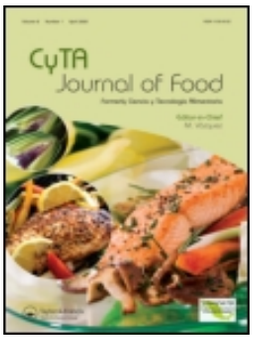

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In vitro cytoprotection of modified casein hydrolysates by plastein reaction on rat hepatocyte cells

Citoprotección *in vitro* de hidrolizados de caseína modificados por reacción de plasteina en células de hepatocitos de rata

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Casein hydrolysate of a degree of hydrolysis of 9.4% was generated from caseinate using papain and modified by plastein reaction with extrinsic phenylalanine or tyrosine addition, and then fractionated by an ethanol-water solvent (6:4, v/v). Compared to casein hydrolysate or mixtures of casein hydrolysate and phenylalanine or tyrosine, two modified hydrolysates prepared thereof showed higher scavenging activity on 2,2-diphenyl-1-picrylhydrazyl (DPPH) or hydroxyl radicals and reducing power. The obtained soluble and precipitated fractions had higher and lower total hydrophobic amino acids especially stronger and weaker three antioxidant properties than the parent substrates, respectively. All hydrolysates particularly soluble fractions protected rat hepatocytes against H_2O_2- or galactosamine-induced injury *in vitro*, resulting in enhanced cell viability, decreased cellular malondialdehyde content, and lactate dehydrogenase leakage. Cytoprotection of the hydrolysates was observed at a dosage-dependence manner. A negative correlation ($P < 0.05$) was found between malondialdehyde content and scavenging activity on hydroxyl radicals or reducing power of the hydrolysates.

Keywords: casein hydrolysate; plastein reaction; solvent fractionation; antioxidant activity; rat hepatocytes; cytoprotection

Hidrolizado de caseína de un grado de hidrólisis de 9,4% se generaron a partir de caseinato usando papaína y modificado por reacción de plasteina con fenilalanina extrínseca o adición de tirosina, y luego fraccionada con un solvente de etanol-agua (6:4, v/v). En comparación con hidrolizado de caseína o mezclas de hidrolizado de caseina y fenilalanina o tirosina, dos hidrolizados modificados preparados de la misma mostraron mayor actividad captadora de radicales DPPH o hidroxilo y poder reductor. Las fracciones solubles y precipitados obtenidos tenían más altas y más bajas aminoácidos hidrófobos totales especialmente más fuertes y más débiles tres propiedades antioxidantes que los sustratos padres, respectivamente. Todos los hidrolizados particularmente las fracciones solubles protegieron los hepatocitos de rata contra H_2O_2 o galactosamina-inducida por lesión *in vitro*, lo que resulta en una viabilidad celular mejorada, disminución de contenido de malondialdehído celular y la perdida de lactato deshidrogenasa. Se observó citoprotección de los hidrolizados de una manera dosis-dependiente. Se encontró una correlación negativa ($p < 0,05$) entre el contenido de malondialdehído y la actividad captadora de radicales hidroxilo o poder reductor de los hidrolizados.

Palabras claves: hidrolizado de caseína; reacción plasteina; fraccionamiento con disolventes; actividad antioxidante; hepatocitos de rata; citoprotección

Introduction

Functional food ingredients are becoming the most popular issue among food chemists as they may beneficially affect the body by improving the health and well-being and/or by reducing the risk of diseases. Aging and disease of the body are proofed with a relation to the free radical generated (Christen, 2000; Kehrer, 1993). Growing evidence shows that intake of antioxidants including antioxidant peptides or proteins and others has a positive effect, as they can protect the body against the radical-induced injury and the radical-linked pathogenesis (Valko et al., 2007), e.g. oxidative injury is considered to be one of the key factors in the development of hepatic disorders (Çetin, Kanbur, Çetin, Eraslan, & Atasever, 2011). Food proteins can be digested by proteases or fermented by edible microorganisms to produce protein hydrolysates, which are more active than the parent proteins. Many of these hydrolysates or the purified peptides, such as those from soybean (Moure, Domínguez, & Parajo, 2006), rice (Adebiyi, Adebiyi, Ogawa, & Muramoto, 2008), and animal-origin (Hernández-Ledesma, Miralles, Amigo, Ramos, & Recio, 2005; Lin, Guo, You, Yin, & Liu, 2012; Tavares et al., 2011; Zhuang & Sun, 2011) proteins, were found to have antioxidant properties, and thus received increasing attention in the recent years (Elias, Kellerby, & Decker, 2008). Among the milk protein products, caseinate and whey products are now produced in large quantities as protein ingredients for food industry. Milk protein hydrolysates were reported to have antioxidant activity (Korhonen, 2009; Zhao, Wu, & Li, 2010) and cytoprotection on the cells toward hydrogen peroxide (H_2O_2)-induced oxidative injury (Kong, Peng, Xiong, & Zhao, 2012). They were also able to enhance cell viability, cellular glutathione content, and catalase activity *in vitro* (Lahart et al., 2011; Phelan, Aherne-Bruce, O'Sullivan, FitzGerald, & O'Brien, 2009).

