# Ameliorative effects of functional crude-chalaza hydrolysates on the hepatosteatosis development induced by a high-fat diet

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**ABSTRACT** Approximately 400 metric tons of egg chalazae, a byproduct in the liquid-egg processing plant, are produced yearly but always regarded as a waste in Taiwan. Our team successfully developed a crude egg chalaza hydrolysate by protease-A digestion (**CCH-A**). Free branched-chain amino acids, 3-aminoisobutyric acid, and β-alanine, and anserine were assayed in the CCH-A used in this study. Besides, the in vitro bile-acid binding ability and inhibitory lipase activity of CCH-As were demonstrated. Then, high-fat diet feeding for 10 wk caused hyperlipidemia, insulin resistance, and hepatosteatosis in hamsters ($P < 0.05$). However, CCH-A co-treatment decreased serum/liver triglyceride levels and lipid accumulation in livers by increasing daily fecal lipid/bile-acid outputs, upregulating fatty-acid β oxidation, and downregulating fatty-acid biosynthesis in livers ($P < 0.05$). CCH-A co-treatment also amended insulin resistance, augmented hepatic antioxidant capacity, and decreased liver damages and inflammatory responses ($P < 0.05$). Taken together, our results do not only demonstrate the hepatoprotective effects of CCH-As against a chronic high-fat dietary habit, achieving effects similar to Simvastatin, but also decrease the environmental burden of handling chalazae in the liquid-egg industry.

**Key words:** crude-chalaza hydrolysate, high-fat diet, lipid homeostasis, insulin resistance, hepatosteatosis

2021 Poultry Science 100:101009
https://doi.org/10.1016/j.psj.2021.01.031

## INTRODUCTION

Global poultry consumption, i.e. chicken, eggs etc., is steadily increased because of the nutritional, convenient, and cheap views (FAO, 2020). In addition, more and more liquid-egg products have been available in the market due to the convenience and food-safety concern. Based on the 6% of total eggs used as liquid-egg production in Taiwan, approximately 400 metric tons of egg chalazae, a byproduct in the liquid-egg processing plant, are produced yearly but always regarded as a waste in Taiwan (Council of Agriculture, Executive Yuan, Taiwan, 2020). However, these byproducts with a large yield but lower utilization result in a heavy environmental burden. How to well utilize these byproducts is a priority not only to the poultry industry but also scientists.

The functional protein hydrolysate has been effective in producing bioactive peptides or increasing free amino acids; besides, protein hydrolysates demonstrate some physiologic functions, such as antioxidant capacity, hypolipidemic effect, hepatoprotection, and so on. For example, chicken-liver hydrolysate via pepsin digestion showed a hypolipidemic effect (Yang et al., 2014) and in vitro or in vivo antioxidant abilities (Chou et al., 2014); and chicken blood hydrolysate via alcalase digestion showed an antihypertensive effect (Wongngam et al., 2020). This is because of the free amino acid profile and bioactive imidazole-ring dipeptides in poultry-sourced protein hydrolysates. Among some free amino acids and bioactive dipeptides in poultry-sourced proteins, branched-chain amino acids (**BCAAs**; valine, leucine, and isoleucine) and carnosine/anserine are often mentioned. The biological effects of BCAAs involve a number of functional effects, including insulin resistance, regulation of glucose metabolism, and enhancement of lipolysis (Kawaguchi et al., 2008). Hypoglycemic effects of BCAAs were also observed by downregulating



Received November 13, 2020.
Accepted January 5, 2021.

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glycolysis, as well as increasing glucose transporter 1 and 4 and glycogen synthase (Nishitani et al., 2005; Doi et al., 2007). Besides, Takahiro et al. (2006) reported that ε-polylysine demonstrates an anti-obesity function in high-fat diet (**HFD**)-fed mice because ε-polylysine acts as an inhibitor of pancreatic lipase activity and intestinal lipid absorption. Carnosine improved insulin sensitivity and lowered glycogen concentration in 2-deoxy-D-glucose-induced hyperglycemic rats (Yamano et al., 2001). Carnosine and anserine also have antiglycation abilities (Szwergold, 2005).

Overnutrition and obesity have been perceived as a problematic issue for both developing and developed countries (Chopra et al., 2002). Modern diet, urbanization, and industrialization have altered people's lifestyle patterns. Modern diet is always calorie- and refined oil/starch-rich but short of micronutrients (i.e., vitamins, minerals, amino acids, and fiber). This modern dietary pattern can cause various diseases, for instance, hypertension, hyperlipidemia, obesity, diabetes, and fatty liver. Over the last few decades, nonalcoholic fatty liver disease (**NAFLD**) has been characterized as a leading cause of chronic liver diseases worldwide. The pathological processes of NAFLD can range from a simple fatty liver (hepatosteatosis) to steatohepatitis, and cirrhosis (irreversible and advanced scarring of the liver). Meanwhile, in western countries, 70 to 90% of the NAFLD population is clinically diagnosed with obesity and type 2 diabetes, and hence, metabolic syndrome sufferers have a high risk for developing liver fibrosis and cirrhosis (Targher et al., 2010). Lipid homeostasis is mainly regulated through exogenous lipid absorption in the intestine, de novo lipogenesis (fatty-acid biosynthesis), and energy expenditure (fatty-acid β oxidation). Generally, triglycerides (**TGs**) in food are hydrolyzed into free fatty acids by pancreatic lipases and then absorbed in the intestine. Excessive fatty acids would then be stored in extrahepatic tissues or taken up by the liver, thus causing obesity and hepatosteatosis, respectively. In addition to the exogenous lipid absorption in the intestine, de novo lipogenesis and energy expenditure occur in the liver. De novo lipogenesis and energy expenditure in the liver are regulated by adenosine 5′-monophosphate-activated protein kinase, sterol regulatory element-binding protein-1 (**SREBP1c**), acetyl CoA carboxylase (**ACC**), fatty acid synthase (**FAS**), peroxisome proliferator-activated receptor alpha (**PPARα**), carnitine palmitoyltransferase-1 (**CPT1**), and uncoupling protein-2 (**UCP2**) (Horton et al., 2002; Su et al., 2015; Lee and Imm, 2018). Su et al. (2015) have indicated that excessive lipid intake can not only cause hepatic lipid accumulation or hepatosteatosis but also increase oxidative stress or inflammatory responses. In addition, Day and James (1998) proposed a "two-hit" hypothesis for the etiology of NAFLD. The first hit is that the free fatty acids in sera are elevated due to insulin resistance, and this promotes the hepatic fatty-acid uptake and TG biosynthesis, thus leading to the occurrence of hepatosteatosis. The second hit is that excessive TG and fatty acids impair hepatic fatty-acid β oxidation and increase oxidative stress, thus leading to oxidative damage in the liver. Moreover, hepatic lipid overload potentially stimulates the inflammatory phase of steatohepatitis to the fibrotic phase of cirrhosis (Argo and Caldwell, 2009).

