Journal of Food Measurement and Characterization
https://doi.org/10.1007/s11694-017-9685-0

ORIGINAL PAPER

Antiproliferative and antioxidative activities of cuttlefish (*Sepia pharaonis*) protein hydrolysates as affected by degree of hydrolysis

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Received: 10 April 2017 / Accepted: 14 November 2017

Abstract

Bioactivities (including antioxidative and antiproliferative properties) of cuttlefish mantle protein hydrolysates (CPH) with the degree of hydrolysis (DH) of 20.9, 25.5, 30.6, 35.3 and 40.6% (shortened as 20, 25, 30, 35 and 40%, respectively) prepared using alcalase were evaluated. The results indicated that the CPH with 20, 30 and 40% DH showed the greatest activity against DPPH radical scavenging [5.2 µmol TE (torolox equivalent)/g sample], reducing power (0.4 absorbance at 700 nm) and total antioxidant capacity (0.6 mg ascorbic acid equivalent/g sample), which were 2.5, 6.5 and 13.8 times higher than the cuttlefish mantle protein isolate (CPI), respectively. The CPH with the DH of 20% had the highest effect against MDA-231 and T47D cancer cell lines with growth inhibition of 78.2 and 66.2%, which were 6.5 and 6 times higher activities compared to the CPI, respectively. The amino acid profile of CPH indicated that glutamine (15.7%) and asparagine (10.9%) were predominant.

Keywords Antioxidant · Antiproliferate · Protein hydrolysate · Cuttlefish · *Sepia pharaonis*

Introduction

Oxidation is an unavoidable process in all living creatures in which reactive oxygen species (ROS) such as free radicals are formed [1]. The ROS can attack biomolecules such as membrane lipids, proteins and DNA leading to many diseases such as cancer [2]. In recent years, extensive scientific evidence has been provided for the existence of biological active peptides and proteins derived from foods that might have beneficial effects upon human health without any side effects [3]. Hydrolysates from marine resources have been reported to show bioactive properties as antioxidative, anticancer, antihypertensive, antithrombotic and immunomodulatory compounds [4, 5]. Moreover, resistance to anticancer drugs has been reported, and there is a growing interest in the identification and characterization of more effective and less toxic natural antitumor agents. The elimination of cancer with natural food ingredients, such as fish protein hydrolysates in the early stages is an integral part of chemoprevention, and measuring the cytotoxic properties of a given food compound against cancer cells provides useful insight into its chemoprotective potential [6].

Among all the potential processes, using enzymes are more reproducible and varied than chemical hydrolysis [7, 8]. It has been reported that the choices of substrate, protease and especially degree of hydrolysis (DH) generally affect the properties of the resulting hydrolysates [7].

The antiproliferative activity of peptides derived from fish protein hydrolysates was rarely studied in which hydrolysates from blue whiting, cod, plaice and salmon [9] tuna dark muscle [10] and jumbo squid [11] have shown to inhibit MCF7/6, MDA-MB-231; MCF-7 and M12.C3F6 cell lines, respectively. However, there is no information

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Published online: 23 December 2017

on antiproliferative activity of hydrolysate from cuttlefish mantle exists. Antioxidative activity of hydrolysates from fish species such as Alaska pollack [12], fresh water carp [13], anchovy sprat [14], sardine, horse mackerel, axillary seabream, bogue and small-spotted catshark [15], etc have been widely assessed, but few studies were carried out to evaluate the antioxidative activity of hydrolysates from cuttlefish, in which, the effects of DH [16] and fractionation [17] on the cuttlefish (*Sepia officinalis*) protein hydrolysates have been investigated.

Large amounts of cuttlefish (*Sepia pharaonis*) are captured annually as a by-catch in Iran and are not eaten in Iran directly, Moreover, the bioactivities of the cuttlefish have not been evaluated. Thus, the aim of this study is to evaluate the antioxidant and antiproliferative properties of hydrolysate from Persian Gulf cuttlefish.

Materials and methods

Chemicals

Alcalase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), potassium ferricyanide, *O*-phthaldialdehyde (OPA) and soluble tetrazolium salt (MTS) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Phosphate Buffered Saline (PBS) tablet, ferric chloride and ascorbic acid were prepared from Merck (Germany). RPMI 1640 Medium, Penicillin–Streptomycin and fetal bovine serum (FBS) were from Gibco™. Sulphuric acid and methanol were purchased from Scharlau (Scharlab, Barcelona, Spain). The MDA-231 and T47D cell lines were purchased from the Pasteur Institute of Iran (Tehran).