Plastein reaction, designed for protein hydrolysates with three mechanisms as condensation, transpeptidation, and physical forces (Andrews & Alichanidis, 1990), was used in the past to modify the functional properties or nutritional value of

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food proteins (Andrews & Alichanidis, 1990; Yamashita, Arai, & Fujimaki, 1976). It was also applied in the recent studies to improve *in vitro* angiotensin I converting enzyme (ACE)-inhibitory (Zhao & Li, 2009) and antioxidant activities (Zhao, Wu, & Li, 2010) of casein hydrolysates. Extrinsic amino acids such as proline, phenylalanine, and tyrosine could be added into the reaction system to confer the products better ACE-inhibitory or antioxidant activities (Li, Li, & Zhao, 2010; Yue, Li, & Zhao, 2013), as some reported powerful ACE-inhibitory or antioxidant peptides had high hydrophobic residues content (Kuba, Tana, Tawata, & Yasuda, 2005; Pan, Luo, & Tanokura, 2005; Torruco-Uco, Chel-Guerrero, Martinez-Ayala, Davila-Ortiz & Betancur-Ancona, 2009). The role of the antioxidants in the cells is critical to keep the oxidative processes in balance, as excessive free radicals should be eliminated effectively by the antioxidant defense system in the cells; otherwise, an oxidative injury thereby occurs, resulting in damage to DNA, proteins, and lipids, and leading to adverse impacts on the body health, e.g. aging, cancer, cardiovascular disease, atherosclerosis, hypertension, and others (Valko et al., 2007). It is of greatest interest to find out some safe and powerful natural antioxidants to enhance the body's antioxidant defenses by dietary supplements (Finkel & Holbrook, 2000), among which are antioxidant proteins or peptides. A detailed investigation of the role of the antioxidant peptides should be carried out to reveal their potential biological impacts on *in vitro* treated cells. Whey peptides were investigated for their protection on lung fibroblast MRC-5 cells against H_2O_2-induced injury (Kong et al., 2012). Casein hydrolysates also had been studied for their biological effects on cellular antioxidant status of a human Jurkat T cell line (Lahart et al., 2011; Phelan et al., 2009). However, cytoprotection of the plastein reaction-modified milk protein hydrolysates (e.g. casein hydrolysate) against induced injury on the cells is not well-investigated yet, particularly the relationship between antioxidant properties and *in vitro* cytoprotection of the applied hydrolysates.

H_2O_2 and galactosamine are widely used to induce acute cell injury. They may result in depletion in antioxidant defense system, damage in cell membrane, and lipid oxidation (El-Beshbishy, 2008; Kong et al., 2012), characterized by some cellular status such as enhanced malondialdehyde (MDA) formation and leakage of lactate dehydrogenase (LDH). Liver is the major organ to bio-transform exogenous chemicals in the body, and protection of liver against injury is very important for health. In the present study, a casein hydrolysate was prepared by *in vitro* digestion of caseinate with papain, and modified by papain-catalyzed plastein reaction in the presence of extrinsic tyrosine (Tyr) or phenylalanine (Phe). The modified hydrolysates were then fractionated by an ethanol-water solvent. Three antioxidant properties such as scavenging activity on 2,2-diphenyl-1-picrylhydrazyl (DPPH) or hydroxyl radicals and reducing power of the obtained hydrolysates were evaluated *in vitro*. Some hydrolysates were investigated for amino acid composition, and for cytoprotection on a rat hepatocyte line (BRL cells) against H_2O_2- and galactosamine-induced injury. Cell viability, MDA formation, and LDH leakage of the treated cells were assayed and compared, to reveal the protective effect of the applied hydrolysates, the relationship between antioxidant properties of the applied hydrolysates, and the evaluated cellular status, and the potential of plastein reaction and solvent fractionation to obtain casein hydrolysates with better antioxidant properties and cytoprotective capacity.

Materials and methods

Materials

Caseinate (crude protein content of 86.0%, on dry basis) was purchased from Beijing Aoboxing Bio-Tech Co. Ltd (Beijing, China). Papain with an activity of 22.5 kU/g was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). L-Phenylalanine and L-tyrosine were purchased from Aladdin-reagent Co. Ltd (Shanghai, China). Dulbecco's modification of Eagle's medium (DMEM), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), DPPH, and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). Other chemicals used were of analytical grade. Highly purified water prepared by Milli-Q PLUS (Millipore Corporation, New York, NY, USA) was used in buffers or solutions preparation.

The rat hepatocyte line (BRL cells) was obtained from Cell Bank of Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences (Shanghai, China).

Preparation, modification, solvent fractionation, and evaluation of the casein hydrolysate

The procedures and conditions used to prepare casein hydrolysate, mixture of the hydrolysate and extrinsic amino acids, modified casein hydrolysate (MCH), and fractionated modified hydrolysates were same to those described in the previous study (Yue et al., 2013). Briefly, two modified hydrolysates (MCH1 and MCH2) were prepared for the case of Phe and Tyr added at 0.74 mol/mol free amino groups of casein hydrolysate. Another MCH was prepared without extrinsic amino acid addition. Two mixtures (Mix1 and Mix2) were prepared by mixing casein hydrolysate with Phe and Tyr, respectively, with the same ratio used in the preparation of MCH1 and MCH2. Fractionation of MCH1 and MCH2 was carried out by an ethanol-water solvent (6:4, v/v) to obtain two soluble and precipitated fractions.

