Research Article

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Protective effects of antioxidant egg-chalaza hydrolysates against chronic alcohol consumption-induced liver steatosis in mice

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Abstract

BACKGROUND: Reactive oxygen species (ROS) overproduction is highly related to some human chronic diseases. There are approximately 400 metric tons of chalazae produced yearly after the processing of the liquid-egg production, which are disposed of as waste. The objectives of this study were to look for the optimal production condition of antioxidant crude chalaza hydrolysates and evaluate the *in vivo* antioxidant capacity via a chronic alcohol consumption mouse model.

RESULTS: Antioxidant crude chalaza hydrolysates (CCH-As) could be produced by protease A at 1:100 ratio (w/w) and 0.5 h hydrolytic period. After our analyses, CCH-As were rich in leucine, arginine, phenylalanine, valine, lysine and antioxidant dipeptides (anserine and carnosine), and the major molecular masses were lower than 15 kDa. Regarding protective effects of CCH-As against oxidative damage in alcoholic-liquid-diet-fed mice, alcohol-fed mice had lower ($P < 0.05$) liver antioxidant capacities, and higher ($P < 0.05$) liver lipid contents, serum lipid/liver damage indices and IL-1β/IL-6 values. CCH-A supplementation reversed ($P < 0.05$) liver antioxidant capacities and reduced ($P < 0.05$) serum/liver lipids in alcohol-fed mice, which may result from increased ($P < 0.05$) fecal lipid output, upregulated ($P < 0.05$) fatty acid β-oxidation and downregulated ($P < 0.05$) lipogenesis in the liver.

CONCLUSION: Taken together, this CCH-A should benefit the liquid-egg industry, while also offering consumers a choice of healthy ingredients from animal sources.

Keywords: alcoholic fatty liver; amino acid profile; *in vitro* and *in vivo* antioxidant effects; lipid metabolism; protease-A-digested crude chalaza hydrolysate

INTRODUCTION

As we know, reactive oxygen species (ROS) overproduction is highly related to some human diseases, i.e. steatohepatitis, atherosclerosis, diabetes, cancers, some neurological disorders and ageing. Alcoholic beverage is one of the earlier drinks in human history and also important in human society. Alcohol is mainly metabolized into acetaldehyde by alcohol dehydrogenase (ADH) and then converted into acetic acid by aldehyde dehydrogenase (ALDH) in the liver.[1] However, excessive alcohol metabolism is always coupled with poisonous metabolites, i.e. ROS generation, imbalance of cellular energy metabolism and lipid aggregation, thus inducing steatohepatitis.[2,3]

The Ministry of Health and Welfare, Executive Yuan, Taiwan (2018) reported that liver-related diseases always rank as one of the ten leading causes of death in Taiwan;[4] the World Health Organization also indicated that more than half of liver diseases globally are highly attributable to alcohol consumption.[5] Hence scientists strive to look for natural agents with antioxidant abilities against chronic alcohol consumption, e.g. citrus[6] and pepsin-digested chicken liver hydrolysate.[7] Recently, it was reported that certain food-derived hydrolysates or peptides indeed demonstrate some health effects, especially antioxidant, lipid-lowering and anti-inflammatory effects. A functional chicken liver hydrolysate obtained by pepsin digestion demonstrates increased antioxidative capacity, lipid homeostasis regulation and hepatoprotective effects against either chronic alcohol consumption or thioacetamide induction in D-galactose-induced mice,[8] high-fat-fed hamsters,[9] alcohol-consuming mice[7] and thioacetamide-injected rats,[10] respectively. Besides, dried egg

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chalazae contain 11% lysozyme, which is as a complex form with ovomucin.[11] It was reported that a high antioxidant and antimicrobial peptide against both Gram-negative and Gram-positive bacteria was isolated and identified as NTDGSTDYGILQINSR from hen-egg white lysozyme hydrolysates via hydrolyzation by a combination of trypsin and papain.[12] Recently, two novel antioxidant peptides, LDEPDPL and NIQTDDFRT, were also derived from the ultrafiltrate of ovomucin hydrolysates with a microbial protease (Protamex®).[13]

According to a statistical report from the Council of Agriculture, Executive Yuan, Taiwan, there were approximately 7 billion eggs produced in 2016, and 6% of them were used as a liquid-egg production.[14] After a calculation (chalazae: 1.6% total egg weight), there are approximately 400 metric tons of chalazae produced yearly after the processing of the liquid-egg production. Hence, to increase the added value of liquid-egg by-products, the objectives of this study were divided into two aspects: (i) to look for the optimal production condition of antioxidant crude chalaza hydrolysates; and (ii) to evaluate the hepatoprotective effects of this hydrolysate against chronic alcohol consumption.

MATERIALS AND METHODS

Egg chalaza collection

Fresh egg chalazae were collected from the liquid-egg producer (Daiegg Co. Ltd, Tainan City, Taiwan), which had passed the certification of Certified Agricultural Standards (CAS) in Taiwan. Collected chalazae were transported to our lab under −20 °C transportation. The eggs used in the liquid-egg production should be safe from concerns of antibiotic and sulfonamide residues. For 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 they were all found to be safe. Upon arrival, the large chalaza blocks were separated into several smaller portions sufficient for a single experiment, followed by storage at −20 °C until used. Prior to an experiment, the smaller blocks were tempered overnight in a refrigerator at 2 °C, resulting in partially frozen chalazae, which were also removed from visible impurities, i.e. eggshells, yolk and some dirt.

Determination of manufacturing condition of crude chalaza hydrolysates (CCHs)

Distilled and deionized water (ddH_2O) was added to partially frozen raw crude egg chalazae at a ratio of 1:2 (chalaza:ddH_2O, w/w), followed by homogenization for 3 min and inactivation of endogenous enzymes of raw materials by temperature treatment at 95 °C for 15 min. After cooling, the enzymatic hydrolysis was started by adding pepsin (Cat. #: 099A3000, 3 000 000 U g^{-1}; Calzyme Laboratories Inc., San Luis Obispo, CA, USA; optimal hydrolytic conditions: pH 2, 37 °C), protease A (63 000 U g^{-1}; Amano Enzyme Ltd, Nagoya, Japan; optimal hydrolytic conditions: pH 6, 50 °C), and prozyme 6 (Lot #: PZL0450509, 748 000 U g^{-1}; Amano Enzyme Ltd, Nagoya, Japan; optimal hydrolytic conditions: pH 8, 45 °C), respectively. The appropriate hydrolytic periods (0, 0.5, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 h) of pepsin, protease A or protease 6 regarding their optimal hydrolyzing condition were investigated. The ratio of enzyme to substrate (crude egg chalaza) was 1:200 (w/w). Then, the optimal enzyme for producing CCHs was determined by using the same enzymatic unit (1000 U g^{-1} crude chalaza) of those three enzymes in the reaction mixture. After the optimal enzyme was chosen, an optimal ratio of crude chalaza enzymatic homogenates was determined via hydrolyzation by different ratios of enzyme to raw material at 1:100, 1:200, 1:300, 1:400, and 1:500 (w/w) with its optimal hydrolytic period and enzyme. After hydrolysis, the chalaza enzymatic homogenates were immediately heated at 95 °C for 15 min. The CCHs were centrifuged at 900 × *g* at 4 °C for 15 min (model 3700, Kubota Co., Tokyo, Japan). After filtering, the supernatant was lyophilized (model CoolSafe 110-9 Pro Freeze Drying, LaboGene Aps, Lynge, Denmark). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging ability, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^+$) radical scavenging activity, reducing powder, yield [(weight of lyophilized CCHs / weight of raw chicken livers) × 100%], and peptide level of final lyophilized CCHs were used to determine the optimal manufacturing conditions.