Sample preparation

Fresh cuttlefish (*S. pharaonis*) from the Persian Gulf (mean weight of 1300 ± 240 g) were supplied by a local fish market (Bandar-e-Abbas, Iran) in the winter of 2015.The samples were immediately frozen at − 20 °C, and then the frozen cuttlefish transported in a Styropor box containing ice to the Department of Seafood Processing, Tarbiat Modares University, Noor, Iran by airplane within 2 h. The frozen cuttlefish were cleaned, skinned, eviscerated and washed at 0–2 °C. The mantles were then kept at − 80 °C until use which was no longer than 2 weeks.

Production of cuttlefish mantle protein hydrolysate (CPH)

The cuttlefish mantle was hydrolysed using alkalase (P4860). The samples were minced with a kitchen mincer (Praskhazar, Tehran, Iran) and pre-incubated at 85 °C for 20 min to inactivate endogenous enzymes. Thereafter, the mantles were mixed with deionized water and homogenized (T 25 D, IKA, Germany) before hydrolysis. To obtain the hydrolysates with 20, 25, 30, 35 and 40% DH, the reactions were carried out according to the following conditions, respectively: pH of 8, 8.5, 7.5, 8.5 and 8; temperature of 40, 45, 55, 45 and 50 °C; reaction time of 120, 90, 150, 150 and 180 min; enzyme concentration of 2, 1.5, 1.5, 2.5 and 2% (v/w).

The mixture was adjusted to the required pH with 0.1 and 0.01 M NaOH. All reactions were done in a shaking incubator (Comecta, Ivymen System, Spain). Samples were incubated at 95 °C for 10 min to stop the enzymatic reaction and were then centrifuged (Universal 320 R, Hettich, Tuttlingen, Germany) at 8000×*g* for 10 min. The supernatants were lyophilized (Alpha 1-2 LDplus freeze dryer, Germany). All hydrolysates were determined for degree of hydrolysis (DH) using protein precipitation with TCA (trichloroacetic acid) per the method of Hoyle and Merritt [18]. Hydrolysates were added with an equal volume of 20% TCA prior to 20 min centrifugation at 6700×*g*. The following equation was applied to calculate the DH:

$$\mathrm{DH}\,(\%) = \left[\mathrm{N_2\ in\ 10\%\ TCA\text{-}hydrolysate\ mixture/\ total\ N_2\ in\ sample}\right] \times 100 \quad (1)$$

Chemical composition and amino acid profile

The chemical composition of the cuttlefish mantle and the hydrolysate were assessed according to the procedures of the Association of Official Analytical Chemists [19]. Moisture, ash, protein, and fat contents were assayed by methods 934.01, 920.153, 954.01, and 991.36, respectively, using a Kjeldahl factor of 6.25 for protein.

The amino acid profile of CPH was determined according to the method of Antoine et al. [20] as described by Sotoudeh et al. [21]. Samples for the analysis of total amino acids were hydrolyzed in 6 M HCl for 24 h at 110 °C and analyzed using HPLC (Knauer, Germany) with a fluorescence detector (RF-530, Knauer). Then, samples were derivatized with *o*-phthaldialdehyde (OPA) and analyzed using a C18 column (Knauer) at the flow rate of 1 ml/min with fluorescence detector (RF-530, Knauer, Germany).

DPPH radical scavenging activity

DPPH radical scavenging activity of CPH was measured using the method of Binsan et al. [22]. To 1.5 ml of sample solution (5 mg hydrolysate/ml distilled water), 1.5 ml of 0.15 mM DPPH in 95% (v/v) methanol were added. The mixture was then mixed vigorously and allowed to stand

at room temperature in the dark for 30 min. Thereafter, the absorbance of the solution was read at 517 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve was prepared using Trolox in the range of 10–60 µM. The activity was expressed as µmol Trolox equivalents (TE)/g sample using the following linear equation:

$$Y = -0.0079X + 0.7409,\ R^2 = 0.9939 \quad (2)$$

where Y is the absorbance at 517 nm and X is the concentration as µmol Trolox equivalents (TE)/g sample.