Seventeen amino acids except for Trp in the samples were assayed by an amino acids analyzer 835-50 (Hitachi, Tokyo, Japan) with the procedure provided by the manufacturer. The sample of 200 mg was hydrolyzed by 6 moL/L HCl at 110ºC for 24 h. Total hydrophobic amino acids (THAA) were calculated as the total of eight amino acids including Ala, Val, Leu, Ile, Phe, Pro, Tyr, and Met.

Scavenging activity on DPPH radicals and reducing power of the samples were assayed by the method of Nsimba, Kikuzaki, and Konishi (2008) and Oyaizu (1988), respectively, while scavenging activity on hydroxyl radicals was evaluated as described by Chung, Osawa, and Kawakishi (1997). All analyses were carried out at a UV-2401PC spectrophotometer (Shimadzu, Kyoto, Japan).

Cell culture and injury

The rat hepatocytes were cultured in the DMEM supplemented with heat-inactivated 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and maintained in monolayer culture at 37ºC in an incubator with 5% CO_2 and 90% relative humidity. The medium was changed every other day. Sub-confluent cells (80%) were passaged using 0.25% trypsin and 0.02% ethylenediamine tetra-acetic acid (EDTA).

To investigate the protective effects of the hydrolysates on the hepatocytes, the cultured cells were seeded in 96-well plates (10^4 cells/mL, 100 μL per well, for cell viability evaluation) or

24-well plates (2×10^5 cells/mL, 1 mL per well for MDA content and LDH leakage analysis). The wells were subjected to different treatments designed as a control group (no H_2O_2, galactosamine or hydrolysate addition), two model groups (treated by 6.5 mmol/L H_2O_2 or 20 mmol/L galactosamine at 37ºC for 1.5 or 8 h without hydrolysate addition), and seven test groups (hydrolysate addition at 0.5, 1, and 2 mg/mL). For the test groups, the cells were incubated at 37ºC for 24 h with the hydrolysates before the injury, and then treated with H_2O_2 or galactosamine addition. The applied hydrolysates were casein hydrolysate, Mix1, Mix2, and four fractionated products of MCH1 and MCH2, respectively.

Assaying of cell viability, MDA content, and LDH leakage

Cell viability was measured by the MTT assaying, in which a blue product formazan is generated by the living cells (Kang et al., 2005). After the cells were treated as described previously, 20 µL of MTT solution (5 g/L) was added into each well. After an incubation of 4 h at 37ºC, the plate was centrifuged at 1800 × *g* for 5 min at 4ºC, and the left MTT solution was removed from the wells by aspiration. Then, 100 µL of DMSO were added into each well to dissolve the formazan generated. The absorbance of each well was recorded on a microplate reader (Bio Rad, Hercules, CA, USA) at a wavelength of 490 nm. Cell viability in each test and model group was expressed as percentage of the control group. The vehicle-treated cells were taken as 100% viable.

MDA content of the cells was measured by a commercial test kit (Jiancheng Biochemical, Inc., Nanjing, China), based on the mechanism that the MDA can react with thiobarbituric acid (TBA) to generate a color compound with maximal absorbance at 532 nm. The assaying procedure was recommended by the kit producer. In brief, the hepatocytes were lyzed by the cell lysis buffer, and centrifuged at 1600 × *g* for 10 min to collect the supernatant. Aliquot of the supernatant (0.1 mL) was mixed with 0.2 mL TBA solution and incubated in a water bath of 95ºC for 40 min. The absorbance was recorded on the microplate reader at a wavelength of 532 nm. The protein content was determined by BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). MDA content was expressed as micromole per gram protein to reflect the lipid oxidation in the cells.

LDH leakage of the cells was assayed by another commercial test kit (Jiancheng Biochemical, Inc.), and used to reflect membrane integrity of the cells (Dvořák et al., 2003). After cell treatment, the culture medium was centrifuged at 1600 × *g* for 10 min and the supernatant was collected. The collected supernatant of 20 µL was analyzed by the kit with the procedure and conditions recommended by the producer. Briefly, 20 µL of sodium pyruvate solution and 25 µL substrate buffer were added, mixed, and incubated for 15 min at 37ºC in the incubator. Subsequently, 2,4-dinitrophenylhydrazine of 25 µL was added and incubated for another 15 min at 37ºC. Eventually, the reaction was stopped by adding 0.4 mol/L NaOH solution at room temperature for 5 min. The absorbance was detected in the microplate reader at a wavelength of 450 nm.

Statistical analysis

All reported data were expressed as means ± standard deviations from three independent trials. Differences between the means of multiple groups were analyzed by one-way analysis of variance (ANOVA) with Duncan′s multiple range tests. Pearson's correlation coefficient was calculated by the bivariate correlations procedure to show the relationship between one antioxidant property of the applied hydrolysates and one evaluated cellular index at the fixed hydrolysate addition. SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the data.

Results and discussion

Antioxidant properties and amino acid composition of the modified casein hydrolysates

The preparation of casein hydrolysate with papain for 2 h resulted in a degree of hydrolysis (DH) of 9.4% and the highest scavenging activity (38.7%) on DPPH radicals at 1 mg/mL. Results given in Table 1 show that, when casein hydrolysate was modified by papain-catalyzed plastein reaction with or without Phe or Tyr addition, or was mixed with Phe or Tyr, three antioxidant properties of the products obtained thereof were totally enhanced.