In order to well utilize egg chalazae produced in the liquid-egg industry, our group indicated that volumes of free BCAAs, lysine, and imidazole-ring dipeptides (anserine) are significantly enhanced in the egg chalazae via protease-A hydrolyzation (crude-chalaza hydrolysate from protease-A digestion [**CCH-A**]), and this CCH-A has been patented as well (Chen and Lin, 2019). Additionally, it has also been proven that this CCH-A can ameliorate an alcoholic fatty liver by downregulating de novo lipogenesis and upregulating fatty-acid β oxidation in livers (Yang et al., 2019), as well as improving oxidative stress-induced cognitive decline by reversing the antioxidant capacity and decreasing inflammatory expressions in the brain (Chan et al., 2020). Hence, the preventive effect of this CCH-A on hepatosteatosis development in a high-fat dietary habit warrants a comprehensive investigation. To elucidate the preventive effect of this CCH-A, the major analyses in this study included: 1) regulation of lipid homeostasis (exogenous lipid absorption, de novo lipogenesis, and energy expenditure), and 2) alleviation of hyperlipidemia and liver injury (antioxidant capacity and inflammatory response).

## MATERIALS AND METHODS

### *Preparation of Functional CCH-As*

Fresh egg crude chalazae were collected from a liquid-egg producer (Daiegg Co. Ltd., Tainan City, Taiwan) and immediately transported to our laboratory at −20°C. Because of safety concerns, the antibiotic and sulfonamide residues in the raw chalazae used in this study were further examined at the Inspection Center of the National Animal Industry Foundation (Pingtung County, Taiwan), and were all found to be safe. The manufacturing procedure of CCH-A powders was carried out according to our USA patent (Chen and Lin, 2019). Protease-A (Amano Enzyme Ltd., Nagoya, Japan) was used to digest the collected crude chalazae. To ensure the contents of bioactive compounds (free amino acids and imidazole-ring dipeptides [carnosine and anserine]), the CCH-A powder used in this study was tested using an amino acid analyzer (Hitachi L8800 amino acid analyzer, Hitachi High-Technologies Co., Tokyo, Japan) at the Food Industry Research and Development Institute (Hsinchu City, Taiwan).

### *In Vitro Lipase Activity and Bile-Acid Binding Ability of CCH-As*

The in vitro lipase activity and bile-acid binding ability of CCH-As were assayed according to earlier reports of Wu et al. (2013) and Yang et al. (2014), respectively, with minor modifications. The in vitro lipase activity

test measures the rate of release of oleic acid from triolein by using a commercial kit (Randox Laboratories Ltd., Antrim, UK). The lipase activities of various CCH-A concentrations (100, 200, 300, 400, and 500 μg/mL) were obtained by measuring the decrease in turbidity at 340 nm multiplied by a factor (which was the activity of standard lipase divided by a decrease of turbidity at 340 nm of standard lipase). The lipase activities for various concentrations of CCH-As were expressed relative to the average values for $ddH_2O$, which was set to 100%. Regarding in vitro bile-acid binding activity, various concentrations of CCH-As (6.25, 12.50, 18.75, 25.00, and 31.25 mg/mL) and cholestyramine (6.25 mg/mL, Sigma Co., St. Louis, MO) were hydrolyzed with pepsin (400 U/mg protein, Sigma Co.) at a ratio of 9:4 (wt./wt.) to CCH-A powders and adjusted to pH 2.0 with 0.01 N HCl at 37°C for 1 h. Then, 2 mmol bile-acid solution (Sigma Co.) and 2 mg/mL porcine pancreatin (Sigma Co.) for digestion were added and incubated at 37°C for a further 1 h; meanwhile, the pH value of the mixture was adjusted to 6.3 with 0.1 N NaOH. Next, the mixture was filtrated by using 3000 Da Vivaspin (VS0191, Satorius Stedim Biotech, Goettingen, Germany) at 10,000 *g* (Centrifuge 3,700, Kubota Co., Tokyo, Japan) for 1 h, and the levels of unbound bile acid in the filtrated aliquot were measured by using a commercial kit (Bile acids, Randox Laboratories Ltd.). The bile-acid binding activities for various concentrations of CCH-As were expressed relative to the average values for cholestyramine (6.25 mg/mL) (Sigma Co.), which was set to 100%.

## Animals and Treatments

The anti-obesity and hypolipidemic effects of CCH-A powders have been proven in our previous study (Chen et al., 2020). In this study, 40 male Golden Syrian hamsters (8-week-old) were purchased from the National Laboratory Animal Center (National Science Council, Taipei, Taiwan). Two hamsters with an ear tag (no. 1 or 2) were housed in each cage at 22 ± 2°C with a 12/12 h light-dark cycle. To induce the development of hepatosteatosis in hamsters, the HFD (12% fat/0.2% cholesterol) was formulated based on a standard rodent diet (5% fat/0% cholesterol, normal diet) (Laboratory Rodent Diet 5001, Land O'Lakes Inc., St. Louis, MO) with lard and cholesterol (Chang et al., 2011). After 1 wk of acclimation, hamsters were randomly divided into 5 groups: 1) CON: normal diet + pure water (oral gavage); 2) HFD: HFD + pure water (oral gavage); 3) HFD + 1X_CCH: HFD + 240 mg CCH-A/kg BW (oral gavage); 4) HFD + 2X_CCH: HFD + 480 mg CCH-A/kg BW (oral gavage); and 5) HFD + Simvastatin: HFD + 6 mg Simvastatin/kg BW (oral gavage) for 10 wk. Simvastatin, which belongs to the group of hypolipidemic medicines called statins, was used as a positive agent (Chen et al., 2020). Animal use and protocol were reviewed and approved by the National Taiwan University Animal Care Committee (NTU105-EL-00163). The liver and blood samples of each hamster were obtained after sacrifice by $CO_2$ asphyxiation. Blood samples were placed at room temperature (25°C) for 1 h. Then, the sera were collected by centrifugation at 3,000 × *g*, 4°C for 15 min, and then stored at −20°C for further analyses. Liver homogenates (10%, w/w) were prepared with PBS (pH 7.0, containing 0.25 M sucrose). Next, the liver homogenates were centrifuged (3,000 × *g*, 4°C, 15 min), and the collected supernatant was stored at −20°C for future analyses. The protein concentration in the supernatant was measured by using a Bio-Rad protein assay kit (#500-0006, Bio-Rad Laboratories Inc., Hercules, CA).

## Determination of Serum Biochemical Values, Intraperitoneal Glucose Tolerance Test (ipGTT), Hepatic Lipids, and Daily Fecal Lipids and Bile-Acid Outputs

The liver damage indices (aspartate aminotransferase and alanine aminotransferase [**ALT**]) in the sera collected from the hamsters at the end of the experiment were determined by an Arkray Spotchem SP-4410 Automatic Dry Chemistry Analyzer (Arkray, Inc., Kyoto, Japan); meanwhile, serum lipid (TG and total cholesterol) and glucose levels were measured by using commercial kits (Randox Laboratories Ltd.). The ipGTT was based on the procedure described by Liu et al. (2015). On the 64th day of the experiment, the hamsters were fasted overnight and injected with glucose (1.0 g/kg BW, i.p.). Blood glucose level was assessed at 30, 60, 90, and 120 min post-glucose injection by using a glucose meter (GM300, Bionime Co. Ltd., Taichung City, Taiwan). Furthermore, the glucose area under the curve (**AUC**) was calculated by the total AUC from the sampling period of 0 to 120 min. Besides, concentrations of hepatic/fecal lipids and fecal bile acids were measured by using commercial kits (Randox Laboratories Ltd.). The feces in the last 3 d of the experiment were collected while daily fecal lipid/bile-acid outputs were obtained by multiplying by the daily fecal output weight (g).

