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Antioxidant Activity and Anticancer Effect of Bioactive Peptides from Rainbow Trout (*Oncorhynchus mykiss*) Skin Hydrolysate

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Abstract
In this study, rainbow trout skin (*Oncorhynchus mykiss*) was hydrolyzed using Alcalase (HA) and Flavourzyme (HF) enzymes and bioactive peptides separated by membrane ultrafiltration method (< 3, 3–30 and > 30 kDa). The antioxidant properties of the protein hydrolyzed (non-fractionated) and anti-cancer (peptide fractions) were evaluated. The results showed that DPPH radical inhibitory power and ferric reducing antioxidant power in hydrolyzed skin protein by Flavourzyme were significantly higher than Alcalase hydrolyzed protein ($p < 0.05$). By increasing protein concentrations, the antioxidant property of the hydrolyzed protein increased. To evaluate anti-cancer activity, MTT assay and HCT-116 cell line were used. Hydrolyzed skin protein with molecular weight less than 3 kDa had the highest inhibitory concentration (IC50). The results proved rainbow trout skin protein hydrolysate showed antioxidant properties and could be used as an antioxidant in food. The isolated fractions on HCT-116 cancer cells have cytotoxic properties and inhibit the growth of these cells in vitro. In order to achieve more accurate results, evaluation of properties is recommended in in vivo conditions.

Keywords Fish protein hydrolysates · Proteolytic enzymes · Antioxidant · Anticancer · Clone cancer

Introduction

Fish has a high volume of waste, including head, tail, fins, spine, viscera and skin which accounts for up to 75% of the total weight (Rustad et al. 2011). In addition to the disposal problem, the waste cause environmental pollution while using biochemical and enzymatic methods, they can be used as essential compounds in the production of various value added products such as bioactive peptides, biosilage, collagen and gelatin (Halim et al. 2016). Fish waste is a rich source of essential amino acids (Pezeshk et al. 2018). Bioactive peptides found in fish waste can be isolated by biochemical hydrolysis (Halim et al. 2018). The enzymes used for enzymatic hydrolysis are of plant, animal and microbial sources (Kristinsson and Rasco 2000). The proteolytic enzymes break down the protein into small peptide components, containing 2–20 amino acids, which in addition to the nitrogen source have functional properties such as antihypertensive, antidepressant, antimicrobial, antioxidant and anticancer (Ug et al. 2018). The specific activity of these peptides depends on the sequence, type of amino acid and peptide length (Choksawangkarn et al. 2018). The most important benefits of peptides as drugs are that peptides have high biological activity and impose less costly treatments on the patient. Normally, such drugs do not have side effects due to low toxicity, any complications for the patient (Ehrenstein and Lecar 1977).

Various forms of active oxygen and free radicals are generated during respiration in humans and other aerobic organisms. Free radicals are unstable compounds that quickly react with other substances or molecules in the body, leading to cellular and tissue damage. Antioxidants are substances that significantly inhibit or delay oxidation and protect the body against oxidative stress (Mittler 2002). Because of the side effects of synthetic antioxidants, researchers are looking for

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natural antioxidants, among which fish protein hydrolysates can be considered as new alternatives (Halim et al. 2016). Pezeshk et al. (2018) reported that the enzymatic hydrolysis in yellowfin tuna (*Thunnus albacores*) viscera has an antioxidant property (DPPH, ABTS, and metal reducing power) (Pezeshk et al. 2018). Razali et al. (2015) reported that the hydrolyzed protein of cobia skin gelatin has reducing property, iron chelating power and the ability to inhibit DPPH. According to cancer progression, there is a very close relationship between inflammation and oxidative stress, a compound that has anti-inflammatory or antioxidant properties that can be as an anticancer agent (Moheghi et al. 2011). Additionally, antioxidants were reported to be potentially used to prevent and treat diseases such as cancer that associated to oxygen species (Leng et al. 2005).

Colon cancer after lung cancer is the second leading cause of death in the world (Leong et al. 1998), and after stomach and intestinal cancer, is the third most common gastrointestinal cancer in Iran, which several factors contribute to the development and spread of colon cancer (Mohebbi et al. 2008). Various studies have shown that the potential of peptides anticancer are mainly due to their antioxidant, antiproliferation and anti-mutated properties. Peptides extracted from aquatics, is cause cell death with stimulating various mechanisms such as apoptosis, antiproliferation and cytotoxicity. Some cancers can be treated with surgery, chemotherapy and radiotherapy, but these treatments have often damaging effects through the use of drugs on healthy cells and tissues (Hubenak et al. 2014). Hence, research on safe anticancer agents in the pharmaceutical industry is important (Halim et al. 2018). Many researchers examined the effects of the protein hydrolysates on different colon tumor-derived and in vitro transformed cell lines. Various colon cancer cell lines, such as HT-29, RKO, KM12L4, DLD-1 and HCT15 (human colon carcinoma); Caco-2, TC7 and HCT-116 (human colorectal carcinoma) have been extensively used for investigating the anticancer activities of protein hydrolysates prepared from a variety of food protein sources (Chalamaiah et al. 2018). Research have shown that peptide with anticancer activity from different sources had low molecular weight between 300 and 1950 Da. Peptides of lower molecular weight have greater molecular mobility and diffusivity than peptides of higher molecular weight (Ishak and Sarbon 2018). Another study on oyster protein hydrolysate found that peptide with molecular weight of 515.29 Da exhibited anticancer activity against human colon carcinoma (HT-29) cell lines (Umayaparvathi et al. 2014).