In vitro study

DPPH scavenging ability assay

DPPH scavenging ability was assayed according to the method described by Chou *et al.*[8] The solution was obtained by mixing 0.8 mL CCH solution (10 mg lyophilized CCH mL^{-1} ddH_2O) with 0.2 mL of 1 mmol L^{-1} DPPH (Sigma Co., St Louis, MO, USA) dissolved in 95% ethanol and kept in the dark for 30 min at room temperature. The supernatant was picked up via centrifugation at 2000 × *g* for 5 min. Absorbance was measured at 517 nm. The blank was treated in the same manner, except that ddH_2O was used instead of CCH solution. Butylated hydroxytoluene (BHT) (Sigma Co.) (1 mg mL^{-1}) was used as a positive control compared to CCHs. The DPPH scavenging ability was calculated from the following equation:

$$\text{DPPH scavenging ability (\%)} = [1 - (A_{blank} - A_{sample} \text{ or } A_{BHT})/A_{blank}] \times 100\%$$

$ABTS^+$ radical scavenging activity assay

$ABTS^+$ radical scavenging activity was assayed based on the method of a previous report,[15] with a slight modification. A free radical, $ABTS^+$, can be generated by mixing ABTS (1 mmol L^{-1}) (Sigma Co.) with H_2O_2 (10%) (Sigma Co.) and peroxidase (44 U mL^{-1} reaction solution) (Sigma Co.). Briefly, a 0.25 mL mixture of ABTS, H_2O_2 and peroxidase was mixed well with 1.5 mL ddH_2O and then transferred to a 96-well plate (200 µL per well). The 96-well plate was placed in a dark room. After 60 min, 20 µL of various CCH solutions (10 mg lyophilized CCH mL^{-1} ddH_2O) was added to the wells. Absorbance was measured at 734 nm after the interaction of sample solution for 10 min. According to a standard curve plotted for Trolox (Sigma Co.), the decrease in absorption at 734 nm after the addition of reactant was used to calculate the $ABTS^+$ radical scavenging activity, which was expressed as µmol Trolox mg^{-1} protein.

Reducing powder

Reducing power was determined according to the method of a previous report,[16] with a slight modification. The antioxidant action of a reducing agent is based on its ability to reduce Fe(III) ion to Fe(II) ion. 0.5 mL CCH solution (10 mg lyophilized CCH mL^{-1} ddH_2O) was mixed with 0.25 mL phosphate-buffered saline (PBS) and 0.25 mL potassium ferricyanide (1%) (Sigma Co.), and the mixture was incubated at 50 °C for 15 min. 0.25 mL trichloroacetic acid solution (10%) (Sigma Co.) was added to the mixture, which was then centrifuged at 900 × *g* for 10 min at room temperature. Then

0.5 mL supernatant was mixed with 0.25 mL distilled water and 500 mL ferric chloride solution (0.1%) (Sigma Co.), and after 10 min the absorbance was measured at 700 nm immediately.

Peptide levels of lyophilized CCHs

The peptide levels of lyophilized CCHs were determined according to the method described by Chou *et al.*[8] Briefly, the *o*-phthaldialdehyde (OPA) reagent was freshly prepared by mixing 25 mL of 100 mmol L^{-1} sodium tetraborate (Sigma Co.), 2.5 mL of 20% sodium dodecyl sulfate (Sigma Co.), 100 μL of 14.3 mol L^{-1} β-mercaptoethanol and 40 mg OPA (Sigma Co.) in 1 mL methanol (Sigma Co.) and ddH_2O to 50 mL. Twenty microliters of CCH solution (10 mg lyophilized CCH mL^{-1} ddH_2O) was mixed with 1.5 mL OPA reagent and allowed to stand for 2 min before the measurement of absorbance at 340 nm. Peptide levels in lyophilized CCHs were calculated with reference to glycine (Sigma Co.) standard curve.

Investigation of protein patterns, and free amino acid and carnosine/anserine content in CCHs obtained from the optimal hydrolysis condition

When the optimal manufacturing conditions for CCH production were determined, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), free amino acid and carnosine/anserine content in CCHs were used to understand the hydrolytic levels and bioactive compounds in CCHs. The protein patterns in raw materials (crude chalaza) and CCHs obtained from the optimal hydrolysis conditions were investigated using an SDS-PAGE method with 4% stacking gel and 15% running gel. Electrophoresis was run in a Mini-Protein 3 cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA) at 80 V for 15 min and then at 120 V for 30 min. After completing electrophoresis, the gels were placed in a fixing solution containing 25% (v/v) isopropanol (Sigma Co.) and 10% (v/v) acetic acid (Sigma Co.) for 15 min, then stained with Coomassie brilliant blue R-250 for 2 h and de-stained with 10% acetic acid.

Free amino acid and carnosine/anserine content in lyophilized CCHs from the most optimal hydrolyzing conditions

Free amino acid and carnosine/anserine contents of dried raw materials (dried crude egg chalaza powder) or CCHs from the most optimal hydrolyzing condition were analyzed at the Food Industry Research and Development Institute (FIRDI, HsinChu, Taiwan) using an amino acid analyzer (model L8800, Hitachi High-Technologies Co., Tokyo, Japan). For the preparation of analyzed items, 5 g lyophilized chalazae or CCH powders were homogenized, respectively, in 20 mL trichloroacetic acid (7%, v/v) for 2 min and then filtered. The precipitates were also homogenized twice with trichloroacetic acid as described above. The data were reported as milligrams of amino acid, anserine or carnosine per 100 g lyophilized dried crude egg chalazae or CCH powders, respectively.

In vivo study

Animals and groupings

After the most optimal hydrolysis condition of CCHs, antioxidant crude chalaza hydrolysates (CCH-As) can be produced by protease A at ratio of 1:100 (w/w) and 0.5 h hydrolytic period. *In vivo* antioxidant effects of CCH-As was evaluated via liver damage induced by oxidative stress in alcoholic-liquid-diet-fed mice. Animal use and protocol were reviewed and approved by National Taiwan University Animal Care Committee (IACUC No. 105–035). Eighteen male C57BL/6J (B6) mice of age 8 weeks were purchased from the Laboratory Animal Center of National Taiwan University (Taipei City, Taiwan). All mice were individually housed in cages in an animal room at 22 ± 2 °C with a 12/12 h light–dark cycle. For induction of chronic alcoholic liver disease development, mice were fed a Lieber–DeCarli regular ethanol diet containing 5% (v/v) ethanol (35% ethanol-derived calories) (Research Diets, Inc., New Brunswick, NJ, USA). After 1 week of acclimation, all mice were randomly divided into three groups: (i) control (CON): isocaloric control liquid diet (47% of calories as carbohydrate, Research Diets, Inc.) and 0.1 mL ddH_2O (oral gavage); (ii) ALC: Lieber–DeCarli regular ethanol diet and 0.1 mL ddH_2O (oral gavage); (iii) ALC + CCH-A: Lieber–DeCarli regular ethanol diet and 100 mg kg^{-1} body weight (BW) protease A digested crude chalaza hydrolysate (CCH-A) in 0.1 mL ddH_2O (oral gavage) for 8 weeks. The final body weight of each mouse at the end of the experiment was recorded. A daily liquid feed (g per mouse per day) intake as a food intake was calculated per mouse on a daily basis. Mice were sacrificed by CO_2; heart, liver, kidney and abdominal fat pad in the abdominal cavity were obtained and weighed individually after sacrificing and stored at −80 °C for further analyses.

