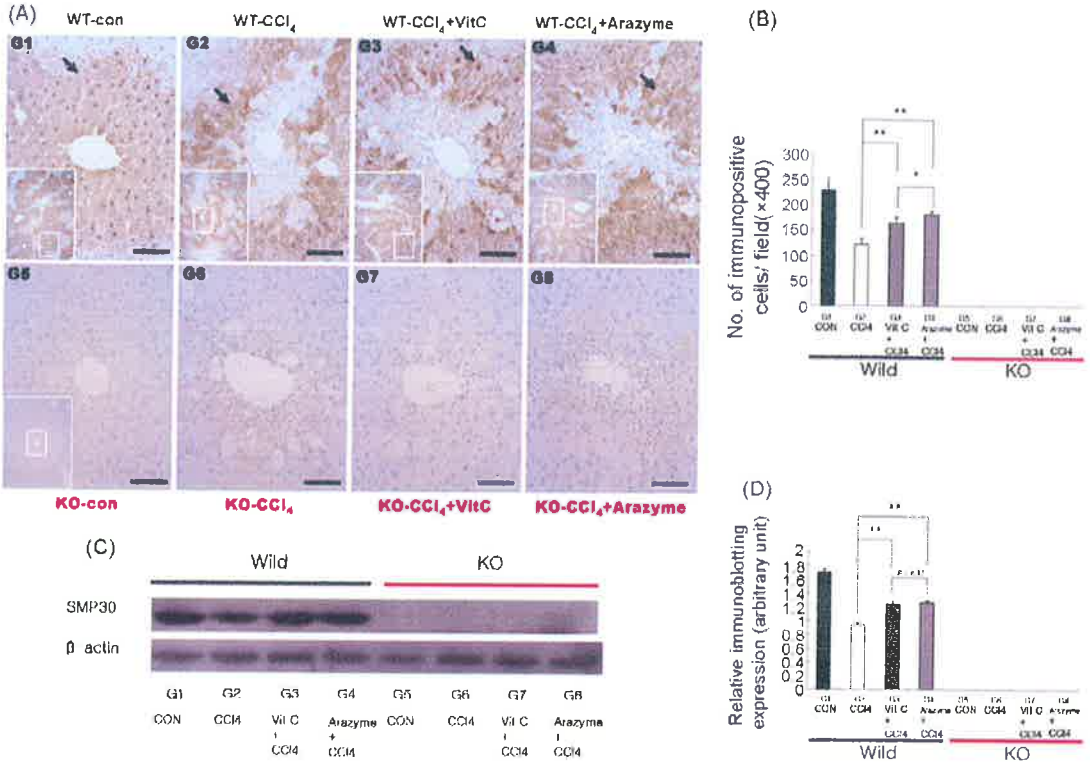


Fig. 3. Immunohistochemical and immunoblot analysis for SMP30. (A) The $CCl_4$-treated WT mice (G2) showed significantly decreased expression of SMP30 compared with the control (G1) WT mice. However, the Arazyme-treated group (G4) and the vitamin C-treated group (G3) demonstrated increased expression of SMP30 in hepatocytes. The Arazyme-treated group (G4) revealed slightly increased expression of SMP30 in comparison with that of the vitamin C-treated group (G3). Scale bars = 100 μm (high magnification), original magnification 66× (low magnification). (B) Estimation of the SMP30 expression level was quantified by counting the SMP30 positive cells in five fields (400×). Data are shown as mean ± S.D. (*$P < 0.05$, **$P < 0.01$). (C) Arazyme-treated group (G4) and vitamin C-treated group (G3) represented increased levels of SMP30 expression in comparison with the $CCl_4$-treated WT mice group (G2). Immunoblotting for SMP30 and β-actin (internal standard) antibodies. (D) Relative immunoblotting expression level of SMP30. Graph represents the relative band density compared with β-actin. Data are shown as mean ± S.D. (*$P < 0.05$, **$P < 0.01$).

which are intermediates involved in TGF-β signaling. Consequently, Arazyme down-regulates TGF-β/Smad mediated liver injury.