Compared to casein hydrolysate, the mixture of casein hydrolysate and extrinsic Phe or Tyr (i.e. Mix1 or Mix2) had an unchanged scavenging activity on DPPH radicals but higher reducing power and scavenging activity on hydroxyl radicals ($P < 0.05$). MCH had an enhanced reducing power and scavenging activity on DPPH radicals but an unchanged scavenging activity on hydroxyl radicals than the two mixtures ($P < 0.05$).

Table 1. Antioxidant properties of casein hydrolysate and its modified or fractionated products.[a]

Tabla 1. Propiedades antioxidantes de hidrolizado de caseína y sus productos modificados o fraccionados.

Peptide samples[b]	Scavenging activity on DPPH radicals (%)[c]	Reducing power	Scavenging activity on hydroxyl radicals (%)
Casein hydrolysate	38.7 ± 0.5 ^{A}	0.497 ± 0.002 ^{A}	17.2 ± 0.2 ^{A}
Mix1	39.7 ± 0.4 ^{AB}	0.521 ± 0.004 ^{B}	21.1 ± 0.1 ^{B}
Mix2	39.7 ± 0.4 ^{AB}	0.529 ± 0.002 ^{B}	21.1 ± 0.1 ^{B}
MCH	42.9 ± 1.3 ^{C}	0.557 ± 0.011 ^{C}	21.4 ± 0.3 ^{B}
MCH1	49.2 ± 0.2 ^{E}	0.634 ± 0.008 ^{F}	22.3 ± 1.9 ^{B}
MCH2	46.7 ± 0.9 ^{D}	0.662 ± 0.007 ^{G}	29.7 ± 2.8 ^{D}
MCH1-SF	56.6 ± 0.3 ^{G}	0.725 ± 0.004 ^{H}	22.6 ± 0.4 ^{B}
MCH1-PF	41.9 ± 0.6 ^{C}	0.581 ± 0.007 ^{D}	21.9 ± 0.5 ^{B}
MCH2-SF	52.0 ± 0.4 ^{F}	0.722 ± 0.007 ^{H}	34.4 ± 0.2 ^{E}
MCH2-PF	40.7 ± 0.4 ^{B}	0.608 ± 0.002 ^{E}	25.0 ± 0.7 ^{C}

[a]Different capital letters after the values as superscripts in same column indicate that one-way ANOVA of the means is significantly different ($P < 0.05$).
[b]MCH, MCH1, and MCH2, modified casein hydrolysates without amino acid addition, with Phe and Tyr addition, respectively; SF and PF, soluble and precipitated fractions by ethanol-water solvent in 6:4 (v/v). Mix1 or Mix2, mixture of casein hydrolysate and Phe or Tyr, respectively.

Table 2. Amino acids compositions (%, in molar) of casein hydrolysate and four fractionated hydrolysates by ethanol-water solvent.[a]

Tabla 2. Composicion de aminoácidos (%, en moles) de hidrolizado de caseína y cuatro hidrolizados fraccionados con disolvente etanol-agua.

Amino acids	Casein hydrolysate	MCH1-SF	MCH1-PF	MCH2-SF	MCH2-PF
Asp	7.11	6.24	6.45	6.39	6.32
Thr	4.75	4.19	4.32	4.23	4.30
Ser	7.17	5.93	6.97	6.16	6.82
Glu	20.07	17.59	18.44	17.96	18.11
Gly	3.24	2.77	2.87	2.87	2.82
Ala	4.24	3.65	3.77	3.81	3.64
Cys	1.47	1.69	1.40	1.54	1.85
Val	6.23	5.67	5.40	5.51	5.70
Met	1.63	1.17	0.16	0.81	1.32
Ile	3.94	3.22	3.26	3.26	3.42
Leu	8.68	7.77	7.61	7.78	7.56
Tyr	3.82	3.54	3.33	14.49	13.70
Phe	3.54	14.81	14.60	3.08	2.88
Lys	7.03	6.31	6.29	6.46	6.31
His	2.59	2.40	2.40	2.27	2.52
Arg	2.49	2.23	2.27	2.24	2.26
Pro	12.00	10.82	10.47	11.14	10.46
Total	100.00	100.0	100.0	100.0	100.0
THAA	42.45	49.49	48.44	49.08	47.37

[a]THAA, total hydrophobic amino acids; MCH1 and MCH2, modified casein hydrolysates in the presence of Phe and Tyr, respectively; SF and PF, soluble and precipitated fraction by ethanol-water solvent in 6:4 (v/v).

MCH1 exhibited improved reducing power and scavenging activity on DPPH radicals than MCH ($P < 0.05$) while MCH2 showed three improved antioxidant properties respect to MCH ($P < 0.05$). These results indicated that it was the plastein reaction but not physical addition of Phe or Tyr that conferred MCH1 and MCH2 better antioxidant properties.