Reducing power assay (RP)

The RP of the peptides was computed using the method of Oyaizu [23]. An aliquot of 2 ml CPH was mixed with 2 ml 0.2 M potassium phosphate buffer (pH 6.6) and 2 ml 0.1% potassium ferricyanide. The mixture was placed in an incubator at 50 °C for 20 min. Thereafter, an equal volume of 10% TCA (2 ml) was added to the solution, followed by 10 min centrifugation at 3000×*g*. Supernatant (2 ml) of each mixture was mixed with 2 ml of distilled water and 0.4 ml of 0.1% ferric chloride in test tubes. After 10 min, the absorbance was measured at 700 nm. Greater absorbance number indicated higher RP.

Total antioxidant capacity (TAC)

This assay is based on the reduction of Mo (VI) to Mo (V) by the sample and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH [24]. An aliquot of 0.1 ml of sample solution at different concentrations was combined in an Eppendorf tube with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated at 90 °C for 90 min. After that, each sample was allowed to cool to room temperature and the absorbance was measured at 695 nm against a control. The control solution consists of 1 ml of reagent solution and 0.1 ml distilled water. The activity of the hydrolysate was expressed as ascorbic acid equivalent.

Antiproliferative activity of cuttlefish mantle protein hydrolysate (CPH)

Breast cancer cell lines, MDA-231 and T47D were cultured at 37 °C in RPMI 1640 enriched with 10% FBS (fetal bovine serum) and 1% antibiotics (penicillin and streptomycin 1:1). Antiproliferative activities were evaluated according to the method of Picot et al. [9]. A concentration of 10 mg/ml CPH in phosphate buffered saline (PBS, 0.1 M pH 7.4) was prepared as a stock solution. The solution was then diluted in RPMI 1640 to reach the final CPH concentration of 2 mg/ml. PBS and SDS were prepared using the same method and applied as a positive and negative control, respectively.

A portion of 10^4 cells was suspended in culture media and put in each well in a 96 well microplate. Thereafter, cancer cells were exposed to CPH at a final concentration of 1 mg/ml. The microplate was incubated in 5% CO_2–95% air for 72 h. At the end of the incubation, 15 µl of MTS (soluble tetrazolium salt) solution was added to each well, and the plate was incubated for a further 4 h to allow MTS metabolism to formazan by the succinate-tetrazolium reductase which is only active in viable cells. A solubilization/stop solution (100 µl) was added to stop the succinate-tetrazolium reductase activity, kill the cells and solubilize formazan crystals for 12 h at 37 °C. Optical densities were read on a microplate reader (Epoch, Biotech, Winooski, Vermont, USA) at 570 nm. The antiproliferative activity of CPH was calculated using the following equation:

$$\text{Growth inhibition} = 100 - \left[100\left(\left(A_{CPH} - A_{NC}\right)/\left(A_{PC} - A_{NC}\right)\right)\right] \quad (3)$$

where ACPH refers to the absorbance of the culture medium added with CPH, ANC refers to the absorbance of the medium containing 2% SDS (negative control) and APC is the absorbance of medium containing PBS (positive control).

Statistical analysis

The production of hydrolysates was carried out twice for certain DHs. All antioxidative and antiproliferative experiments were run in 12 and 6 replicates, respectively. The mean values ± standard deviation were calculated. The one-way ANOVA and Duncan's multiple-range test were done using SPSS software (Version 16.0, Chicago, IL, USA) to evaluate the significance of differences among mean values with a $P < 0.05$.

Results and discussion

Chemical composition and amino acid content

The mean (± SD) percentages of total protein, fat, moisture and crude ash for fresh cuttlefish mantle in this study were 18.2 ± 1.3, 1.4 ± 0.0, 74.9 ± 0.4 and 4.9 ± 0.1, respectively. The variation in the chemical composition of fish is closely related to their nutrition, living area, fish size, catching season, seasonal and sexual variations, as well as other environmental conditions [25]. Chemical compositions and amino acid content of hydrolysates with different DHs were determined and no significant differences were observed. Protein content in CPH was 76.0% which was similar to other

cuttlefish studies in which protein contents were reported in the range of 71.2–78.9% [26]. High protein content in the hydrolysates could be attributed to solubilization of proteins during hydrolysis and removal of insoluble solid matter by centrifugation [27].