### 3.4. Expression of CYP2E1 in the Arazyme-treated group and vitamin C-treated group

In an acute $CCl_4$-induced liver injury, $CCl_4$ is metabolized by the liver CYP 2E1 and its hepatotoxicity is dependent on this metabolism. Thus, the pattern of CYP2E1 expression in the $CCl_4$-induced injured liver of Arazyme and vitamin C-treated group was observed by performing an immunohistochemical examination (Fig. 5A and B). Surprisingly, only the Arazyme-treated groups of the SMP30 KO mice and WT mice (G4, G8) showed a positive reaction on the injured hepatocytes around the central vein. On the other hand, the $CCl_4$-treated groups (G2, G6) and vitamin C-treated group (G3, G7) revealed a negative reaction for CYP 2E1.

### 3.5. Proteomic analysis of Arazyme-related proteins in $CCl_4$-induced liver injuries of SMP30 KO and WT mice

To investigate the expression pattern of Arazyme-related antioxidant proteins in the acutely injured liver, proteomic analysis was performed. Fifteen proteins revealed significant changes in the expression level between the Arazyme-treated groups (G4, G8) and the non-Arazyme-treated groups (G2, G6); 10 of these proteins were considered to be antioxidant proteins (Fig. 6A and B and Table 1). Seven antioxidant-related proteins—SMP30, sorbitol dehydrogenase, dihydropteridine reductase (DHPR), NADH dehydrogenase, glutathione *S*-transferase kappa 1-1 (GSTK1-1), dehydrofolate reductase (DHFR) and phospholipid hydroperoxide glutathione peroxidase (PHGPx), were significantly increased in the Arazyme-treated groups (G4, G8) compared with the non-Arazyme-treated groups (G2, G6) in both WT and SMP30 KO mice (Fig. 7A). Notably, in the WT mice, GSTK1-1 and PHGPx were the most highly induced proteins, and they were expressed approximately twofold more in the Arazyme-treated group compared with the non-Arazyme-treated group. Other proteins, including NADH dehydrogenase and DHFR, showed up-regulated expression that was more than 1.5-fold higher than that of the non-treated group (Fig. 7B). SMP30 and sorbitol dehydrogenase also showed a slightly increased level of expression in the Arazyme-treated group. However, no significant alteration in the expression level of DHPR was observed (Fig. 7B). Surprisingly, in the SMP30 KO mice, DHPR was the most significantly increased protein in the Arazyme-treated group; it showed a 4.5-fold increase in

Table 1
Remarkable changed proteins by $CCl_4$ and Arazyme treatment in wild-type mice and SMP30 knock-out mice