## Determination of Cytokine Levels and Antioxidant Capacities in Livers

Tumor necrosis factor-alpha (**TNF-α**) and interleukin-1 beta (**IL-1β**) were used to evaluate the proinflammatory level of livers of HFD-fed hamsters. Liver TNF-α and IL-1β concentrations were measured by ELISA kits, following the commercial manufacturer's instructions (BioLegend Inc., San Diego, CA). TNF-α and IL-1β levels were expressed as pg/mL protein by using standard curves. Thiobarbituric acid reactive substances (**TBARS**), Trolox equivalent antioxidant capacity, and reduced glutathione (**GSH**) levels, as well as activities of superoxide dismutase and catalase (**CAT**), and glutathione peroxidase (**GSH-Px**) in livers were represented as the liver antioxidant capacity. These

assays were based on the methods of our previous study (Lin et al., 2017).

### Histopathological Analysis

For hematoxylin and eosin (**H&E**) staining, the liver tissues were placed in a 3.7% formaldehyde solution up to 48 h and then kept dehydrated in graded alcohol (30, 50, 75, and 95%), cleared in xylene, and embedded in paraffin wax. The blockers were sliced (5-μm thick) by using a microtome, deparaffinized in xylene, dehydrated in graded alcohol, and stained with H&E solution. Photomicrographs were taken by using a Leica DM500 microscope (Leica Microsystems, Singapore) with an IHD-4600 camera system (Sage Vision Co. Ltd., New Taipei City, Taiwan) and ToupView 3.7 software (ToupTek Co. Ltd., Hangzhou, China). Histologic grade and stage of hepatic steatosis were determined according to a report by Dixon et al. (2004). The samples were categorized into a 5-grade system based on the proportion of hepatocytes affected (0: no steatosis, 1: <5% of hepatocytes affected; 2: 5 to 25% of hepatocytes affected; 3: 25 to 75% of hepatocytes affected; 4: >75% of hepatocytes affected). Finally, different scores were given to each group after an evaluation.

### Western Blotting

The liver homogenates from hamsters with the no. 1 ear tag were mixed with 5× western sample dye and heated at 95°C for 10 min (n = 4 per group). After cooling, the samples were loaded into SDS-PAGE gel (4% stacking gel and 10% running gel), and then transferred to polyvinylidene (**PVDF**) membranes (Bio-Rad Laboratories Inc.). The membranes were blocked with 5% skim milk for 2 h at 4°C. Next, anti-PPARα (ab24509, 1:3000 dilution, Abcam, Cambridge, UK), anti-CPT1 (ABS65, 1:5000 dilution, Merck Millipore Co., Darmstadt, Germany), anti-UCP2 (662047, 1:5000 dilution, Merck Millipore, Calbiochem), anti-SREBP1c (sc-365513, 1:3000 dilution, Santa Cruz Biotechnology, Inc., Dallas, TX), anti-ACC (#3662, 1:5000 dilution, Cell Signaling Technology Inc., Danvers, MA), anti-FAS (#3180, 1:3000 dilution, Cell Signaling Technology Inc.), and anti-GAPDH mouse polyclonal antibody (1:5000 dilution, Thermo Fisher Scientific Inc., Waltham, MA) were treated with PVDF overnight at 4°C. Subsequently, the PVDF membrane was rinsed with washing buffer (PBS with Tween-20), and the membrane was incubated with secondary antibody (conjugated anti-mouse IgG horseradish peroxidase, 1:10,000 dilution, Thermo Fisher Scientific Inc.) for 2 h at 4°C. The protein bands were detected by a chemiluminescence kit (Immobilon Western, Millipore, Billerica, MA) and analyzed by the ChemiDoc MP Imaging System (Bio-Rad Laboratories Inc.). Image J software (National Institutes of Health, Bethesda, MD) was used to quantify the optical density of protein bands with the value of the GAPDH band as a reference, and then compared with the CON group, which was set to 1.

### Statistical Analyses

The experiment was conducted by using a completely random design. Data were reported as mean ± SEM and analyzed using an analysis of variance. When a significant difference ($P < 0.05$) was obtained, the differences between treatments were distinguished by using the Least Significant Difference test. All statistical analyses of data were carried out by using SAS (SAS Institute Inc., Cary, NC, 2002).

## RESULTS

### Free Amino Acids/Imidazole-Ring Dipeptides in CCH-As, and In Vitro Suppressive Ability of CCH-As on Dietary-Lipid Absorption

Regarding bioactive compounds in CCH-As, free amino acids and imidazole-ring dipeptides in CCH-As prepared for this study were analyzed by using an Amino Acid Analyzer at the Food Industry Research and Development Institute. In 100 g CCH-A powder, there was 1,720.23 mg leucine, 741.28 mg isoleucine, 638.55 mg valine, 916.39 mg lysine, 980.46 mg phenylalanine, 1,072.13 mg arginine, 299.59 mg histidine, 143.16 mg glycine, 342.19 mg alanine, 85.48 mg β-alanine, 165.00 mg 3-aminoisobutyric acid, and 66.06 mg anserine (Supplementary Table 1). However, carnosine was not detectable in the CCH-As used in this study. In vitro bile-acid binding ability and lipase activity were used to evaluate the suppressive effects of CCH-As on bile-acid reabsorption and exogenous lipid absorption, respectively (Figure 1). Although incomparable to the efficacy of cholestyramine on bile-acid binding ability, CCH-As indeed increased the bile-acid binding ability with a dose-dependent efficacy ($P < 0.05$). When the CCH-A dosages were beyond 18.75 mg/mL, the bile-acid binding abilities were higher than 50% relative to cholestyramine (Figure 1A). In addition, the CCH-As showed a weak suppressive ability on lipase activities (9–12%); however, 500 μg CCH-As/mL can decrease 18% lipase activity compared to no addition of CCH-As (Figure 1B).