Fractionation of MCH1 and MCH2 by the ethanol-water solvent resulted in two soluble fractions higher while two precipitated fractions lower scavenging activities and reducing power than the parent substrates ($P < 0.05$) (Table 1), but one exception was the scavenging activity on hydroxyl radicals of the two fractions of MCH1 ($P > 0.05$). Soluble and precipitated fractions of MCH1 or MCH2 had high Phe or Tyr content than casein hydrolysate, owing to extrinsic Phe or Tyr addition in the reaction system; more important, soluble fractions had high THAA content than the precipitated fractions (49.49 *vs.* 48.44, or 49.08 *vs.* 47.37%) (Table 2).

The hydrolysates/peptides from milk proteins have scavenging activity on DPPH, superoxide, and other radicals (Hogan, Zhang, Li, Wang, & Zhou, 2009), thus can prevent oxidation (Sakanaka, Tachibana, Ishihara, & Juneja, 2005) and increase catalase activity in the cells (Lahart et al., 2011). Plastein reaction improved antioxidant properties of squid hepatopancreas (Ono, Kasai, Sugano, Ohba, & Takahashi, 2004) and casein (Zhao et al., 2010) hydrolysates efficiently. Addition of extrinsic hydrophobic amino acids to the reaction system also enhanced antioxidant properties of plastein reaction-modified casein hydrolysates (Zhao, Wang, & Li, 2011). Antioxidant properties of protein hydrolysates are governed by the size, structure, and amino acid sequences other than DH of the peptides (Lahart et al., 2011). Condensation and transpeptidation in plastein reaction can modify the size of the peptides and generate new peptide sequences. Thus, MCH1 and MCH2 totally showed higher antioxidant properties than casein hydrolysate, Mix1 and Mix2. At the same time, MCH1 and MCH2 also showed better antioxidant properties than MCH, due to extrinsic Phe and Tyr addition. Fractionation of casein hydrolysates by a solvent consisting of water and one of miscible organic solvents could lead to soluble or precipitated fractions higher or lower ACE inhibition, respectively (Sun & Zhao, 2012). This previous result offered a support to the present result, i.e. the obtained fractions had different antioxidant properties. The hydrolysate with much hydrophobic amino acids has higher scavenging activity on DPPH radicals (Wang, Tang, Chen, & Yang, 2009). The soluble fractions (MCH1-SF and MCH2-SF) were rich in THAA than the precipitated counterparts (MCH1-PF and MCH2-PF), reasonably had better antioxidant properties. Solvent fractionation thus can be used as a practical approach to obtain protein hydrolysates with better antioxidant properties.

Protective effects of the casein hydrolysates on rat hepatocytes against the induced injuries

Seven hydrolysates were selected to investigate their prevention against H_2O_2- or galactosamine-induced injury to a rat hepatocyte line (BRL cells) observed as cell viability, MDA content and LDH leakage. The results are given in Tables 3 and 4. The cells in the model groups showed lower cell viability, greater MDA content, and LDH leakage than those in the control groups, indicating the occurrence of induced jury. Based on the different index values of the cells in the model groups and test groups, all applied hydrolysates exhibited protection on the cells toward the carried out injury. Casein hydrolysate and soluble fraction of MCH2 (i.e. MCH2-SF) totally showed the lowest and highest protection on the cells, respectively, indicating the potential of plastein reaction to improve cytoprotection of protein hydrolysates.

When cells were directly damaged by H_2O_2 or galactosamine, cell viability was decreased to 29 or 33.5%. If the cells were pretreated by the applied hydrolysates at three levels before the injury, cell viability was totally increased about 9−139% because cell viability in the test groups was about 37.2−56.9% (H_2O_2 injury) or 36.6−80.1% (galactosamine injury) (Tables 3 and 4). This fact reveals that the applied hydrolysates protected the cells against the toxicity caused by H_2O_2 and galactosamine. Among the applied

Table 3. Protective effects of the investigated hydrolysates on rat hepatocytes against H_2O_2-induced injury.[a]

Tabla 3. Los efectos protectores de los hidrolizados investigados en hepatocitos de rata frente a la lesión inducida por H_2O_2.