| Spot no. | Coverage (%)[a] | | Accession no.[b] | Protein name | Protein level | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | $CCl_4$-treated WT[c] | Arazyme-treated WT[d] | $CCl_4$-treated SMP30 KO[e] | Arazyme-treated SMP30 KO[f] |
| 1 | **29.1** | **33407/5.2** | Q64374 | Regucalcin (RC) (Senescence marker protein 30) (SMP30) | Down | Up | Down | Up |
| 3 | **14.1** | **61918/7.9** | P70166 | Cytoplasmic polyadenylation element-binding protein 1 (CPE-binding protein 1) (CPE-BP1) (mCPEB-1) (mCPEB) | Down | Up | Down | Up |
| 4 | **9.3** | **64987/8.1** | Q91XQ5 | *N*-Acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (EC 2.8.2.33) (GalNAc4S-6ST) (B-cell RAG-associated gene protein) | Down | Up | Down | NC[g] |
| 5 | **22** | **24753/8.5** | 61103 | Protein FAM3C precursor | Down | Up | Down | Up |
| 6 | **13.3** | **41136/8.2** | Q9EQC1 | 3 beta-hydroxysteroid dehydrogenase type 7 (3 beta-hydroxysteroid dehydrogenase type VII) (3-beta-HSD VII) (3-beta-hydroxy-delta(5)-C27 steroid oxidoreductase) (EC 1.1.1.-) (C(27) 3-beta-HSD) | Down | Up | Down | NC |
| 8 | **23.2** | **40092/6.6** | Q64442 | Sorbitol dehydrogenase (EC 1.1.1.14) (L-iditol 2-dehydrogenase) (fragment) | Down | NC | Down | Up |
| 9 | **17.4** | **25870/7.7** | Q8BVI4 | Dihydropteridine reductase (EC 1.5.1.34) (HDHPR) (quinoid dihydropteridine reductase) | Down | NC | Down | Up |
| 10 | **30.7** | **21984/7.7** | Q9CQJ8 | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9 (EC 1.6.5.3) (EC 1.6.99.3) (NADH-ubiquinone oxidoreductase B22 subunit) (Complex I-B22) (CI-B22) | Down | Up | Down | Up |
| 11 | **15.9** | **25704/9.0** | Q9DCM2 | Glutathione *S*-transferase kappa 1 (EC 2.5.1.18) (GST 13–13) (glutathione *S*-transferase subunit 13) (GST class-kappa) (GSTK1-1) (mGSTK1) | Down | Up | Down | Up |
| 12 | **23.5** | **21606/8.6** | P00375 | Dihydrofolate reductase (EC 1.5.1.3) | Down | Up | Down | Up |
| 14 | **21.3** | **22182/8.7** | O70325 | Phospholipid hydroperoxide glutathione peroxidase, mitochondrial precursor (EC 1.11.1.12) (PHGPx) (GPX-4) | Down | Up | Down | Up |
| 18 | **25.2** | **25970/7.7** | P10649 | Glutathione *S*-transferase Mu 1 (EC 2.5.1.18) (GST class-mu 1) (glutathione *S*-transferase GT8.7) (pmGT10) (GST 1-1) | Down | Up | Down | NC |
| 19 | **45.9** | **25702/7.6** | P19639 | Glutathione *S*-transferase Mu 3 (EC 2.5.1.18) (GST class-mu 3) (glutathione *S*-transferase GT9.3) | Down | Up | Down | Up |
| 20 | **28.6** | **14957/8.5** | P62962 | Profilin-1 (Profilin I) | Down | Up | Down | Up |
| 21 | **35.7** | **14266/8.6** | P58019 | CD59B glycoprotein precursor (membrane attack complex inhibition factor) (MACIF) (MAC-inhibitory protein) (MAC-IP) (protectin) | Down | Up | Down | Up |

MW and *pI*: obtained from the MS-fit search of proteinprospector database.

[a] Coverage (%): percentage in coverage of the matched peptides.

[b] Accession no.: protein no. from SwissProt database (2007.04.19).

[c] $CCl_4$-treated WT: only $CCl_4$-treated WT mice group. Down-regulation of proteins compared with $CCl_4$ non-treated WT group.

[d] Arazyme-treated WT: $CCl_4$ and Arazyme-treated WT mice group. Up-regulation of proteins compared with only $CCl_4$-treated WT group.

[e] $CCl_4$-treated SMP30 KO: only $CCl_4$-treated SMP30 KO mice. Down-regulation of proteins compared with $CCl_4$ non-treated KO group.

[f] Arazyme-treated SMP30 KO: $CCl_4$ and Arazyme-treated SMP30 KO mice. Up-regulation of proteins compared with only $CCl_4$-treated KO group.

[g] NC: no change between Arazyme-treated group and Arazyme non-treated group.