Sample collection and liver homogenate preparation

Blood samples were collected by orbital sinus, and then kept at room temperature for 1 h. Then, sera were centrifuged (3000 × *g*) at 4 °C for 15 min (model 3740; KUBOTA, Tokyo, Japan), and the collected supernatant was stored at −80 °C for further analyses. Serum triglyceride (TG), cholesterol (TC), aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) levels were assayed using commercial enzymatic kits with an ESPOTCHEM™ EZ SP4430 biochemistry analyzer (ARKRAY Inc., Kyoto, Japan). For preparation of liver homogenates, 0.5 g liver was homogenized on ice in 4.5 mL phosphate buffered saline (PBS, pH 7.0, containing 0.25 mol L^{-1} sucrose) and centrifuged (900 × *g*, 15 min, 4 °C). The protein concentration in the supernatant was quantified using a Bio-Rad protein assay kit (Cat. 500–0006, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Measurements of proinflammatory cytokines and antioxidative capacities in livers

Levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) in livers were determined using enzyme-linked immunosorbent assay (ELISA) kits purchased from R&D Systems Inc. (Minneapolis, MN, USA). Briefly, liver homogenate, control buffer, washing buffer, standard solution and substrate solution were prepared before the analyses. Fifty microliters of assay diluent was added to a 96-well plate, and then 50 μL standard or homogenate was added and the mixture incubated for 2 h at room temperature. The plate was then washed with washing buffer and then 100 μL conjugated buffer was added to the plate, which was incubated for 2 h at room temperature, and the washing process was repeated. Finally, 100 μL substrate solutions were added to the wells, then incubated for 30 min at room temperature in a dark room, and 100 μL stop solution was added. After reaction for 30 min, the optical density value of each well was immediately measured using an ELISA reader (Model Synergy H1 hybrid reader, Bio-Tech Instruments, Inc., Winooski, VT, USA). Hepatic TNF-α, IL-1β and IL-6 levels were all expressed as pg mg^{-1} protein. 2-Thiobarbituric acid reactive

substances (TBARS), reduced glutathione (reduced GSH) and Trolox equivalent antioxidative capacity (TEAC) values, as well as activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in livers, were analyzed according to the methods of previous reports.[7,10] TBARS and reduced GSH values were calculated by taking the extinction coefficients of malondialdehyde (MDA) to be 1.56×10^5 L mol^{-1} cm^{-1} at 535 nm and TNB to be 1.36×10^4 L mol^{-1} cm^{-1} at 412 nm, respectively. TEAC value was measured by the decrease in absorption at 734 nm after the addition of reactant via a standard curve for Trolox on scavenging ABTS$^+$ capacity. The decrease in absorption at 734 nm after the addition of reactant was used to calculate TEAC value. A standard curve was plotted for trolox on scavenging ABTS+ capacity and was calculated as the TEAC value. SOD activity was detected by its inhibitory effect on purpurogallin of pyrogallol oxidation product, and was recorded at 420 nm in 3 min. The change in absorbance per minute under 420 nm wavelength was calculated to obtain inhibition (%) by the following formula: 1 − [(sample or standard ΔA/min) / (max. speed Δi/min)], where max. speed was set to reaction of ddH_2O loading, indicating almost no inhibition of pyrogallol autoxidation. A standard curve was plotted for SOD, which was used for calculation of activities of SOD in liver tissues. CAT activity was calculated by taking the extinction coefficient of H_2O_2 to be 39.4 L mol^{-1} cm^{-1} at 240 nm and 25 °C and expressed as units mg^{-1} protein. GPx activity was measured using the RANSEL assay kit (Randox Laboratories Ltd, Crumlin, UK). GPx activity of the liver was measured as the decrease in absorbance at 340 nm wavelength between 0 and 3 min and expressed as units mg^{-1} protein.

Lipid profile analyses in livers or feces

TG and TC concentrations in livers or feces were assayed according to the methods reported by Chang *et al.* (2011).[17] Briefly, liver or fecal lipid was extracted by chloroform and methanol (2:1, v/v). The extract was dried under N_2 and resuspended in isopropanol via an ultrasonic cleaner (model DC150H, Taiwan Delta New Instruments, New Taipei City, Taiwan). TG and TC concentrations in livers or feces were measured using commercial kits (Randox Laboratories Ltd).

Histopathological analyses

Liver tissues were fixed in neutral buffered formalin and then dehydrated in graded alcohol (30%, 50%, 70%, 95% and 99.5%). After impregnating the tissues in xylene, they were embedded in paraffin for sectioning using a microtome and cut into 5 µm thick sections. On completion of de-paraffinization with xylene, tissues were dehydrated in graded alcohol and stained with hematoxylin and eosin (H&E). Microscopic analysis was conducted using a Zeiss Axioskop 340 microscope and AxioCam ERc 5s camera system, with AxioVision Release 4.8.2 (06-2010) 341 software (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). The steatosis scores of livers were evaluated according to a report by Hübscher.[18] The samples were categorized into three levels based on severity.

Determination of mRNA expression of lipogenesis and β-oxidation in livers

The transcription of RNA to cDNA was carried out using commercial kits (GoScript™ Reverse Transcriptase, Promega Co., Madison, WI, USA). Real-time polymerase chain reaction (PCR) was carried out using commercial kits (Fast SYBR Green Master Mix, Applied Biosystems, Molecular Probes Inc., Eugene, OR, USA). All the primers used were designed: *Gapdh* (F: 5′-AACCTGCCAAGTATGATGA-3′, R: 5′-GGAGTTGCTGTTGAAGTC-3′; Accession No. NM_001289726); *Srebp1-c* (F: 5′-CCTAGAGCGAGCG TTGAACT-3′, R: 5′-GAACTCCCTGTCTCCGTCA-3′; Accession No. NM_011480.3); *Fas* (F: 5′-GACTCGGCTACTGACACGAC-3′, R: 5′-CGA GTTGAGCTGGGTTAGGG-3′; Accession No. NM_007988.3); *Acc* (F: 5′-GGAGGCTGCATTGAACACAAG-3′, R: 5′-CGACGGTGAAATCTCT GTGC-3′; Accession No. NM_133904.2); *Ppar-α* (F: 5′-TGACACCTTC CTCTTCCCAAA-3′, R: 5′-CGTCGGACTCGGTCTTCTTG-3′; Accession No. NM_001113418.1); *Cpt1* (F: 5′-CTGAGCCATGAAGCCCTCAA-3′, R: 5′-CACACCCACCACCACGATAA-3′; Accession No. NM_013495.2); *Ucp2* (F: 5′-CCTCCCTTGCCACTTCACTT-3′, R: 5′-AGGCATGAACC CCTTGTAGA-3′; Accession No. NM_011671.5). The qPCR was performed according to instructions (Fast SYBR® Green Master Mix; Table III-5) and detected the fluorescence signal by using a StepOne real-time PCR system (Applied Biosystems, Foster, CA, USA). Details of the method and calculation of the fold increases over CON group were according to the instruction manual of the StepOne real-time PCR system. *Gapdh* was used as a housekeeping gene with the mRNA gene expressions of CON group setting at 1.0.