Lipid content in the hydrolysates from cuttlefish mantle decreased from 1.4 to 0.2% which was less than the raw material because the majority of lipids in cuttlefish mantle was removed during centrifugation. Moisture and ash contents of CPH was 4.5 and 17.6% respectively. These are related to the production procedure including freeze dryer and usage of added acid or base for adjustment of pH [27].

The amino acid (AA) content of hydrolysates prepared from cuttlefish mantle are shown in Table 1. According to the results, 95.5% of the AA in CPH were detected in which glutamine (15.7%), asparagine (10.9%) and isoleucine (8.7%) predominated. The essential amino acids (EAA) were 41.4%, of the total and EAA/total AA ratio was 0.4. The EAA/non-EAA ratio was 0.8 which was similar to the findings of Jhaveri et al. [28] who reported this ratio as 0.7 in cod, 0.7 in whiting fish, 0.7 in mackerel and 0.6 in squid. The ratio was reported as 0.8 for Beluga (*Huso huso*) [29] and 1.1 for roe from Beluga [25].

Table 1 Amino acid profile of hydrolysates prepared from cuttlefish mantle using alcalase

Amino acid	Level (%)
Histidine (His)[a]	0.5 ± 0.0
Isoleucine (Ile)[a]	8.7 ± 0.0
Leucine (Leu)[a]	4.8 ± 0.0
Lysine (Lys)[a]	1.2 ± 0.0
Methionine (Met)[a]	5.1 ± 0.1
Phenylalanine (Phe)[a]	4.9 ± 0.0
Tyrosine (Tyr)	3.4 ± 0.0
Threonine (Thr)[a]	5.6 ± 1.1
Arginine (Arg)[a]	7.3 ± 0.0
Valine (Val)[a]	3.1 ± 0.0
Asparagine (Asn)	10.9 ± 0.0
Glutamine (Gln)	15.7 ± 0.0
Serine (Ser)	4.8 ± 0.0
Glycine (Gly)	3.0 ± 0.0
Alanine (Ala)	8.2 ± 0.0
Taurine (Tau)	8.0 ± 0.0
Total	95.5
Total essential amino acids	41.4
Savory amino acids[b]	26.6
Sweet amino acids[c]	11.2
Essential/total	0.4
Essential/non-essential	0.8
Savory amino acids/total	0.3
Sweet amino acids/total	0.1
Essential amino acids/non-essential	0.7

[a]Essential amino acids

[b]Asparagine + glutamine

[c]Glycine + alanine

Antioxidant activities

The antioxidative properties of hydrolysate prepared from cuttlefish mantle including DPPH radical scavenging (Fig. 1a), reducing power (Fig. 1b) and total antioxidant capacity (Fig. 1c) were determined.

The activity of CPH with different DHs including 20.9, 25.5, 30.6, 35.3 and 40.6% (shortened as 20, 25, 30, 35 and 40%, respectively) against DPPH radical are depicted in Fig. 1a. As the results shown, DPPH radical scavenging activity of the hydrolysates were higher than that of the cuttlefish mantle protein isolate (CPI) ($P < 0.05$). The free radical quenching activity of CPH decreased with increasing the DH except for an increase from 25 to 30% DH ($P > 0.05$). With increasing reaction time, more peptide bonds were cleaved and resulted in smaller peptides that were presumed to be more hydrophilic. Those peptides had less ability to interact with DPPH radicals, which are oil-soluble [30–32]. Hamzeh et al. [33] reported that hydrolysates from splendid squid (*Loligo formosana*) protein isolate with a MW of ~ 1381 Da had higher antioxidative activity than those with MW of ~ 404 Da. The results of this study was in agreement with the findings of Intarasirisawat et al. [31] and Klompong et al. [34].

The RP assay is usually applied for assessment of an antioxidant capability to reduce the Fe^{3+}/ferric cyanide complex to the Fe^{2+} complex which can be determined by measuring the formation of Perl's Prussian blue at 700 nm [31, 35]. As shown in Fig. 1b, CPH with a DH of 30% showed the greatest ability to reduce iron(III) followed by hydrolysates with the DH of 20, 25, 40, 35% and non-hydrolyzed CPI, respectively ($P < 0.05$). The ability of CPH varied in RP, suggesting the presence of specific peptide/amino acid composition [35, 36].