Samples [b]	Addition level (mg/mL)	Cell viability (%)	LDH leakage (U/L)	MDA content (μmol/g protein)
Casein hydrolysate	0.5	41.1 ± 0.6 ^{B}	537 ± 13 ^{J}	27.3 ± 0.1 ^{K}
	1.0	46.5 ± 0.7 ^{DE}	460 ± 1 ^{G}	25.3 ± 0.7 ^{J}
	2.0	48.4 ± 0.2 ^{EF}	432 ± 5 ^{F}	23.4 ± 0.3 ^{H}
Mix1	0.5	37.2 ± 0.7 ^{A}	550 ± 3 ^{K}	25.6 ± 0.4 ^{J}
	1.0	42.2 ± 0.3 ^{BC}	541 ± 4 ^{J}	23.1 ± 0.2 ^{H}
	2.0	46.6 ± 1.0 ^{DE}	392 ± 4 ^{D}	21.5 ± 0.5 ^{G}
Mix2	0.5	44.6 ± 0.3 ^{CD}	501 ± 2 ^{I}	24.2 ± 0.4 ^{I}
	1.0	48.4 ± 2.6 ^{EF}	480 ± 5 ^{H}	20.8 ± 0.5 ^{F}
	2.0	51.5 ±0.6 ^{GH}	412 ± 4 ^{E}	19.1 ± 0.2 ^{BC}
MCH1-SF	0.5	48.2 ± 0.4 ^{EF}	551 ± 2 ^{K}	22.9 ± 0.4 ^{H}
	1.0	49.4 ± 0.9 ^{FG}	498 ± 4 ^{I}	20.9 ± 0.3 ^{F}
	2.0	54.2 ± 0.9 ^{I}	318 ± 2 ^{B}	18.9 ± 0.4 ^{B}
MCH1-PF	0.5	41.9 ± 1.0 ^{B}	543 ± 2 ^{JK}	24.1 ± 0.1 ^{I}
	1.0	49.1 ± 0.7 ^{EFG}	400 ± 3 ^{D}	23.0 ± 0.2 ^{H}
	2.0	53.2 ± 1.1 ^{HI}	375 ± 2 ^{C}	20.0 ± 0.2 ^{DE}
MCH2-SF	0.5	47.7 ± 0.3 ^{EF}	435 ± 2 F	21.9 ± 0.3 ^{G}
	1.0	48.8 ± 0.7 ^{EF}	392 ± 1 ^{D}	19.5 ± 0.2 ^{CD}
	2.0	56.9 ± 0.8 ^{J}	294 ± 13 ^{A}	16.5 ± 0.2 ^{A}
MCH2-PF	0.5	39.9 ± 1.1 ^{B}	462 ± 5 ^{G}	23.5 ± 0.1 ^{H}
	1.0	44.5 ± 0.9 ^{CD}	400 ± 2 ^{D}	20.1 ± 0.2 ^{E}
	2.0	47.2 ± 0.5 ^{EF}	372 ± 3 ^{C}	18.6 ± 0.5 ^{B}

[a]LDH and MDA, lactate dehydrogenase and malondialdehyde. Cell viability and LDH leakage for the cells in model group are (29.0 ± 0.4)% and (568 ± 6) U/L, while MDA content for the cells in control and model group is (8.9 ± 0.6) and (27.9 ± 0.3) μmol/g protein, respectively. Different capital letters after the values as superscripts in same column indicate that one-way ANOVA of the means is significantly different ($P < 0.05$).
[b]MCH1 and MCH2, modified casein hydrolysates in the presence of Phe and Tyr, respectively; SF and PF, soluble and precipitated fraction by ethanol-water solvent in 6:4 (v/v). Mix1 or Mix2, mixture of casein hydrolysate and Phe or Tyr, respectively.

Table 4. Protective effects of the investigated casein hydrolysates on rat hepatocytes against galactosamine-induced injury.[a]

Tabla 4. Efectos protectores de los hidrolizados de caseína investigados en hepatocitos de rata frente a la lesión inducida por galactosamina.

Samples[b]	Addition level (mg/mL)	Cell viability (%)	LDH leakage (U/L)	MDA content (μmol/g protein)
Casein hydrolysate	0.5	36.6 ± 1.0 ^{A}	287 ± 4 ^{J}	19.2 ± 0.3 ^{K}
	1.0	40.3 ± 2.5 ^{A}	260 ± 25 ^{I}	17.1 ± 0.8 ^{I}
	2.0	47.7 ± 2.9 ^{BC}	212 ± 23 ^{EFGH}	14.7 ± 0.6 ^{EFG}
Mix 1	0.5	40.0 ± 2.9 ^{A}	225 ± 1 ^{FGH}	17.6 ± 0.1 ^{IJ}
	1.0	44.7 ± 2.0 ^{B}	203 ± 8 ^{CDEF}	15.1 ± 0.7 ^{FG}
	2.0	51.0 ± 5.3 ^{CD}	201 ± 18 ^{BCDEF}	15.1 ± 0.3 ^{FG}
Mix 2	0.5	51.9 ± 2.1 ^{CD}	229 ± 9 ^{GH}	18.0 ± 0.4 ^{J}
	1.0	55.4 ± 1.6 ^{EEF}	208 ± 5 ^{DEFG}	15.2 ± 0.9 ^{G}
	2.0	60.0 ± 0.5 ^{FGH}	206 ± 27 ^{DEFG}	14.7 ± 0.5 ^{EFG}
MCH1-SF	0.5	55.3 ± 2.1 ^{DEF}	266 ± 8 ^{IJ}	15.0 ± 0.3 ^{FG}
	1.0	58.2 ± 2.9 ^{EFG}	202 ± 20 ^{BCDEF}	13.9 ± 0.7 ^{E}
	2.0	77.9 ± 1.1 ^{J}	178 ± 3 ^{AB}	11.7 ± 0.2 ^{ABC}
MCH1-PF	0.5	54.4 ± 1.6 ^{DE}	262 ± 9 ^{I}	16.2 ± 0.3 ^{H}
	1.0	61.1 ± 2.7 ^{GH}	210 ± 19 ^{DEFGH}	14.6 ± 0.5 ^{EFG}
	2.0	62.8 ± 3.8 ^{GH}	194 ± 3 ^{ABCDE}	12.7 ± 0.1 ^{D}
MCH2-SF	0.5	53.8 ± 3.9 ^{DE}	233 ± 2 ^{H}	14.0 ± 0.1 ^{E}
	1.0	70.2 ± 0.3 ^{I}	192 ± 1 ^{ABCDE}	12.2 ± 0.2 ^{BCD}
	2.0	80.1 ± 2.2 ^{J}	175 ± 4 ^{A}	11.2 ± 0.1 ^{A}
MCH2-PF	0.5	50.3 ± 3.3 ^{CD}	198 ± 5 ^{ABCDE}	14.3 ± 0.1 ^{EF}
	1.0	53.1 ± 0.6 ^{D}	186 ± 9 ^{ABCD}	12.3 ± 0.2 ^{CD}
	2.0	63.3 ± 3.1 ^{H}	181 ± 2 ^{ABC}	11.5 ± 0.2 ^{AB}