### Effects of CCHs on Growth Performance, Serum Lipid and Glucose Values, Intraperitoneal Glucose Tolerance, and Fecal Lipid/Bile-Acid Outputs of Experimental Hamsters

After 10 wk of the experiment, no differences in final BW and food and water intake were observed among all groups ($P > 0.05$) (Table 1). The HFD group had higher serum TG, total cholesterol, and glucose levels than the CON group ($P < 0.05$). Supplementing CCH-A and Simvastatin decreased serum TG and glucose values ($P < 0.05$). However, there was only a lower tendency toward serum total cholesterol values in CCH-A-

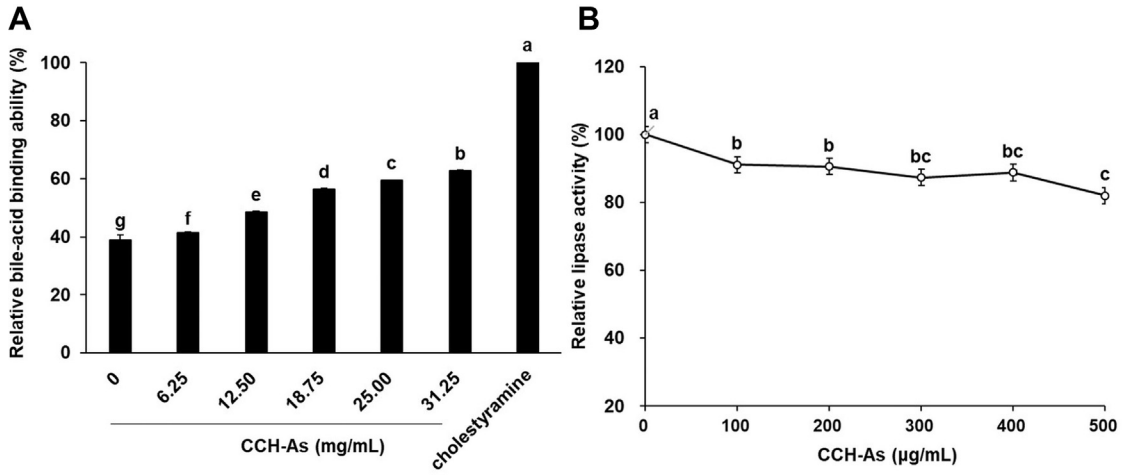


**Figure 1.** Relative (A) bile-acid binding ability and (B) lipase activity of CCH-As. The data are given as mean ± SEM (n = 3). Data bars or data points in each tested concentration without a common letter are significantly different ($P < 0.05$). Abbreviation: CCH-As, crude-chalaza hydrolysates from protease-A digestion.

supplemented groups while supplementing Simvastatin decreased ($P < 0.05$), becoming similar to that of the CON group. In the ipGTT (Figure 2A), the blood glucose levels of all groups peaked at 30 min after an injection with glucose, and then reduced gradually. There were differences in the blood glucose levels among groups at 90 and 120 min ($P < 0.05$). At 90 and 120 min after injection of glucose, the increased blood glucose

**Table 1.** Effects of CCH-As and Simvastatin on growth performance, serum biochemical values, and daily fecal lipid/bile-acid outputs, liver weight/size, liver lipids, liver cytokines, and liver antioxidant capacity of experimental hamsters.

| | CON[1] | HFD[1] | HFD+1X_CCH[1] | HFD+2X_CCH[1] | HFD + Simvastatin[1] |
|---|---|---|---|---|---|
| | Growth performance | | | | |
| Initial BW (g) | 123.00 ± 2.91$^{a}$ | 124.12 ± 1.83$^{a}$ | 123.20 ± 2.27$^{a}$ | 125.48 ± 1.45$^{a}$ | 125.21 ± 1.70$^{a}$ |
| Final BW (g) | 142.50 ± 2.45$^{a}$ | 147.81 ± 3.30$^{a}$ | 145.94 ± 2.50$^{a}$ | 148.69 ± 2.28$^{a}$ | 145.53 ± 2.85$^{a}$ |
| Food intake (g/hamster/day) | 8.47 ± 0.05$^{a}$ | 8.45 ± 0.02$^{a}$ | 8.38 ± 0.10$^{a}$ | 8.69 ± 0.17$^{a}$ | 8.57 ± 0.18$^{a}$ |
| Water intake (mL/hamster/day) | 9.98 ± 0.36$^{a}$ | 10.75 ± 0.27$^{a}$ | 10.11 ± 0.39$^{a}$ | 10.44 ± 0.54$^{a}$ | 10.04 ± 0.20$^{a}$ |
| | Serum biochemical values | | | | |
| Triglyceride (mg/dL) | 158.75 ± 9.02$^{b,c}$ | 215.38 ± 12.35$^{a}$ | 185.13 ± 6.71$^{b}$ | 177.88 ± 8.22$^{b}$ | 144.38 ± 10.21$^{c}$ |
| Total cholesterol (mg/dL) | 134.88 ± 5.28$^{b,c}$ | 153.25 ± 3.66$^{a}$ | 142.25 ± 4.71$^{a,b}$ | 143.00 ± 3.47$^{a,b}$ | 123.50 ± 4.05$^{c}$ |
| Glucose (mg/dL) | 82.75 ± 3.02$^{c}$ | 118.63 ± 6.43$^{a}$ | 104.63 ± 5.06$^{b}$ | 98.75 ± 2.89$^{b}$ | 92.75 ± 5.47$^{b,c}$ |
| AST (U/L) | 53.75 ± 5.19$^{a}$ | 51.13 ± 5.47$^{a}$ | 53.50 ± 4.53$^{a}$ | 47.00 ± 2.09$^{a}$ | 42.00 ± 1.66$^{a}$ |
| ALT (U/L) | 74.88 ± 4.14$^{c,d}$ | 104.00 ± 5.54$^{a}$ | 87.75 ± 2.97$^{b}$ | 85.63 ± 4.48$^{b,c}$ | 68.50 ± 4.47$^{d}$ |
| | Daily fecal lipid/bile-acid output | | | | |
| Triglyceride (mg/hamster/day) | 6.98 ± 1.01$^{c}$ | 9.44 ± 0.27$^{b}$ | 12.61 ± 0.81$^{a}$ | 11.59 ± 0.67$^{a,b}$ | 10.71 ± 0.67$^{a,b}$ |
| Cholesterol (mg/hamster/day) | 6.61 ± 0.62$^{b}$ | 7.83 ± 0.62$^{a,b}$ | 9.56 ± 0.85$^{a}$ | 9.70 ± 0.90$^{a}$ | 9.78 ± 0.49$^{a}$ |
| Bile acid (μmol/hamster/day) | 0.95 ± 0.13$^{b}$ | 1.15 ± 0.12$^{b}$ | 1.75 ± 0.11$^{a}$ | 1.69 ± 0.19$^{a}$ | 1.55 ± 0.10$^{a}$ |
| | Liver | | | | |
| Weight (g/hamster) | 4.68 ± 0.12$^{a}$ | 5.07 ± 0.11$^{a}$ | 4.80 ± 0.09$^{a}$ | 4.83 ± 0.13$^{a}$ | 4.60 ± 0.11$^{a}$ |
| Size (g/100 g BW hamster) | 3.29 ± 0.05$^{b}$ | 3.43 ± 0.06$^{a}$ | 3.29 ± 0.04$^{b}$ | 3.25 ± 0.04$^{b}$ | 3.16 ± 0.04$^{b}$ |
| | Liver lipids | | | | |
| Triglyceride (mg/g liver) | 14.17 ± 1.03$^{c}$ | 33.65 ± 1.16$^{a}$ | 22.98 ± 1.18$^{b}$ | 22.39 ± 1.26$^{b}$ | 20.73 ± 0.79$^{b}$ |
| Cholesterol (mg/g liver) | 6.11 ± 0.45$^{b,c}$ | 7.51 ± 0.40$^{a}$ | 6.72 ± 0.32$^{a,b}$ | 6.73 ± 0.50$^{a,b}$ | 5.33 ± 0.44$^{c}$ |
| | Liver cytokines | | | | |
| TNF-α (pg/mg protein) | 2.34 ± 0.16$^{b}$ | 3.33 ± 0.12$^{a}$ | 2.33 ± 0.13$^{b}$ | 2.67 ± 0.10$^{b}$ | 2.50 ± 0.17$^{b}$ |
| IL-1β (pg/mg protein) | 17.20 ± 0.95$^{b}$ | 25.87 ± 0.87$^{a}$ | 17.48 ± 0.74$^{b}$ | 18.99 ± 0.83$^{b}$ | 18.55 ± 0.85$^{b}$ |
| | Liver antioxidant capacity | | | | |
| TBARS (nmol MDA eq./mg protein) | 0.42 ± 0.06$^{b}$ | 0.62 ± 0.05$^{a}$ | 0.45 ± 0.02$^{b}$ | 0.47 ± 0.05$^{b}$ | 0.45 ± 0.03$^{b}$ |
| Reduced GSH (μmol/mg protein) | 32.56 ± 1.63$^{c}$ | 28.20 ± 1.68$^{d}$ | 36.91 ± 1.28$^{a,b}$ | 33.19 ± 1.28$^{b,c}$ | 38.86 ± 1.60$^{a}$ |
| SOD (unit/mg protein) | 3.89 ± 0.23$^{a}$ | 3.28 ± 0.19$^{a}$ | 3.62 ± 0.26$^{a}$ | 3.61 ± 0.20$^{a}$ | 3.69 ± 0.23$^{a}$ |
| Catalase (unit/mg protein) | 53.07 ± 2.47$^{c}$ | 66.49 ± 1.77$^{b}$ | 71.71 ± 3.47$^{a,b}$ | 72.26 ± 3.39$^{a,b}$ | 77.51 ± 2.28$^{a}$ |
| GSH-Px (unit/mg protein) | 17.91 ± 0.70$^{b}$ | 15.32 ± 0.77$^{b}$ | 21.83 ± 1.07$^{a}$ | 21.77 ± 1.70$^{a}$ | 21.08 ± 0.94$^{a}$ |