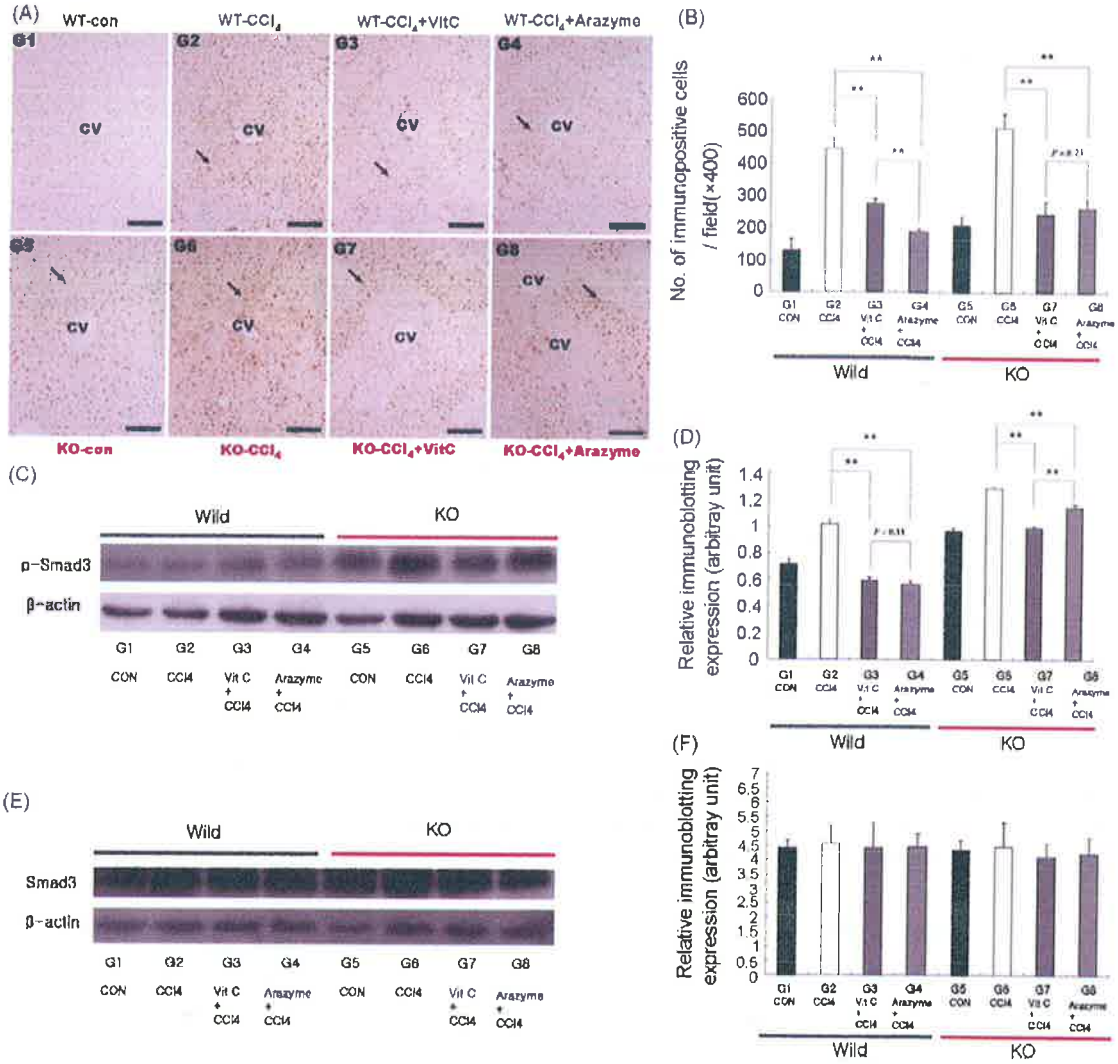


Fig. 4. Immunohistochemical analysis for p-Smad2/3 and immunoblotting for p-Smad3 and Smad3. (A) Arazyme-treated groups (G4, G8) and the vitamin C-treated groups (G3, G7) indicated decreased p-Smad2/3 expression in comparison with that of the $CCl_4$-treated groups (G2, G6). The expression of p-Smad2/3 produced a stronger positive reaction in SMP30 KO mice than in WT mice. Scale bars = 100 μm. (B) Immunohistochemical expression of p-Smad2/3. Estimation of the level of p-Smad2/3 expression was quantified by counting p-Smad2/3 positive cells in five fields (400×). Data are shown as mean ± S.D. (*$P < 0.05$, **$P < 0.01$). (C) The $CCl_4$-treated groups (G2, G6) showed more significantly increased expression of p-Smad3 compared with the control group (G1, G5). The Arazyme-treated groups (G4, G8) and vitamin C-treated groups (G3, G7) revealed more decreased expression of p-Smad3 than those of the $CCl_4$-treated groups (G2, G6). Immunoblotting for SMP30 and β-actin (as an internal standard) antibodies. (D) Relative immunoblotting expression level of p-Smad3. Graph represents the relative band density to β-actin. Data are shown as mean ± S.D. (*$P < 0.05$, **$P < 0.01$). (E) Smad3 revealed no significant changes in the quantity of expression in all the WT mice groups and SMP30 KO mice groups. Immunoblotting for SMP30 and β-actin (as an internal standard) antibodies. (F) Relative immunoblotting expression level of Smad3. Graph represents the relative band density to β-actin. Data are shown as mean ± S.D. (*$P < 0.05$, **$P < 0.01$).