The TAC is a method in which the ability of an antioxidant in reducing molybdenum(VI) to molybdenum(V) is evaluated as indicated by the formation of a green phosphate/Mo(V) complex at acidic condition [37]. CPH with 40% DH showed the highest TAC followed by CPH with 25, 20, 30 and 35% DH. Similar to other antioxidative assays, CPH had greater TAC than CPI. The results suggested that CPH could act as electron donor. Higher TAC in CPH with the DH of 40 might be attributed to presence of more active amino acids or peptides as well as different amino acid sequence and length of the obtained peptides [31, 36].

The results showed that enzymatic hydrolysis of cuttlefish mantle protein could improve its bioactivities. However, the antioxidative activity of the hydrolysates is inherent to

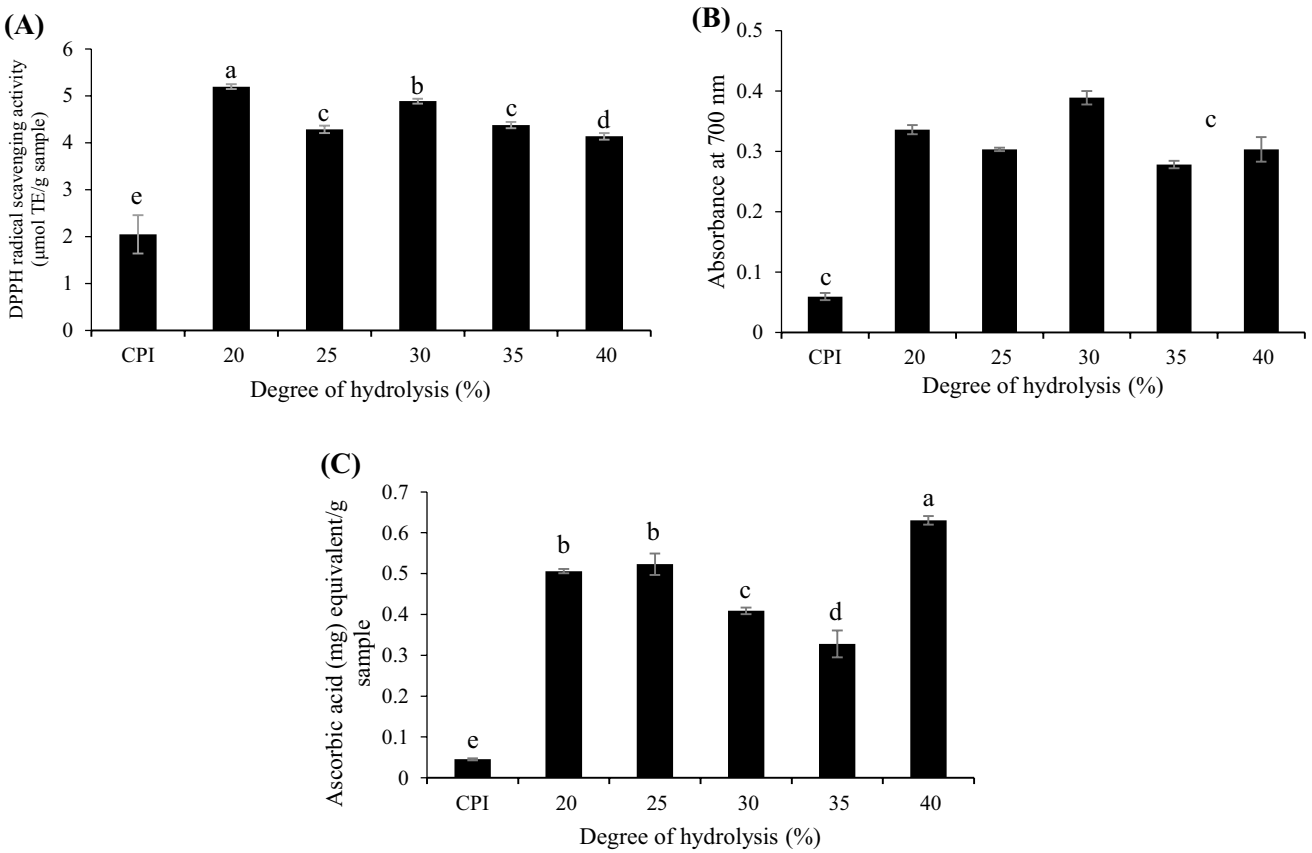

Fig. 1 DPPH radical scavenging capacity (**a**), reducing power (**b**) and total antioxidant capacity (**c**) of cuttlefish mantle protein hydrolysate with 20, 25, 30, 35 and 40% DH (mean ± SD, N = 3) as compared to cuttlefish mantle protein isolate (CPI)