$^{a–d}$Mean values in each tested parameter without a common letter are significantly different ($P < 0.05$).

The data are given as mean ± SEM (n = 8, except food/water intake and daily fecal lipid/bile-acid outputs, n = 4).

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCH-As, crude-chalaza hydrolysates from protease-A digestion; GSH, glutathione; GSH-Px, glutathione peroxidase; IL-1β, interleukin-1 beta; MDA, malondialdehyde; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TNF-α, tumor necrosis factor-alpha.

[1]CON: normal diet + pure water (oral gavage); HFD: high-fat diet + pure water (oral gavage); HFD+1X_CCH: high-fat diet+240 mg CCH-A/kg BW (oral gavage); HFD+2X_CCH: high-fat diet+480 mg CCH-A/kg BW (oral gavage); HFD + Simvastatin: high-fat diet+6 mg Simvastatin/kg BW (oral gavage).

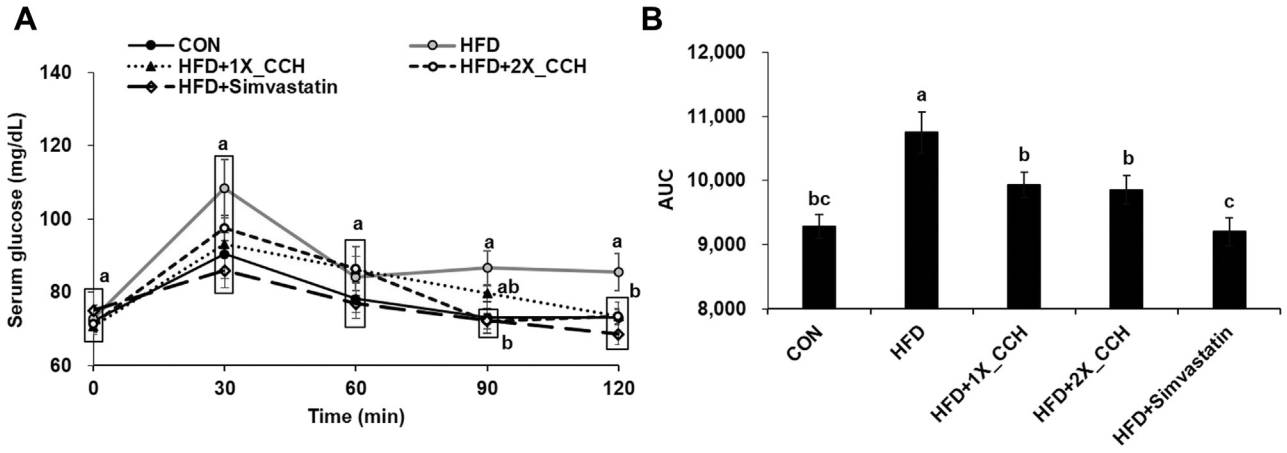


**Figure 2.** Effects of CCH-As and Simvastatin on (A) blood glucose levels and (B) AUC in the intraperitoneal glucose tolerance test of experimental hamsters. The data are given as mean ± SEM (n = 8). Data points in each tested time and data bars without a common letter are significantly different ($P < 0.05$). CON: normal diet + pure water (oral gavage); HFD: high-fat diet + pure water (oral gavage); HFD+1X_CCH: high-fat diet+240 mg CCH-A/kg BW (oral gavage); HFD+2X_CCH: high-fat diet+480 mg CCH-A/kg BW (oral gavage); HFD + Simvastatin: high-fat diet+6 mg Simvastatin/kg BW (oral gavage). Abbreviations: AUC, area under the curve; CCH-As, crude-chalaza hydrolysates from protease-A digestion.

level of HFD-fed hamsters was decreased by supplementing CCH-As and Simvastatin ($P < 0.05$). After calculating the glucose AUC from 0 to 120 min after the injection of glucose, the HFD group had the largest AUC among groups ($P < 0.05$). Supplementing CCH-As and Simvastatin decreased the AUC, achieving a level similar to that of the CON group ($P < 0.05$) (Figure 2B). For daily fecal lipid outputs (Table 1), the HFD increased daily fecal lipid outputs ($P < 0.05$), but there was a tendency toward higher daily fecal TG and cholesterol outputs in HFD-fed hamsters supplemented with CCH-As and Simvastatin. More daily bile-acid outputs were measured in HFD+1X_CCH, HFD+2X_CCH, and HFD + Simvastatin groups than those in CON and HFD groups ($P < 0.05$).