[a]LDH and MDA, lactate dehydrogenase and malondialdehyde. Cell viability and LDH leakage for the cells in model group are (33.5 ± 1.4)% and (289 ± 3) U/L, while MDA content for the cells in control and model group is (8.2 ± 0.3) and (19.5 ± 0.6) μmol/g protein, respectively. Different capital letters after the values as superscripts in same column indicate that one-way ANOVA of the means is significantly different ($P < 0.05$).
[b]MCH1 and MCH2, modified casein hydrolysates in the presence of Phe and Tyr, respectively; SF and PF, soluble and precipitated fraction by ethanol-water solvent in 6:4 (v/v). Mix1 or Mix2, mixture of casein hydrolysate and Phe or Tyr, respectively.

hydrolysates, four fractions enhanced cell viability about 37.6−86.9% (H_2O_2 injury) or 50.1−139% (galactosamine injury) (Tables 3 and 4). The more hydrolysates were added, the higher cell viability was observed at a dosage-dependence manner. Among these hydrolysates, MCH2-SF at 2 mg/mL showed the greatest protection on the cells (cell viability of 56.9% or 80.1%), followed by MCH1-SF at 2 mg/mL (cell viability of 54.9% or 77.9%). That is, the soluble fractions had better protection than the precipitated ones.

The cells injured directly by H_2O_2 or galactosamine gave a LDH leakage of 568 or 289 U/L. Once being pretreated by the applied hydrolysates, the cells showed lowered LDH leakage in 294−551 (H_2O_2 injury) or 175−284 (galactosamine injury) U/L (Tables 3 and 4), resulting in a decreasing level about 1−48%. Four fractions decreased the LDH leakage about 3.0−48.2% (H_2O_2 injury) or 8.0−39.4% (galactosamine injury). MCH2-SF at 2 mg/mL exhibited the most powerful protection, followed also by MCH1-SF at 2 mg/mL. The protection of the applied hydrolysates on LDH leakage of the cells was also observed to follow a dosage-dependent relationship.

MDA content in the cells of the control group was 8.2−8.9 μmol/g protein. Upon being injured directly by H_2O_2 or galactosamine, MDA content in the cells of the model groups was enhanced to 27.9 or 19.5 μmol/g protein, showing significant lipid oxidation in the cells. When the cells were pretreated by the applied hydrolysates, MDA content in the cells of the test groups was 16.5−27.3 (H_2O_2 injury) or 11.2−19.2 (galactosamine injury) μmol/g protein (Tables 3 and 4), totally giving a decrease about 1−42%. Four fractions decreased MDA content in the cells about 13.6−40.9% (H_2O_2 injury) or 16.9−42.6% (galactosamine injury). Similar to the results mentioned above, MCH2-SF at 2 mg/mL showed the most powerful antioxidation, and a dosage-effect relationship was also found for these investigated hydrolysates.

Growing evidence indicates that oxidative stress is a key factor responsible for cell death. In normal cells, oxidative stress can be effectively eliminated by natural antioxidant defense system; but excessive oxidative stresses will cause an irreparable injury to the cells (Jeon et al., 2002). The role of antioxidants including antioxidant peptides against oxidative stress has thus received increased attention in the recent years. Casein hydrolysates are considered as direct free-radical scavengers (Chiu & Kitts, 2004). The investigated casein hydrolysate, two mixtures and four fractions had reducing properties, and showed scavenging activity toward DPPH and hydroxyl radicals; consequentially, addition of them to cell culture was helpful to eliminate the generated radicals, and resulted in an improved survival ratio of the cells.

LDH leakage as a result of the breakdown of the membrane barrier is an important indicator to reflect cell membrane integrity. LDH leakage is well correlated with cell damage and membrane peroxidation (Ienaga, Park, & Yokozawa, 2012), also was used in the present study to show the resulted damage to cell membrane. Some natural antioxidants could decrease LDH leakage in the H_2O_2-injured cells (Hu, Han, Huang, & Wang, 2011). When Jurkat T cells were incubated with three casein hydrolysates, LDH leakage was reduced (Lahart et al., 2011). Similar finding held for tertiary-butyl-hydroperoxide-injured hepatocytes when being protected by an antioxidant protein (Sarkar & Sil, 2010). Consistent with these reported studies, the investigated hydrolysates in the present study had antioxidant properties, thus showed ability to decrease LDH released from the injured cells (i.e. cytoprotection).