Statistical analysis

The experiment was conducted using a completely random design (CRD). When a significant difference was detected at 0.05 probability level using analysis of variance (ANOVA), differences between treatments were tested using the least significant difference (LSD) test. All statistical analyses of data were performed using SAS (2002; SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Manufacturing procedures of functional CCHs with optimal hydrolysis conditions

In vitro assays for antioxidative capacities of CCHs produced by different hydrolytic periods, enzymes and ratios of enzyme to substrate are demonstrated in Fig. 1. At the same ratio of enzyme (pepsin, 3 000 000 U g^{-1}; protease A, 63 000 U g^{-1}; prozyme 6 748 000 U g^{-1}) to substrate (1:200, w/w), hydrolyzed crude egg chalazae had higher DPPH scavenging ability, ABTS$^+$ radical scavenging ability and reducing powder than raw crude egg chalazae (non-digested crude chalaza, at 0 h hydrolytic period) (Fig. 1A). In comparison with CCH using three enzymes, protease A hydrolyzation for 0.5 h resulted in the highest ($P < 0.05$) DPPH scavenging ability, but pepsin hydrolyzation dramatically enhanced ($P < 0.05$) DPPH scavenging ability in comparison to other enzymes beyond 2 h hydrolyzation. As the hydrolytic period goes on, either prozyme 6 or protease A showed higher ($P < 0.05$) ABTS$^+$ radical scavenging ability and reducing powder, respectively; however, pepsin hydrolyzation was always lower ($P < 0.05$) for both ABTS$^+$ radical scavenging ability and reducing powder. Hence, in avoidance of over-hydrolyzation and overall antioxidant capacity recession, a 0.5 h hydrolytic period for three enzymes should be appropriate for further choices of enzyme hydrolyzation and the ratio of enzyme to substrate. Continuously, under 0.5 h hydrolyzation and at the same enzymatic activity (1000 U g^{-1} crude chalaza) (Fig. 1B), protease A produced the highest ($P < 0.05$) DPPH and ABTS$^+$ radical scavenging ability of CCHs compared with the other two enzymes, while CCHs produced by both protease A and prozyme 6 had higher ($P < 0.05$) reducing powder than that produced by pepsin. Hence protease A was chosen as the optimal enzyme for determination of an approximate ratio of enzyme to substrate.

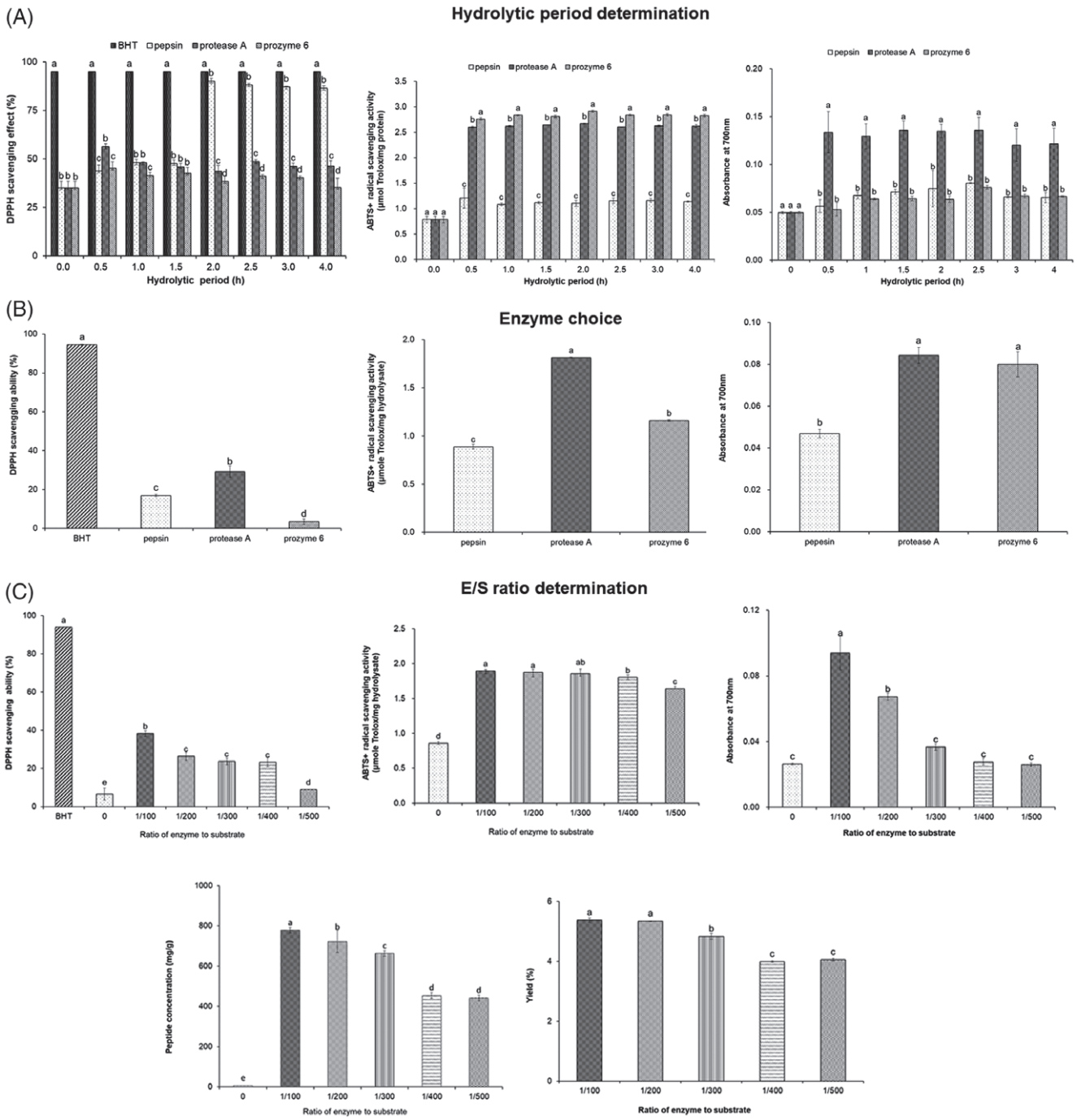
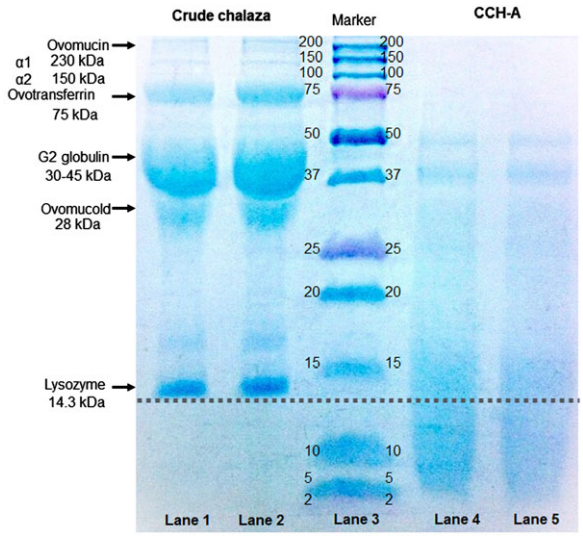


Figure 1. Determination of manufacturing parameters of functional crude chalaza hydrolysates: (A) hydrolytic period, (B) enzyme choice and (C) ratio of enzyme to substrate. Values are means ± SD ($n = 3$). Data bars without a common letter in each tested parameter are significantly different ($P < 0.05$).