expression level in the Arazyme-treated group in contrast with that of the WT mice (Fig. 7C). Sorbitol dehydrogenase showed a twofold increase in expression level in the Arazyme-treated group, and other proteins, including NADH dehydrogenase, GSTK1-1, DHFR and PHGPx, were also increased slightly in the Arazyme-treated group (Fig. 7C). In both WT and SMP30 KO mice, seven proteins were significantly increased in the Arazyme-treated group, indicating that Arazyme has a protective effect against $CCl_4$-induced acute liver injury that is mediated by up-regulation of the expression level of antioxidant proteins. These proteins are thought to be important factors that protect the liver from unstable free radicals and ROS-induced by $CCl_4$ treatment.

## 4. Discussion

In a previous study about Arazyme, it was observed that Arazyme hydrolyzes inflammatory proteins such as kinogen, bradykinin and substance P known to play an important role in inflammation process (Kwak et al., 2007). Therefore, Arazyme was elucidated to have anti-inflammatory effect via the hydrolytic activity on bradykinin and substance P, and Arazyme also showed favorable effect in bovine mastitis (Bersanettia et al., 2005). This hydrolytic activity of Arazyme could be at least part of the molecular mechanism as anti-inflammatory agent. However, any other mechanisms and effects of Arazyme have not been studied sufficiently so far. In this study, the pro-

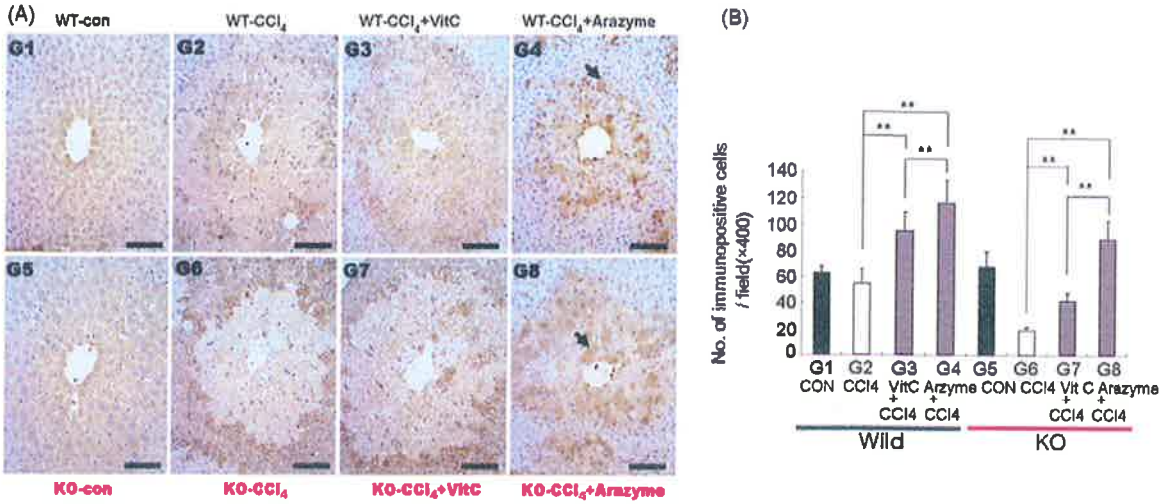


Fig. 5. The expression of CYP2E1 in acutely damaged livers of WT mice and SMP30 KO mice. (A) Immunohistochemical expression level of CYP2E1. The Arazyme-treated group (G4, G8) in all the mice groups showed positive reaction on the injured hepatocytes around the central vein. On the other hand, the $CCl_4$-treated groups (G2, G6) and vitamin C-treated groups (G3, G7) revealed negative reaction. The control groups (G1, G5) indicated normally weak positive reaction. Scale bars = 100 μm. (B) Estimation of the CYP2E1 expression level was quantified by counting the CYP2E1 positive cells in five fields (400×). Data are shown as mean ± S.D. (*$P < 0.05$, **$P < 0.01$).

tective effect of Arazyme was observed in the $CCl_4$-induced liver injury group using WT mice and SMP30 KO mice. Three possible mechanisms for the protective effect of Arazyme can be proposed: (1) Arazyme inhibits the decrease in SMP30 expression caused by acute liver injury; (2) Arazyme inhibits TGF-β/Smad signaling via down-regulation of the expression level of p-Smad3 and p-Smad2/3; and (3) Arazyme up-regulates the expression of antioxidant proteins. These three mechanisms were observed in the study, and will be discussed more specifically below.