### *Effects of CCH-As on Serum Liver Damage Indices/Cytokines, Liver Weight/Size, Lipids, and Antioxidant Capacity, as Well as the Molecular Mechanism of Lipid Homeostasis and Histological Pathology of HFD-Fed Hamsters*

No alteration in liver weights was observed among groups ($P > 0.05$). Compared to the CON group, the HFD group had an enlarged and increased liver size and lipids (TG and cholesterol), respectively ($P < 0.05$). Supplementing CCH-As and Simvastatin decreased liver sizes and liver TG levels in HFD-fed hamsters ($P < 0.05$) (Table 1). However, there was a tendency toward lower liver cholesterol levels in the HFD-fed hamsters supplemented with CCH-As. Histological observations (H&E stains) also illustrated more lipid accumulation (arrowheads) in the liver sections of HFD-fed hamsters, but supplementing CCH-As and Simvastatin reduced lipid droplets (Figure 3A); meanwhile, the increased liver steatosis score in the HFD group was reduced ($P < 0.05$). In lipid metabolism, protein expressions related to fatty-acid β oxidation (PPARα, CPT1, and UCP2) in the livers of HFD-fed hamsters were generally upregulated by supplementing CCH-As (UCP2, $P < 0.05$) (Figure 4). Protein expression levels related to fatty-acid biosynthesis (SREBP1c, ACC, and FAS) were higher in the HFD group than those in the CON group ($P < 0.05$), but supplementing CCH-A or Simvastatin downregulated these ($P < 0.05$). Regarding liver damage indices, except serum aspartate aminotransferase values, the HFD group had higher serum ALT values and liver TNF-α and IL-1β levels than the CON group ($P < 0.05$), but supplementing CCH-As and Simvastatin apparently decreased those values ($P < 0.05$), achieving levels similar to those of the CON group ($P > 0.05$) (Table 1). Moreover, HFD increased TBARS values and decreased reduced GSH levels in the livers of hamsters ($P < 0.05$), but supplementing CCH-As and Simvastatin improved these values ($P < 0.05$). Only increased GSH-Px activities in livers were measured in HFD-fed hamsters supplemented with CCH-As and Simvastatin ($P < 0.05$), but a tendency toward higher CAT activities was assayed in CCH-As co-treated groups.

## DISCUSSION

Based on our knowledge, egg is a very nutritional food item, especially for its protein content. Chalazae are the major components that hold the yolk at the right position in eggs but are considered as waste in the liquid-egg industry. In Taiwan, there are approximately 400 metric tons of chalaza production per year in the liquid-egg industry. Hence, it is imperative to determine how to well utilize this byproduct. The functional protein hydrolysate has been effective in producing bioactive peptides or increasing free amino acids; besides, protein hydrolysates demonstrate some physiologic functions, such as antioxidant capacity, hypolipidemic effect, hepatoprotection, and so on. Bioactive

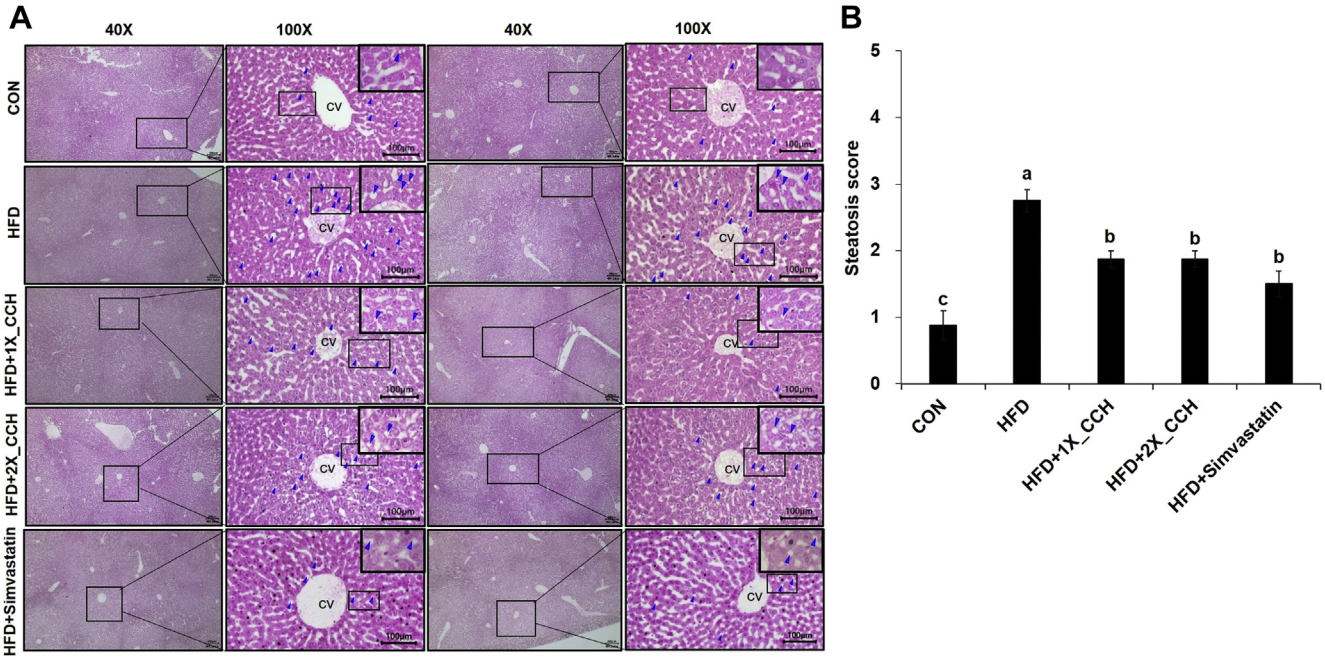


**Figure 3.** Effects of CCH-As and Simvastatin on (A) pathological section analyses (H&E stain) and (B) the steatosis scores of liver tissues of experimental hamsters. Three histological images of each experimental group were illustrated with 40, 100, and 400× magnification. The areas indicated with the black line were 400× magnified and shown in the left-upper corner of 100× magnified illustration. The blue arrowheads indicated the lipid droplets. The data are given as mean ± SEM (n = 8). Data bars without a common letter are significantly different ($P < 0.05$). CON: normal diet + pure water (oral gavage); HFD: high-fat diet + pure water (oral gavage); HFD+1X_CCH: high-fat diet+240 mg CCH-A/kg BW (oral gavage); HFD+2X_CCH: high-fat diet+480 mg CCH-A/kg BW (oral gavage); HFD + Simvastatin: high-fat diet+6 mg Simvastatin/kg BW (oral gavage). Abbreviations: CCH-As, crude-chalaza hydrolysates from protease-A digestion; CV, central vain; H&E, hematoxylin and eosin.

compounds (BCAAs, lysine, and imidazole-ring dipeptides [anserine]) are significantly enhanced in the egg chalazae from the liquid-egg industry via protease-A hydrolyzation (CCH-A), and it has also been proven that this CCH-A can ameliorate an alcoholic fatty liver by downregulating de novo lipogenesis and upregulating fatty-acid β oxidation in livers (Yang et al., 2019), as well as improving oxidative stress-induced cognitive decline by reversing the antioxidant capacity and decreasing inflammatory expressions in the brain (Chan et al., 2020).