Moreover, in *in vitro* antioxidative assays of CCHs produced by various ratios of protease A to substrate (1:100–1:500, w/w) (Fig. 1C), protease A hydrolyzation dramatically increased ($P < 0.05$) DPPH and ABTS$^+$ radical scavenging ability and reducing powder in comparison to raw crude egg chalazae. With regard to protease A:raw crude chalaza mass ratio, CCHs produced by a ratio of 1:100 had the highest ($P < 0.05$) *in vitro* antioxidative abilities (DPPH scavenging ability and reducing powder) than other ratios. Meanwhile, this ratio (1:100) also had the highest ($P < 0.05$) peptide contents (778.65 mg g^{-1} dried CCH powder) and yield (5.38%). The degree of protein denaturation or protein hydrolyzation are related to the process and preparation of enzymatic hydrolysis. It has been reported that food protein via enzymatic hydrolyzation can show some health benefits, which are highly dependent on their inherent amino acid composition or peptide sequence.[7,8,16] Various *in vitro* assays are applied to investigate antioxidant properties. DPPH and ABTS$^+$ radical scavenging activities are more widely employed for screening *in vitro* antioxidant activities. One peculiarity of these two assays is that they are applicable to both lipophilic and hydrophilic compounds.[19] In addition, the principle of reducing power assay is to evaluate the ability of antioxidant to donate electrons.[20] Based on current data, CCHs produced

(A)



(B)

Amino acid	Crude chalaza (mg/100g powder)	CCH-A (mg/100g powder)
L-Leucine	3.01	1675.41
L-Arginine	-	1179.76
L-Phenylalanine	6.96	1054.68
L-Valine	4.73	1044.22
L-Lysine	-	984.47
L-Isoleucine	0.72	737.49
L-Methionine	0.58	532.24
L-Threonine	-	423.57
Tryptophan	-	267.21
L-Histidine	-	350.01
Total EAA	16.00	8249.06
Total BCAA	8.46	3457.12
Asparagine	-	473.56
L-Tyrosine	0.36	456.55
L-Serine	0.77	452.43
o-Phosphoserine	3.33	231.69
L-Glutamic acid	0.52	179.67
L-Aspartic acid	0.70	118.24
Glycine	3.21	141.41
L(-)-Proline	-	76.73
L-2-Aminoadipic acid	-	75.40
DL-3-Aminoisobutyric acid	-	72.70
γ-Aminobutyric acid	-	66.07
L-Ornithine	12.51	61.41
β-Alanine	-	42.53
L-Cystathionine	0.23	37.25
Sarcosine	-	32.44
Taurine	-	35.41
Total NEAA	21.63	2553.49
Dipeptide	Crude chalaza (mg/100 g powder)	CCH-A (mg/100g powder)
L-Carnosine	-	48.06
L-Anserine	-	106.84

* EAA: essential amino acid; NEAA: nonessential amino acid; BCAA: branched chain amino acid; - : not detected.

Figure 2. (A) SDS-PAGE of egg chalazae and crude protease A-digested chalaza hydrolysates (CCH-As) (crude chalaza in lanes 1 and 2, marker in lane 3, and CCH-A in lanes 4 and 5); (B) content of free amino acids and dipeptide (L-carnosine and L-anserine) in dried crude chalazae and CCH-A, respectively.

by a protease A:raw crude egg chalaza ratio of 1:100 and 0.5 h hydrolytic period had higher efficacies on yield, peptide levels and antioxidative effects.

Composition of CCHs produced by protease a at a ratio of 1:100 (w/w) for 0.5 h (CCH-As)

The protein pattern, and free amino acid and functional dipeptide contents of CCH-As were analyzed by SDS-PAGE and amino acid analyzer, respectively (Fig. 2). In comparison to raw crude egg chalazae, the major patterns of raw crude egg chalazae illustrated intensive bands at approximate 200 and 150 kDa (ovomucin subunits), 75 kDa (ovotransferrin), 32–45 kDa (G2 globulin), 30 kDa (ovomucoid) and 14 kDa (lysozyme), but the protein bands of CCH-As majorly gathered at 50, 37 and <15 kDa (Fig. 2A). After hydrolyzation, total free essential amino acid (EAA, 8249.06 mg 100 g^{-1} powder), branched-chain amino acid (BCAA; leucine + iosleucine + lysine = 3457.12 mg 100 g^{-1} powder) and nonessential amino acid (NEAA, 2553.49 mg 100 g^{-1} powder) in CCH-As were significantly increased 515.6, 408.6, and 118.1 fold, respectively, compared to those in lyophilized raw crude egg chalazae (Fig. 2B). Meanwhile, L-carnosine and L-anserine also were only detected in CCH-As rather than lyophilized raw crude egg chalazae.

Based on the illustration of SDS-PAGE, protein patterns of the crude egg chalazae used in this study included α1 and α2 ovomucin, ovotransferrin, G2 globulin, ovomucoid and lysozyme, as similarly demonstrated in previous studies.[21,22] As we know, biofunctional properties of food-derived bioactive hydrolysates or peptides are dependent on amino acid composition or its sequence, generated from fermented and enzymatic hydrolyzation. Chou *et al.* reported that pepsin-digested chicken liver hydrolysates, where the molecular mass was lower than 10 kDa, not only showed *in vitro* antioxidant abilities but also increased TEAC levels, as well as activities of antioxidant enzymes, i.e. SOD, CAT and GPX, in liver and brain tissues of D-galactose-injected mice.[8] It has been reported that this antioxidant property is mostly due to either the existence of hydrophobic amino acids in the peptide or positively charged amino acids at the C-terminus.[8,23] Hydrophobic amino acids such as cysteine, histidine, tryptophan, tyrosine, phenylalanine, methionine, leucine, valine, glycine and proline have been reported to enhance the scavenging activities.[24] It has been mentioned that the phenolic group of tyrosine can act as a hydrogen donor;[25] meanwhile, acidic amino acids, i.e. aspartic and glutamic acids, also display strong antioxidant properties.[26] Besides, both carnosine and anserine are natural histidine-containing dipeptides that show antioxidant and lipid-lowering abilities. Chen *et al.* indicated that because of an imidazole moiety the antioxidant biofunction of carnosine or anserine is attributed to a scavenging ability of free radicals and ROS, or prevention of oxidative damage by interrupting lipid peroxidation, which is involved in hydrogen donation for lipid radical trapping.[27] Hence, based on the hydrolyzed protein patterns and amino acid or dipeptide composition in our CCH-As, it can be conjectured that CCH-As should offer an *in vivo* antioxidant potential beyond the nutritional values.

In vivo hepatoprotective effects of CCH-As

Effects of CCH-As on body weight, size of organs and abdominal fat pad, serum biochemical values, liver damage status, and hepatic and fecal lipids in chronic alcohol-fed mice

With regard to an *in vivo* antioxidant effect of our manufactured CCH-As, chronic alcoholic liquid-diet-fed mice were applied as

Table 1. Final body weight, sizes of organs and abdominal fat pad, serum biochemical values, liver pro-inflammatory cytokine and liver/fecal lipids of experimental mice