The first mechanism of the Arazyme hepatoprotective effect, the inhibition of the decrease of SMP30 expression, was shown.

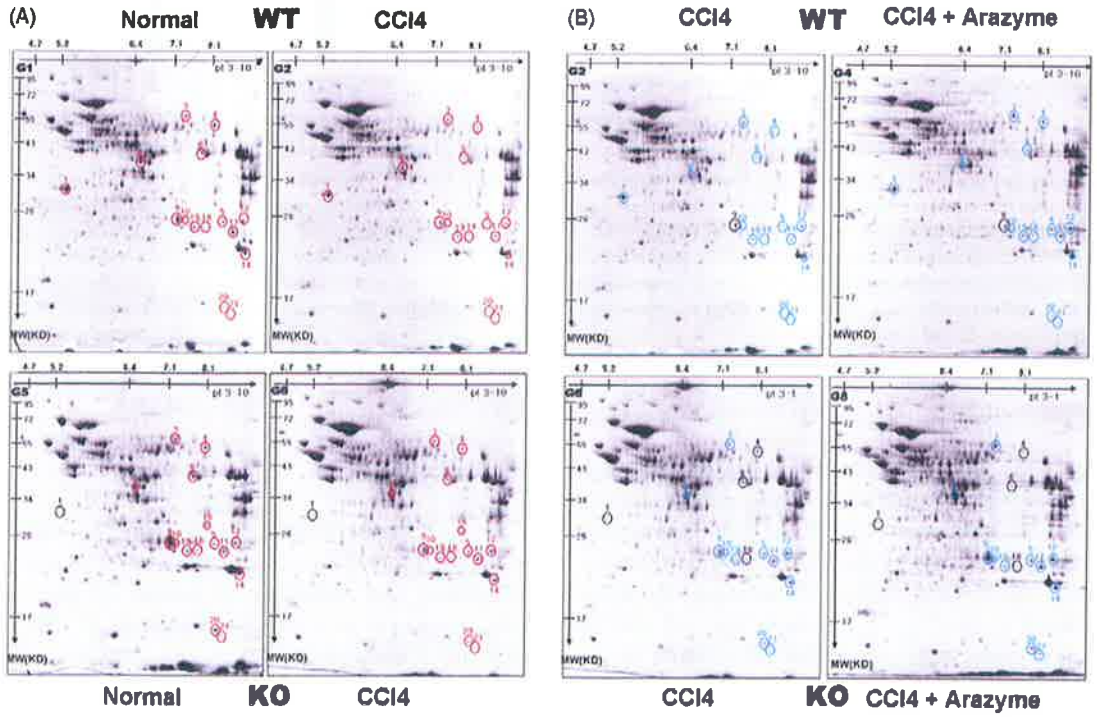


Fig. 6. Remarkably up-regulated antioxidant-related proteins between the Arazyme-treated group and non-treated group in the WT mice and SMP30 KO mice. (A) Comparative image analysis of the WT mice and SMP30 KO mice (control group and $CCl_4$-treated group). Fifteen proteins were down-regulated after a $CCl_4$ administration. Down-regulated proteins were indicated as a red circle. (B) Comparative image analysis of the WT mice and SMP30 KO mice ($CCl_4$-treated group and Arazyme-treated group with $CCl_4$). Fifteen proteins were up-regulated after a $CCl_4$ administration. Up-regulated proteins are indicated as a blue circle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