Over the last few decades, NAFLD has been characterized as a leading cause of chronic liver diseases worldwide. The pathological processes of NAFLD can range

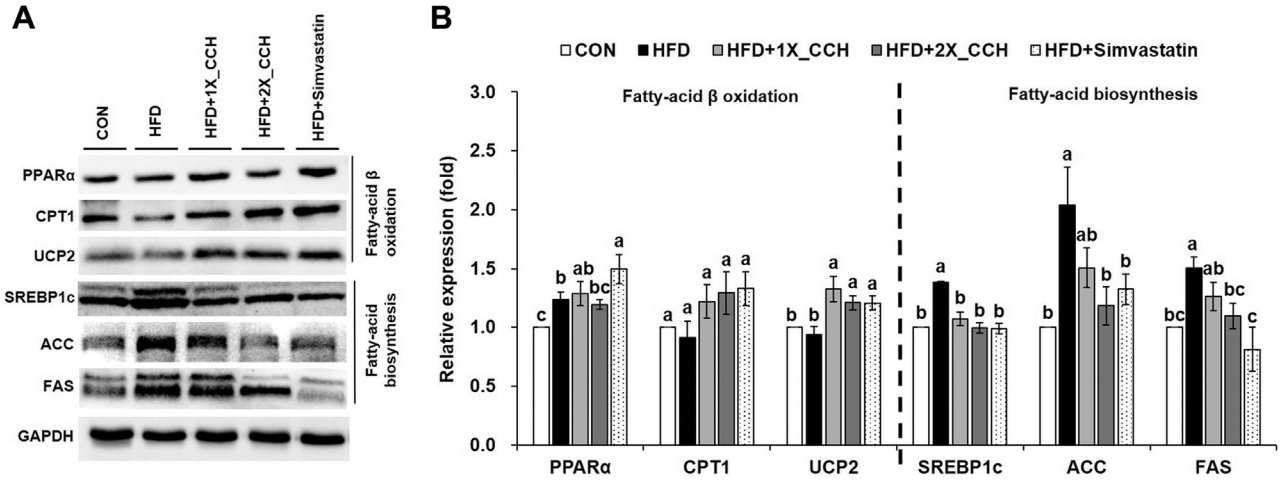


**Figure 4.** Effects of CCH-As and Simvastatin on protein expressions. (A) Western blotting illustration and (B) the quantification results related to fatty-acid β oxidation and fatty-acid biosynthesis in the livers of experimental hamsters. The data are given as mean ± SEM (n = 4). Data bars in each tested protein expression without a common letter are significantly different ($P < 0.05$). CON: normal diet + pure water (oral gavage); HFD: high-fat diet + pure water (oral gavage); HFD+1X_CCH: high-fat diet+240 mg CCH-A/kg BW (oral gavage); HFD+2X_CCH: high-fat diet+480 mg CCH-A/kg BW (oral gavage); HFD + Simvastatin: high-fat diet+6 mg Simvastatin/kg BW (oral gavage). Abbreviations: ACC, acetyl CoA carboxylase; CCH-As, crude-chalaza hydrolysates from protease-A digestion; CPT1, carnitine palmitoyltransferase-1; FAS, fatty acid synthase; PPARα, peroxisome proliferator-activated receptor alpha; SREBP1c, sterol regulatory element-binding protein-1; UCP2, uncoupling protein-2.

from hepatosteatosis to steatohepatitis, and eventually to cirrhosis. Besides, a "two-hit" hypothesis for the etiology of NAFLD was proposed by Day and James (1998). First, the free fatty acids in sera are elevated due to insulin resistance, and hepatic fatty-acid uptake and TG biosynthesis are promoted, thus leading to hepatosteatosis. Then, the excessive TG and fatty acids impair hepatic fatty-acid β oxidation and increase oxidative stress, thus leading to oxidative damage to the liver. Leucine, isoleucine, and valine belong to BCAAs, and they have several protective effects against metabolic syndromes, including amelioration of insulin resistance, regulation of glucose metabolism, and enhancement of lipolysis (Kawaguchi et al., 2008). Alamshah et al. (2017) reported that phenylalanine might have a therapeutic utility in the treatments of obesity and diabetes because it could improve glucose tolerance and reduce food intake by modulating secretions of gut hormones peptide YY and glucagon-like peptide 1, thus reducing plasma ghrelin. Besides, 3-aminoisobutyric acid (β-aminoisobutyric acid) could attenuate the development of obesity and fatty liver by stimulating browning of white adipose tissues and fatty-acid β oxidation in the liver (Roberts et al., 2014). β-Alanine is a precursor of carnosine, an imidazole-ring dipeptide, which has strong antioxidant and hypoglycemic effects (Yamano et al., 2001; Szwergold, 2005). Regarding the recycling utilization of agricultural byproducts, the value of crude egg chalazae, a byproduct, in liquid-egg processing can be enhanced by means of nutraceutical development via protease-A digestion (Chen and Lin, 2019). Meanwhile, the volumes of free BCAAs, phenylalanine, BAIBA, β-alanine, and anserine are also assayed as 3100.06, 980.46, 165.00, 85.48, 66.06 mg/100 g lyophilized CCH-As (Supplementary Table 1).

Aranha et al. (2008) observed that the bile-acid levels (i.e., deoxycholic, chenodeoxycholic, and cholic acids) were increased in the liver tissue of steatohepatitis patients. Thus, it has been suggested that bile acids worsen the state of NAFLD in humans. Bile-acid sequestrants (e.g., cholestyramine), which bind bile acids in the gastrointestinal tract to prevent its reabsorption (Fernandez et al., 2000), have been used for many years to treat hypercholesterolemia and dyslipidemia. Regarding dietary-lipid absorption, pancreatic lipase is essential for efficiently hydrolyzing triacylglycerols to 2-monoacylglycerol and fatty acids. Inhibition of lipase activity is a possible treatment for obesity, hyperlipidemia, and liver TG accumulation (Kumar and Alagawadi, 2013; Wu et al., 2013; Yang et al., 2014). Strong bile-acid binding ability can disturb enterohepatic circulation and thereby increase fecal cholesterol and bile-acid outputs, and the hydrophobic level of protein hydrolysates is highly related to bile-acid binding ability (Iwami et al., 1986). Hence, it can be hypothesized that the bile-acid binding ability of our CCH-As is due to the hydrophobic amino acids (e.g., BCAA, methionine, phenylalanine, alanine, and glycine) (Supplementary Table 1). In addition, Yang et al. (2014) reported that inhibition of in vitro lipase activity of chicken liver hydrolysates is due to its lysine contents (Kido et al., 2003). Owing to the regulation of glucose and lipid metabolism of those amino acids and in vitro suppressive ability of dietary-lipid absorption, we hypothesize that our CCH-As could have potential in the amelioration of hepatosteatosis development in a high-fat dietary habit.

Hypolipidemia and insulin resistance are triggers for hepatosteatosis development. Several reports have indicated that certain amino acids, peptides, and protein hydrolysates can regulate lipid and glucose metabolism. BCAAs and phenylalanine have been proposed to amend insulin resistance and regulate glucose metabolism (Kawaguchi et al., 2008; Alamshah et al., 2017). Szwergold (2005) reported that anserine possesses an antiglycation ability. Besides, more daily fecal lipid/bile-acid outputs are highly correlated to lower serum lipids and central obesity (Yang et al., 2010, 2014). The anti-obesity and cardioprotective effects of our CCH-As have been observed due to reduced BW and perirenal adipose tissue sizes, as well as improved serum lipids, cholesterol profile, and oxidative level (Chen et al., 2020). Based on the current data, it could be summarized that the hypolipidemic and hypoglycemic effects of CCH-As in HFD-fed hamsters are due to the free amino acids/dipeptides (BCAAs, phenylalanine, carnosine, and anserine) (Supplementary Table 1), as well as bile-acid binding activity and inhibitory lipase activity (Figure 1).