	CON	ALC	ALC+CCH-A
Final body weight (g)	21.84 ± 0.33a	23.86 ± 1.24a	19.73 ± 2.78a
Liquid feed intake (g per mouse per day)	9.92 ± 0.28a	9.81 ± 0.01a	9.71 ± 0.08a
Organ size (g 100 g^{-1} body weight)			
Heart	0.51 ± 0.02a	0.48 ± 0.03a	0.50 ± 0.05a
Liver	4.06 ± 0.32b	4.87 ± 0.28a	4.32 ± 0.72ab
Kidney	1.27 ± 0.11a	1.24 ± 0.15a	1.27 ± 0.18a
Abdominal fat pad	1.19 ± 0.23b	2.99 ± 0.33a	0.93 ± 1.03b
Serum biochemical value			
TG (mg dL^{-1})	92.80 ± 3.11b	105.00 ± 9.54a	92.00 ± 2.83b
TC (mg dL^{-1})	83.60 ± 0.55b	117.80 ± 16.41a	81.08 ± 17.61b
AST (U L^{-1})	123.50 ± 11.32b	185.13 ± 36.51a	120.00 ± 40.10b
ALT (U L^{-1})	23.00 ± 2.23b	86.00 ± 2.45a	25.20 ± 3.27b
ALKP (U L^{-1})	72.60 ± 8.88b	89.80 ± 21.92a	64.00 ± 28.14b
Liver pro-inflammatory cytokine			
TNF-α (pg mg^{-1} protein)	270.20 ± 43.16b	900.91 ± 174.42a	863.09 ± 189.81a
IL-1β (pg mg^{-1} protein)	52.64 ± 7.88b	158.64 ± 83.24a	59.20 ± 37.08b
IL-6 (pg mg^{-1} protein)	159.85 ± 87.63c	1488.54 ± 344.43a	874.57 ± 139.50b
Liver lipid profile			
TG (mg g^{-1} liver tissue)	37.17 ± 7.95b	63.16 ± 13.39a	29.25 ± 2.38b
TC (mg g^{-1} liver tissue)	1.68 ± 0.27b	2.38 ± 0.37a	2.18 ± 0.31a
Fecal lipid profile			
TG (mg g^{-1} feces)	1.02 ± 0.01b	0.63 ± 0.27b	2.72 ± 0.88a
TC (mg g^{-1} feces)	1.05 ± 0.05b	1.27 ± 0.11b	1.96 ± 0.29a

Values are means ± SD ($n = 6$). Means without a common letter in each tested parameter are significantly different ($P < 0.05$).
CON, control liquid diet + 0.1 mL saline; ALC, ethanol liquid diet + 0.1 mL saline; ALC+CCH-A, ethanol liquid diet +100 mg CCH-A kg^{-1} BW in 0.1 mL ddH_2O.

an animal model. No ($P > 0.05$) differences in final body weight and liquid feed intake among groups were measured (Table 1). Moreover, larger ($P < 0.05$) sizes of liver and abdominal fat pad were observed in the ALC group than those in the CON group, while CCH-A supplementation (ALC+CCH-A group) reduced ($P < 0.05$) the size of the abdominal fat pad, but only a slight tendency toward lower liver size was calculated. In serum biochemical values, the ALC group had higher ($P < 0.05$) TG, TC, AST, ALT and ALKP values than the CON group, but these values were reduced ($P < 0.05$) by CCH-A supplementation, even becoming similar ($P > 0.05$) to those of the CON group. Moreover, chronic alcohol consumption also dramatically increased ($P < 0.05$) TNF-α, IL-1β and IL-6 levels in livers compared to control liquid diets (CON group), where IL-1β and IL-6 levels were decreased ($P < 0.05$) by supplementing CCH-As. Focusing on the pathological changes of livers due to chronic alcohol consumption and CCH-A supplementation, an alcoholic liquid diet apparently induced macro-lipid droplets in livers (Fig. 3A), and meanwhile had an increased ($P < 0.05$) steatosis score (Fig. 3B), but fewer droplets (only micro-lipid droplets left) in livers were observed in the CCH-A-supplemented group (ALC+CCH-A group), which caused a lower ($P < 0.05$) steatosis score. Meanwhile, both TG and TC levels in livers of mice were increased ($P < 0.05$) in the ALC group compared to those in the CON group, but CCH-A supplementation only decreased ($P < 0.05$) liver TG levels (Table 1). There were no ($P > 0.05$) differences in fecal TG and TC levels between CON and ALC groups, but CCH-A supplementation increased ($P < 0.05$) them (Table 1).

During the process of alcohol metabolism, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) convert alcohol to acetaldehyde and acetaldehyde to acetic acid, respectively. Meanwhile, NADH is produced and then the NADH/NAD+ ratio is increased.[1] Zakhari (2006)[1] also indicated that chronic alcohol consumption downregulates fatty acid β-oxidation in mitochondria; meanwhile, export of hepatic triglycerides is insufficient to counteract the accumulation, thus stimulating glycerol synthesis to form triglycerides in the liver. The higher triglyceride accumulation results in fatty liver or steatosis. Apart from ADH, ethanol oxidation is also through the hepatic microsomal ethanol oxidizing system, which involves cytochrome P450 2E1 (CYP2E1). The CYP2E1 pathway produces acetaldehyde (a toxin to the mitochondria), generates release of free radicals and enhances oxidative stress. These sequential injuries trigger liver inflammatory agents such as lipopolysaccharides and further produce pro-inflammatory (TNF-α) in the Kupffer cells.[6] Dairy supplementation enriched in BCAAs (leucine, valine, and isoleucine) has been proposed to be of benefit to inhibition of lipid peroxidation and NO scavenging activity.[28] It was reported that pepsin-digested chicken liver hydrolysates rich in BCAAs can decrease serum TG and TC, and have a better cholesterol profile (ratio of LDL-C:HDL-C) in high-fat diet fed hamsters.[9] Similarly, it was also reported that pepsin-digested egg white possibly inhibits chylomicron assembly to alter intestinal cholesterol uptake, increase fecal neutral steroid excretion and decrease lymphatic cholesterol transport in rats.[29] In addition, amino acid residues with strong cationic properties at physiological pH