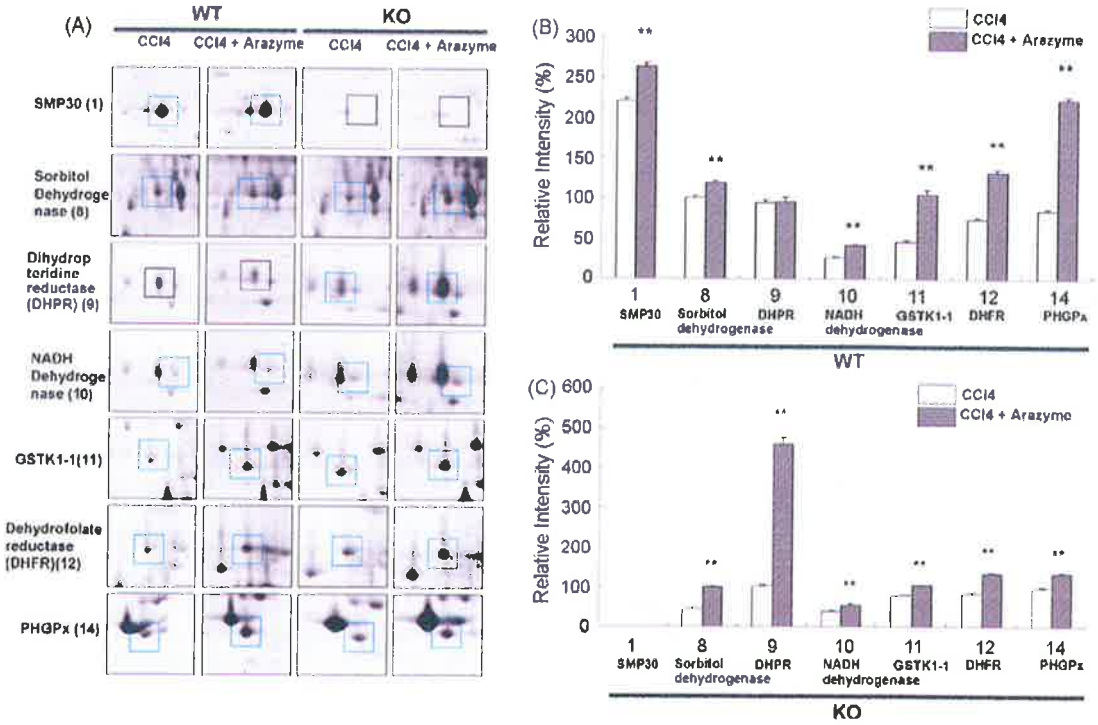


Fig. 7. Proteomic analysis for Arazyme-related proteins. (A) Significant up-regulation of antioxidant proteins in the Arazyme-treated group compared with the non-treated group in WT and SMP30 KO mice. (B) Relative intensity of up-regulated proteins in WT mice. Data are shown as mean ± S.D. (*$P < 0.05$, **$P < 0.01$ vs. $CCl_4$ group). (C) Relative intensity of up-regulated proteins in SMP30 KO mice. Data are shown as mean ± S.D. (*$P < 0.05$, **$P < 0.01$ vs. $CCl_4$ group).

It was observed that Arazyme had a stronger protective effect in WT mice than in the SMP30 KO mice in all groups. This reconfirms that SMP30 has a predominantly protective effect on damaged hepatocytes by maintaining calcium homeostasis and vitamin C biosynthesis. A recent study also reported that SMP30 has a protective action against oxidative stress without influencing the antioxidant enzyme expression (Son et al., 2006). This first mechanism of Arazyme, increasing the level of expression of SMP30, is the critical factor for the protection of injured cells. Finally, the elevated level of SMP30 expression induced by Arazyme might play an important role in liver recovery.

The second mechanism of the Arazyme hepatoprotective effect, the inhibition of TGF-β/Smad signaling via down-regulation of the level of expression of p-Smad3 and p-Smad2/3, was confirmed. TGF-β plays an important role as an acute inflammation mediator recruiting several inflammatory cells (Date et al., 2000; Tahashi et al., 2002). It has been shown that TGF-β is released from necrotic hepatocytes, and this may be the first signal that activates adjacent quiescent HSC (Liu et al., 2006). The multiple biological actions of TGF-β, which include development, cell growth, cell differentiation, cell adhesion, migration and extracellular matrix deposition, play a pivotal role in acute and chronic liver injuries. The effects of TGF-β are induced when the protein binds to its receptor on the target cell surface (Date et al., 2000). P-Smad3 and p-Smad2/3 are the key factors that regulate the TGF-β/Smad pathway. Increased expression of p-Smad2/3 and p-Smad3 are induced by elevated TGF-β (Date et al., 2000), and previous studies have reported that acute liver injury induced by a single administration of $CCl_4$ causes increased expression of TGF-β (Date et al., 2000; Jeong et al., 2004). The expression of p-Smad2/3 in the nuclei of hepatocytes at an early stage of liver injury has also been reported (Jeong et al., 2004). In the present study, a decreased level of expression of p-Smad2/3 and p-Smad3 was detected in the Arazyme-treated groups compared with the non-Arazyme-treated groups.