In addition to exogenous lipid absorption, the disturbance of fatty-acid β oxidation and fatty-acid biosynthesis in livers is another reason for hepatosteatosis development (Chang et al., 2011; Su et al., 2015; Liu et al., 2020). SREBP1c is a transcription factor for initiating fatty-acid biosynthesis (e.g., ACC and FAS) rate limiting enzymes (Horton et al., 2002). PPARα, a ligand-activated transcription factor for many downstream proteins (e.g., CPT1 and UCP2), triggers fatty-acid β oxidation in the livers; hence, the PPARα signaling cascade has been identified as a potential therapeutic target in patients with NAFLD (Pawlak et al., 2015). Kainulainen et al. (2013) have reported that BCAA supplementation overcomes fatigue during exercise by upregulation of fatty-acid β oxidation. Similarly, a BCAA-rich black-vinegar supplement showed enhancements of PPARα target proteins (CPT1 and UCP2), thus increasing energy expenditure and decreasing lipid accumulation in the livers of HFD-fed rats (Liu et al., 2020). Moreover, the CCH-As containing a certain volume of BCAAs demonstrated a decrease of lipid accumulation against chronic alcohol consumption via upregulating fatty-acid β oxidation (Yang et al., 2019). It has been reported that acidic amino acids (aspartic acid and glutamic acid) (Saiga et al., 2003), hydrophobic amino acids (BCAA, glycine, alanine) (Ren et al., 2008), and imidazole-ring amino acids (histidine) or dipeptide (anserine) (Chen et al., 1998; Wu et al., 2003) in CCH-As display antioxidant activities. Some protein hydrolysates also reveal strong antioxidant abilities in vitro and in vivo (Manso et al., 2008; Chou et al.,

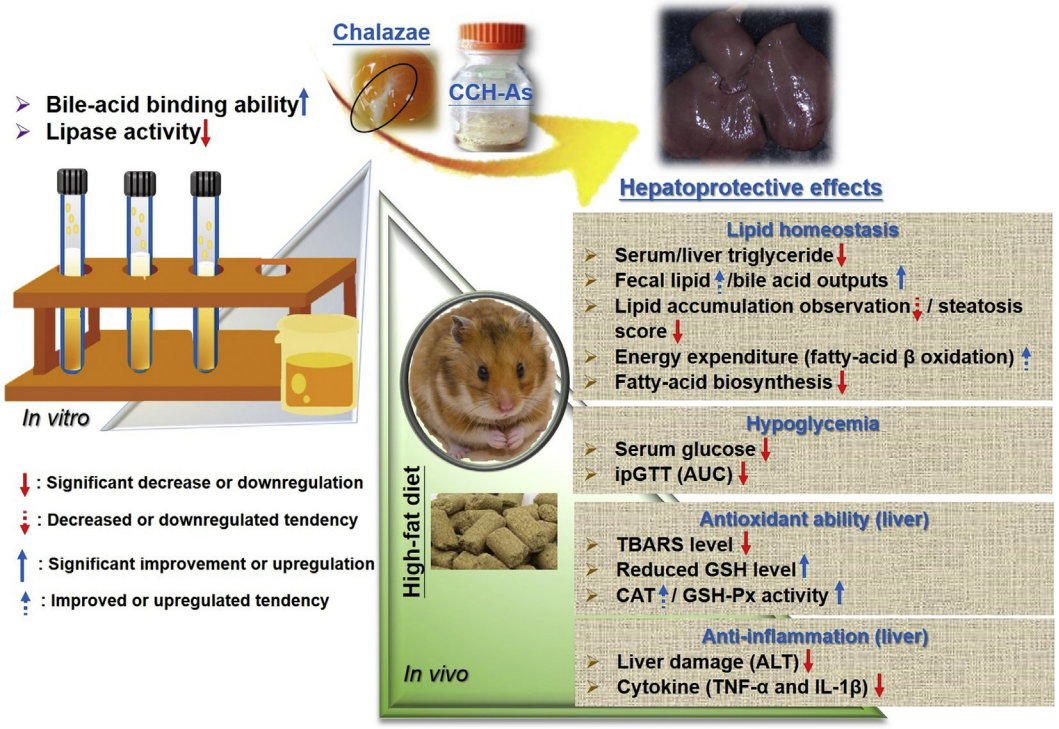


**Figure 5.** Hepatoprotection of CCH-As against a high-fat dietary habit. Abbreviations: ALT, alanine aminotransferase; AUC, area under the curve; CAT, catalase; CCH-As, crude-chalaza hydrolysates from protease-A digestion; GSH, glutathione; GSH-Px, glutathione peroxidase; IL-1β, interleukin-1 beta; ipGTT, intraperitoneal glucose tolerance test; TBARS, thiobarbituric acid reactive substances; TNF-α, tumor necrosis factor-alpha.

2014). Furthermore, lipid accumulation in livers stimulates hepatic oxidative stress and causes liver damage. Hence, the lower TBARS value in livers of HFD-fed hamsters could be due to the free amino acid profile in CCH-As (Supplementary Table 1), and is also counteracted by higher reduced GSH contents and increased CAT and GSH-Px activities, lower lipid contents, and lipid accumulation (steatosis score) in the livers (Table 1 and Figure 3). Furthermore, those improvements in oxidative stress and lipid accumulation in livers result in lower serum ALT value and hepatic TNF-α and IL-1β levels (Table 1).

## CONCLUSION

Based on the current results, the preventive effect of CCH-As against hepatosteatosis development induced by chronic high-fat dietary habits is summarized in Figure 5. First, in vitro suppressed lipase activity and elevated bile-acid binding ability of CCH-As were detected. After 10 wk of feeding an HFD, CCH-A supplementation showed some benefits: 1) decreased serum/liver TG levels and lipid accumulation in livers by increasing daily fecal lipid/bile-acid outputs and energy expenditure (fatty-acid β oxidation), and decreasing fatty-acid biosynthesis; 2) improved insulin resistance; 3) augmented antioxidant capacity in the liver; and 4) reduced liver damage and inflammatory response. Based on the results of this study, this CCH-A could not only enhance the added value of egg chalazae by means of nutraceutical development via ensuring the functionalities but also decrease the environmental burden of handling agricultural byproducts in the egg industry.

## ACKNOWLEDGMENTS

We acknowledge funding for this research from the Ministry of Science and Technology, Taiwan (Project: MOST 105-2313-B-002-031 and MOST 106-2313-B-002-040-MY3) and Council of Agriculture, Executive Yuan, Taiwan (Project: 109AS-2.2.2-AD-U2 (1), 109AS-12.1.1-ST-a1, and MOST 109-3111-Y-067E-001).

## DISCLOSURES

There are no conflicts of interest to declare.

## SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2021.01.031.

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