their characteristics amino acid sequence and chain length of peptides, leading to varying degree of antioxidative activity [16, 38]. The results were in agreement with the findings of Sun et al. [38] who reported no correlation between DH and antioxidative activity in hydrolysates prepared from porcine hemoglobin.

Antiproliferative activities

The cytotoxic effects of CPH with different DH on two breast cancer cell lines are shown in Fig. 2: MDA-231 (Fig. 2a) and T47D (Fig. 2b). Generally, the CPH had greater antiproliferative activity than CPI ($P < 0.05$) in which, CPH with 20% DH was the most effective hydrolysate against the

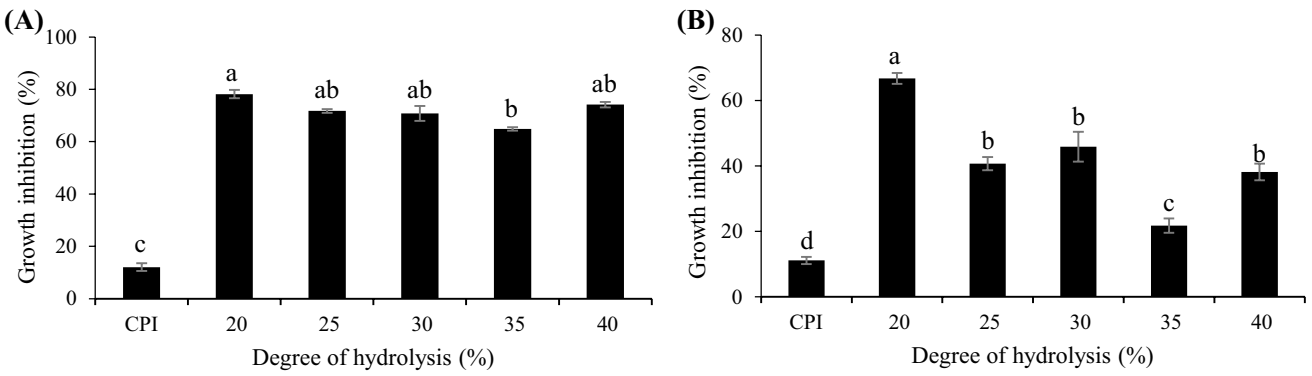

Fig. 2 Antiproliferative effect of cuttlefish mantle hydrolysate with different DHs against MDA-231 (**a**) and T47D (**b**) cell lines (mean ± SD, N = 3) as compared to cuttlefish mantle protein isolate (CPI)

growth of both cancer cells ($P < 0.05$). CPH with 35% DH had the least effect on the growth inhibition of MDA-231 and T47D ($P < 0.05$).

Free radicals are very unstable and react rapidly with other cells in the body which causes health problem such as cancer and other chronic diseases [39]. The peptides in CPH were shown to act as radical scavengers (Fig. 1a). Therefore, they could be helpful to lower the risk of cancer. Moreover, peptides in CPH can directly kill cancer cells or induce cell apoptosis [40]. CPH with different DH showed different abilities to inhibit the growth of cancer cells. The DH affects the size and the amino acid composition of peptides, which could modulate the biological activity of peptides formed during hydrolysis [41].

Conclusions

Hydrolysates prepared from CPH could be a source of bioactive peptide having antioxidative and antiproliferative effects. The CPH showed various antioxidative activities depending on the DH. The hydrolysate with 20% DH had the greatest antiproliferative activity against MDA-231 and T47D cancer cell lines. However, further study using hydrolysates with DH lower than 20% should be conducted to understand the effects of these hydrolysates on antiproliferative activities.

Acknowledgements The authors would like to thank Tarbiat Modares University for the financial support and Prof. Joe M. Regenstein for the editing.

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