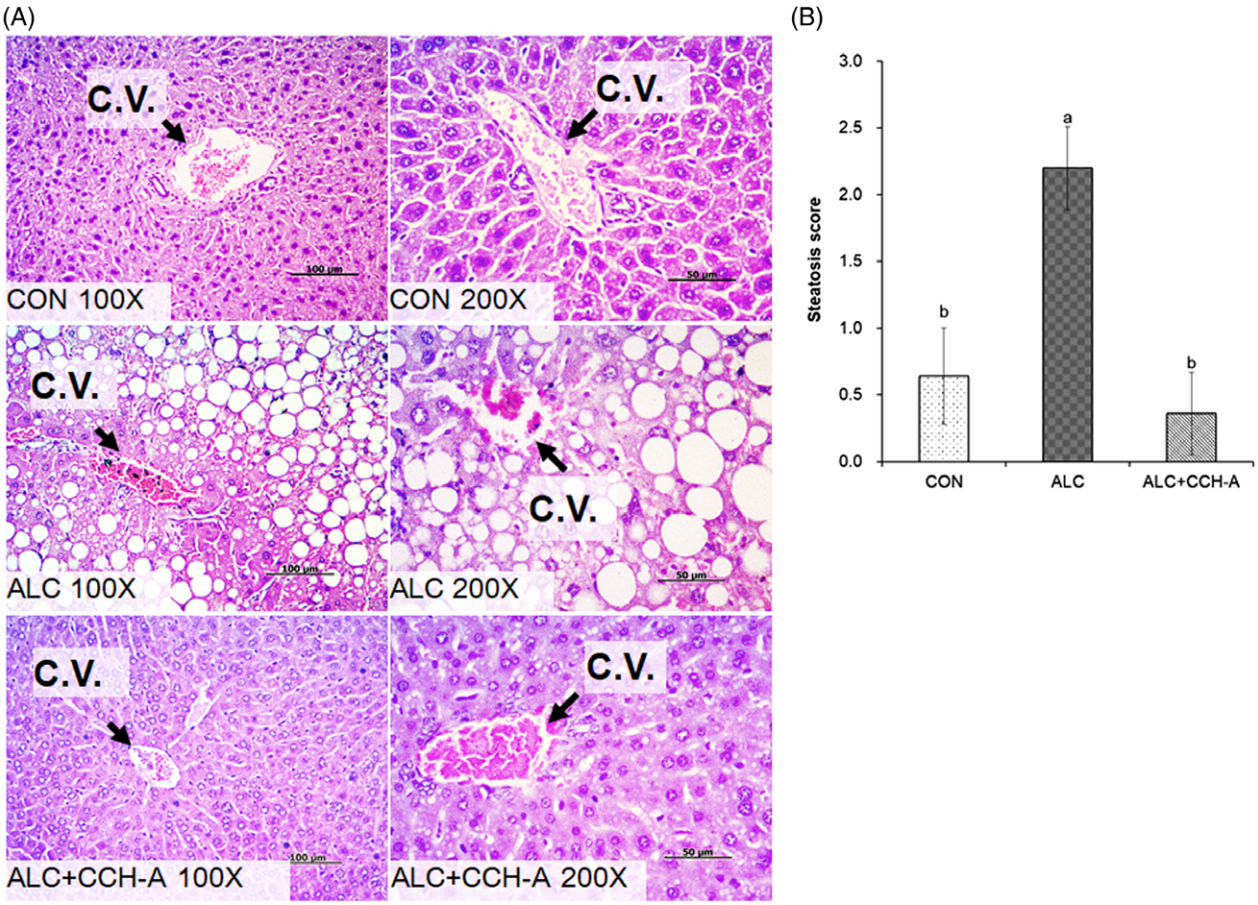


Figure 3. Pathological analyses in liver tissues: (A) H&E stain and (B) steatosis scores of the experimental mice. CV, central vein. Values are means ± SD ($n = 6$). Data bars without a common letter are significantly different ($P < 0.05$). CON, control liquid diet + 0.1 mL saline; ALC, ethanol liquid diet + 0.1 mL saline; ALC+CCH-A, ethanol liquid diet + 100 mg CCH-A kg^{-1} BW in 0.1 mL ddH_2O.

(e.g. lysine and arginine) were also found to have the ability to interact with the carboxylic group of bile acids and to enhance sequestration and fecal excretion.[30] Moreover, pepsin-digested chicken-liver hydrolysates, which also contain substantial amounts of free lysine and arginine, can ameliorate the development of alcoholic livers in mice.[7] Similarly, lysine and arginine are also major free amino acids in our CCH-As; hence it is speculated that the decreased serum and liver lipids are related to the higher fecal lipid output (Fig. 2 and Table 1). Some amino acids, i.e. histidine, glycine and taurine, have the ability to reduce expression of the pro-inflammatory transcription factor NF-κB and downstream cytokine levels.[31,32] It has been mentioned that glycine lowers the gastric emptying rate of ethanol, resulting in the suppression of ethanol absorption from the gastrointestinal tract; by this means it ameliorates liver damage.[33] Hence the hepatoprotection of CCH-As against chronic alcohol consumption may be related to its free amino acid constitution.

Effects of CCH-As on molecular mechanisms of lipogenesis and fatty acid β-oxidation as well as antioxidant capacities in livers of chronic alcohol-fed mice

Observation of the liver lipid contents (Table 1) and histopathological results (Fig. 3) among groups enabled detection of the gene expressions related to lipogenesis (Fig. 4A) and fatty acid β-oxidation (Fig. 4B) in livers. Higher ($P < 0.05$) gene expressions of *Srebp1-c* and *Fas* expressions were observed in ALC group when compared to those in CON group, but *Lxr-α* and *Acc* expression among groups was not influenced ($P > 0.05$). CCH-A supplementation significantly downregulated ($P < 0.05$) *Fas* expression in alcohol diet-fed mice, while there was only a tendency toward lower *Srebp1-c* expression in the ALC+CCH-A group compared to that in the ALC group. In fatty acid β-oxidation in livers, *Pparα* expression in the ALC group was significantly downregulated ($P < 0.05$) compared to that in the CON group. However, the relative expression of *Pparα*, *Rxr-α*, *Cpt1* and *Ucp2* in livers of alcohol fed mice was upregulated ($P < 0.05$) by supplementing CCH-As. Chronic alcohol consumption (ALC group) significantly increased ($P < 0.05$) the TBARS value (Fig. 5A) and lowered ($P < 0.05$) the TEAC level (Fig. 5C) in livers compared to the control liquid diet (CON group), although there was no ($P > 0.05$) difference in reduced GSH content in livers between CON and ALC groups (Fig. 5B). Alternatively, CCH-A supplementation decreased ($P < 0.05$) TBARS value and enhanced ($P < 0.05$) reduced GSH content and TEAC level in livers of alcoholic-diet fed mice. With regard to the activities of antioxidant enzymes, SOD activities were not ($P > 0.05$) influenced among groups (Fig. 4D). The ALC+CCH-A group had the highest ($P < 0.05$) CAT activity, followed by ALC and CON groups (Fig. 5E). Besides, the increased ($P < 0.05$) GPx activity in ALC+CCH-A group was only found and not in CON and ALC groups (Fig. 5F).

Increased *de novo* lipogenesis is one of stimuli for alcohol-induced steatosis.[2,3,7,33] The change in NADH:NAD^+ ratio in alcohol catabolism upregulates SREBP1c, thus triggering *de*

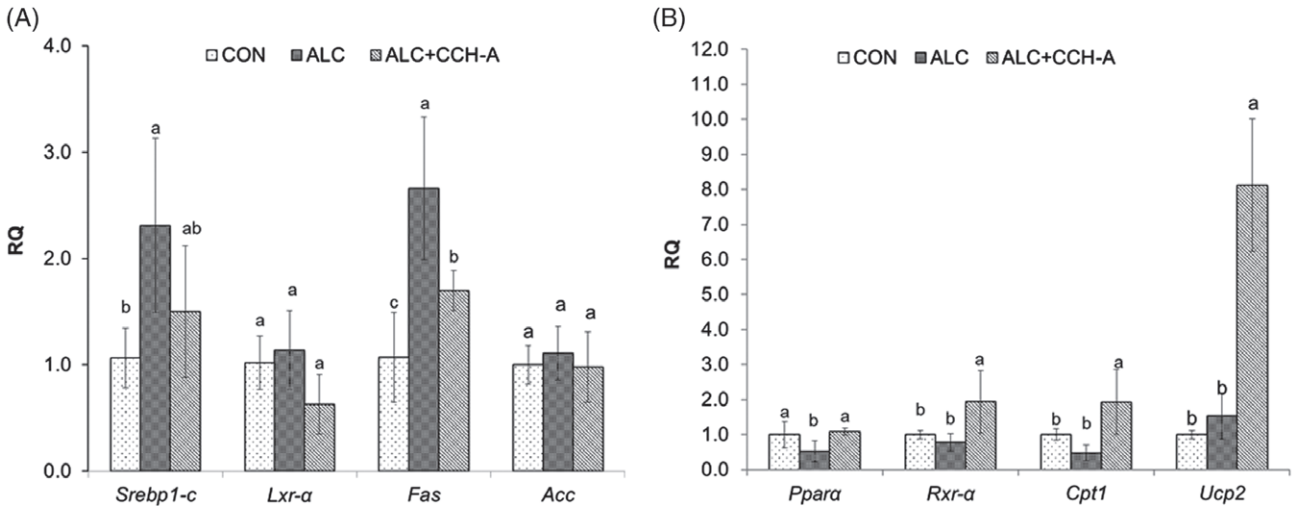


Figure 4. Relative gene expressions of (A) lipogenesis and (B) β-oxidation in livers of experimental mice. Values are means ± SD ($n = 6$). Data bars without a common letter in each tested parameter are significantly different ($P < 0.05$). CON, control liquid diet + 0.1 mL saline; ALC, ethanol liquid diet + 0.1 mL saline; ALC+CCH-A, ethanol liquid diet + 100 mg CCH-A kg^{-1} BW in 0.1 mL ddH_2O.