The third mechanism of the Arazyme hepatoprotective effect, in which it up-regulates the level of expression of antioxidant proteins, was demonstrated by proteomic analysis in the present study. Increased expression of sorbitol dehydrogenase, DHPR, NADH dehydrogenase, GSTK1-1, DHFR and PHGPx was observed in the Arazyme-treated group. In particular, GSTK1-1 and PHGPx showed the most significant up-regulation, indicating a twofold increase in expression. GSTs are enzymes that detoxify electrophilic xenobiotics and inactivate endogenous substances such as quinines, α,β-unsaturated aldehydes and hydroperoxides formed as secondary metabolites during oxidative stress (Bilska et al., 2007). PHGPx, a 20-kDa monomeric seleno-enzyme that was first identified by Ursini et al., is able to catalyze the reduction of diacylphospholipid hydroperoxides directly. PHGPx mainly reduces membrane lipid hydroperoxides that are produced during lipid peroxidation (Antunes et al., 1995). DHFR catalyzes the regeneration of tetrahydrobiopterin from its oxidized form, dihydropterin, in several cell types (Chalupsky and Cai, 2005). When tetrahydrobiopterin is oxidized to dihydropterin, dihydropterin may cause uncoupling of vascular endothelial nitric oxide synthase to produce

superoxide. Dihydropterin, the oxidized form of tetrahydrobiopterin, is recycled by dihydrofolate reductase in several cell types; consequently, DHFR inhibits the production of superoxide (Chalupsky and Cai, 2005). In SMP30 KO mice, DHPR showed the greatest increase in the Arazyme-treated group. DHPR, an enzyme that catalyzes the reduction of dihydropteridine to tetrahydropteridine, has the same function as DHFR (Hyun et al., 1995). It also prevents the oxidation of tetrahydrobiopterin and controls the production of superoxide (Hyun et al., 1995). It was observed that the WT mice in the Arazyme-treated group showed a greater increase in the expression ratio of antioxidant proteins than the SMP30 KO mice, except for the expression of sorbitol dehydrogenase and DHPR (Fig. 6B and C). These results indicate that Arazyme had a stronger protective effect in the WT mice, and that these up-regulated antioxidant proteins might be pivotal factors in antioxidative defense mechanisms against ROS and free radicals formed by $CCl_4$ administration.

The results of the present study suggest that Arazyme has strong protective effects against acute liver injury and oxidative stress caused by hepatotoxins that are mediated by three different mechanisms. Additionally, in the result of CYP2E1 expression, the negative expression of CYP2E1 in the $CCl_4$-treated groups (G2, G6) and vitamin C-treated groups (G3, G7) was caused by $CCl_4$-induced severe centrilobular necrosis of the hepatocytes, but the Arazyme-treated groups (G4, G8) were protected by Arazyme and a few injured hepatocytes around the central vein of the Arazyme-treated groups could express the CYP 2E1 to metabolize the $CCl_4$. Based on these findings, Arazyme has a stronger protective effect than vitamin C against $CCl_4$-induced liver damage.

Consequently, Arazyme revealed a similar or stronger degree of protective efficacy compared with vitamin C in all experiments. We confirmed the mechanisms of hepatoprotective effect of Arazyme using histopathological examination, immunohistochemistry, immunoblotting and proteomic in present study. These three mechanisms proposed here suggest a possibility for the development of a new therapeutic agent for the treatment of acute liver injuries, although we still do not know the precise relationship between these three mechanisms and hydrolytic activity.

## Acknowledgements

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