The radicals generated during cell oxidative injury can attach the polyunsaturated fatty acids in cell membrane, resulting in cell membrane damage and formation of the degraded products of the oxidized lipids (e.g. MDA). MDA content thus is a sensitive marker of cellular lipid peroxidation (Farmer & Davoine, 2007). Casein hydrolysates inhibited lipid oxidation effectively (Rival, Fornaroli, Boeriu, & Wichers, 2001). The present result showed that the applied hydrolysates especially MCH2-SF and MCH1-SF had stronger protection on the hepatocytes, based on significant decrease in cellular MDA. Similar conclusion had been given to caseinophosphopeptides and whey protein hydrolysates for the oxidative injury of Caco-2 cells and lung fibroblast MRC-5 cells (García-Nebot, Cilla, Alegría, & Barberá, 2011; Kong et al., 2012).

Correlation between antioxidant properties and cytoprotection of the casein hydrolysates

Relationship between one of the measured antioxidant properties of the applied hydrolysates and one of the evaluated cellular indices of the hepatocytes at the fixed hydrolysate addition was analyzed, and the results are given in Table 5. MDA content in the hepatocytes showed a most likely negative correlation ($P < 0.05$ or 0.01) to the scavenging activity on hydroxyl (but

Table 5. Correlation coefficients between cell viability, cellular lactate dehydrogenase (LDH) leakage or malondialdehyde (MDA) content and scavenging activity (SA) on DPPH or hydroxyl (OH) radicals, and reducing power (RP) of the evaluated seven casein hydrolysates.

Tabla 5. Coeficientes de correlación entre la viabilidad celular, eliminación de lactato deshidrogenasa celular (LDH) o contenido de malondialdehído (MDA) y la actividad de atrapamiento (SA) sobre los radicales hidroxilo (OH) o DPPH, poder reductora (RP) de los siete hidrolizados de caseína evaluados.

Treatment	Sample level (mg/mL)	Antioxidant properties	Cell viability	LDH Leakage	MDA content
H_2O_2-induced injury	0.5	SA on DPPH	0.828*	–0.137	–0.721
		SA on OH	0.496	–0.806*	–0.825*
		RP	0.767*	–0.392	–0.874*
	1.0	SA on DPPH	0.564	–0.068	–0.531
		SA on OH	0.197	–0.546	–0.772*
		RP	0.488	–0.334	–0.710
	2.0	SA on DPPH	0.760*	–0.896**	–0.608
		SA on OH	0.620	–0.831*	–0.869*
		RP	0.746	–0.971**	–0.790*
Galactosamine-induced injury	0.5	SA on DPPH	0.608	–0.173	–0.660
		SA on OH	0.546	–0.437	–0.805*
		RP	0.726	–0.070	–0.870*
	1.0	SA on DPPH	0.646	–0.379	–0.517
		SA on OH	0.818*	–0.663	–0.850*
		RP	0.783*	–0.578	–0.763*
	2.0	SA on DPPH	0.898**	–0.778*	–0.698
		SA on OH	0.771*	–0.786*	–0.723
		RP	0.971**	–0.936**	–0.885**

*$P < 0.05$.
**$P < 0.01$.

not DPPH) radicals and reducing power of the applied hydrolysates, although there were two exceptions. Cell viability and LDH leakage of the hepatocytes showed potentially positive and negative correlation to the three antioxidant properties of the applied hydrolysates, respectively, but the coefficients were not significant ($P > 0.05$) in most of the cases. This fact points out two important roles of the investigated hydrolysates to perform the cytoprotection. That is, they were hydroxyl radical scavengers and electron donors, which were reflected by their scavenging activities and reducing power, respectively. The present result reveals that scavenging activity on hydroxyl radicals and reducing power were two suitable markers to reflect *in vitro* cytoprotection on the hepatocytes of the hydrolysates against the induced injury.

Tavilani, Goodarzi, Vaisi-raygani, Salimi and Hassanzadeh (2008) found a negative correlation between superoxide dismutase or catalase activity and MDA content in spermatozoa from normozoospermic samples. When lung fibroblast MRC-5 cells were pretreated by a whey protein hydrolysate, MDA content in the cells after injury was strongly negatively correlated to the measured activity of superoxide dismutase, catalase, and glutathione peroxidase (Kong et al., 2012). The two results provided support to the present result. Unfortunately, potential correlation between the antioxidant properties of the applied hydrolysates and the cellular enzyme activities was not studied in the two mentioned studies. It is thus strongly suggested that potential correlation between antioxidant properties *in vitro* of the proteins and peptides and their cytoprotection should be detailed investigated.

Conclusions

Plastein reaction of casein hydrolysate by papain with extrinsic phenylalanine or tyrosine addition might lead to the modified hydrolysates having higher scavenging activity on DPPH or hydroxyl radicals and reducing power. Solvent fractionation of the modified hydrolysates conferred the soluble fractions better antioxidant properties. These hydrolysates had cytoprotection on rat hepatocytes *in vitro* against H_2O_2- or galactosamine-induced injury, among which were the soluble fractions having the stronger effect. More important, MDA content in the hepatocytes was negatively correlated to the scavenging activity on hydroxyl radicals and reducing power of the applied hydrolysates. Plastein reaction (especially with extrinsic amino acid addition) coupled with solvent fractionation is potential means to confer some added values such as better antioxidant properties and cytoprotection capacity on casein and other protein hydrolysates.

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