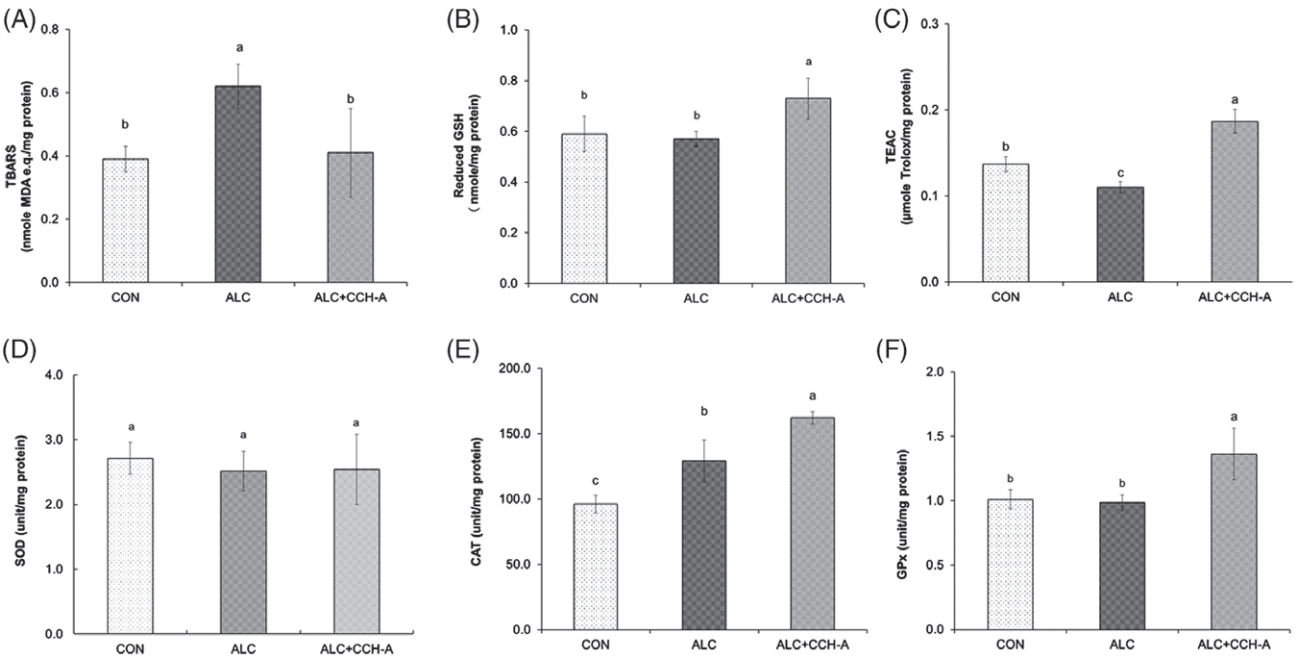


Figure 5. Antioxidant capacities of livers of experimental mice: (A) TBARS value; (B) reduced GSH level; (C) TEAC level; (D) SOD activity; (E) CAT activity; (F) GPx activity. Values are means ± SD ($n = 6$). Data bars without a common letter in each tested parameter are significantly different ($P < 0.05$). CON, control liquid diet + 0.1 mL saline; ALC, ethanol liquid diet + 0.1 mL saline; ALC+CCH-A, ethanol liquid diet + 100 mg CCH-A kg^{-1} BW in 0.1 mL ddH_2O.

novo lipogenesis. Simultaneously, chronic alcohol consumption also suppresses fatty acid β-oxidation.[34] BCAA supplementation clinically showed an anti-fatigue ability during exercise due to an enhancement of fatty acid β-oxidation.[35] Besides, a lipid-lowering effect by downregulating lipogenesis or upregulating fatty acid β-oxidation was observed in high-fat diet or alcohol-fed rodents supplemented with taurine[31] and carnosine.[9] Functional chicken liver hydrolysates containing amounts of BCAAs, taurine and carnosine also demonstrated an ameliorative effect on the development of hepatosteatosis induced by chronic alcohol consumption.[7] This ameliorative effect was attributed to a regulation of lipid metabolism. Hence a certain amount of BCAAs (3457.12 mg 100 g^{-1}), taurine (35.41 mg 100 g^{-1}) and carnosine (48.06 mg 100 g^{-1}) in the CCH-As used in this study (Fig. 2B); meanwhile, downregulation of lipogenesis-related gene expressions (Fig. 4A) and upregulation of fatty acid β-oxidation-related gene expression (Fig. 4B) may contribute to the improvements in liver lipids (Table 1) and hepatopathological changes (Fig. 3) in chronic alcohol consumption. As we know, an excess of ROS, due to excessive production or impaired antioxidant capacities, is harmful and leads to inflammatory responses. Long-term administration (17 weeks) of pepsin-digested egg white hydrolysates to spontaneously hypertensive rats can improve the plasma antioxidant capacity (oxygen radical absorbance capacity).[36] It has been also indicated that gelatin hydrolysates from the skin of Pacific cod (*Gadus macrocephalus*) demonstrate protective effects against

oxidation-induced DNA damage in RAW264.7 cells.[37] Moreover, histidine-containing peptides, i.e. carnosine and anserine, were assayed in our CCH-As (Fig. 2B) as well, and they are demonstrated to possess antioxidant ability due to their ability to scavenge free radicals and ROS.[9,27] As mentioned above, hydrophobic amino acids also display high antioxidant properties.[23] According to a report from Senthilkumar *et al.*,[38] glycine supplementation can elevate the activities of SOD, CAT, GSH, GPx and glutathione reductase in the erythrocyte membrane, plasma and hepatocytes of rats suffering from alcohol-induced liver injury. Moreover, the *in vitro* antioxidant assays of CCH-As are demonstrated in Fig. 1. Hence it can be proposed that the antioxidant properties of CCH-As against alcohol-induced oxidative stress in livers should result from free amino acid and dipeptide constitution in CCH-As (Figs 2B and 5).

CONCLUSION

Antioxidant crude chalaza hydrolysates (CCH-As) can be produced by protease A hydrolyzation at ratio of 1:100 (w/w) and 0.5 h hydrolytic period. The amounts of free essential and branched amino acids in CCH-As were dramatically increased – more than 500 and 400 fold, respectively – compared with those in raw crude egg chalazae, while antioxidant dipeptides (carnosine and anserine) were only analyzed in CCH-As. Moreover, CCH-A supplementation ameliorates the development of alcoholic fatty liver in mice. This hepatoprotection was mainly due to enhancing liver antioxidant capacities, lowering liver lipids and inflammatory cytokine secretions, as well as regulating lipid metabolism (downregulation of lipogenesis and upregulation of fatty acid β-oxidation). Therefore, this CCH-A not only increases the value of byproducts in liquid egg processing but also possesses health effects beyond its nutritional values.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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