Hence, this study aimed to investigate (i) fractionated rainbow trout skin hydrolysate (TSPH) of different molecular weights (3, 3–30 and 30 kDa) on anticancer activity (i.e. cell cytotoxic assay MTT method); and (ii) protein hydrolyzed (non-fractionated) for antioxidant activity (i.e. DPPH radical scavenging and reducing power).

Materials and Methods

Fresh rainbow trout (*Oncorhynchus mykiss*) with an average weight of 530 ± 45 g were obtained from Borzo Aquaculture (Sari, Iran). The fish was placed in ice with the fish/ice ratio of 1:2 (w/w) and transported within less than 1 h to the Laboratory of Caspian Sea Ecology Research Center. Then fish skins were separated and cut into small pieces and stored as a raw material at − 20 °C until used. Alcalase enzyme (endoproteinase extracted from *Bacillus licheniformis*) and Flavourzyme (Endo and exopeptidase extracted from *Aspergillus oryzae*) were provided by Novosim Company (Denmark) via its agency in Iran and were stored at 4 °C until used.

Preparations of Rainbow Trout Skin Protein Hydrolysate

First, rainbow trout skins were thawed at ambient temperature, and then 50 g was weighed and was transferred to 250 ml Erlenmeyer. 100 ml distilled water (weight ratio-volume 1–2) was added to each sample erlenmeyer. In order to inactivate the internal enzymes of the skin, samples were heated for 20 min at 85 °C (Guerard et al. 2002). In the first step, the Alcalase enzyme with 1% protein content was added. Hydrolysis was done for 90 min at pH 8.5 and 58 °C, and slurry was cooled, after deactivate of the enzyme at 90 °C for 10 min. The Flavourzyme enzyme with 1% protein content was added to the second erlenmeyer. Before adding the enzyme, pH and temperature were settled in the range of 7 and 50 °C, respectively (Table 1). This hydrolysis step was also done for 90 min. Hydrolysis was terminated by heating the samples at 90 °C for 10 min using a water bath. After cooling, samples were centrifuged at 10,000 × *g* at 4 °C for 10 min (Ketnawa and Liceaga 2017). Supernatants were filtered, lyophilized (Dura-stop, NY, USA) and stored at − 20 °C until further analyses (Ketnawa and Liceaga 2017). Protein hydrolysate powder was prepared by Alcalase and Flavourzyme kept in Polyethylene bag under vacuum at room temperature in desiccator until used.

Ultrafiltration

Hydrolyzed protein powder was dissolved in 10% (w/v) and then, using 3 and 30 kDa Amicon filters (Amicon Ultra-15;

Table 1 Hydrolysis condition of trout skin

Enzyme	pH	Temperature (°C)	Time (min)
Alcalase	8.5	58	90
Flavourzyme	7	50	90

Millipore Co., Billerica, MA, USA), at 25 °C for 30 min with speed of 12,000×*g* centrifuged (Sigma 2-16kl, Spain). F-I, F-II and F-III represented the fractions with MW distribution of more than 30, 3–30 and < 3-kDa, respectively. Then hydrolyzed protein solution and fractions were recovered, lyophilised, and then of which, the antioxidant and anticancer activities were determined.

Chemical Composition of Fish Skin

The AOAC method was used to measure the approximate composition of the samples. To measure moisture from the oven at 105 °C, a constant weight of the sample was used. To determine the ash, the wet specimen was poured into a Chinese plant and burned in a furnace at 550 °C for 5 h. The protein content was obtained by Kjeldahl method. Total fat was extracted with Soxhlet (AOAC 2002).

Degree of Hydrolysis

The degree of hydrolysis (DH) was measured using trichloroacetic acid (TCA) of 20% (v/v). For this purpose, an equal volume of the protein solution dissolved in a TCA solution was blended and centrifuged at 5 °C (6700×*g*) after stirring at 20 °C. Then, the amount of protein in the solution phase was measured by the Lowry method (Lowry et al. 1951) and the degree of hydrolysis was calculated by the following equation.

$$\mathrm{DH} = \frac{\mathrm{TCH10\%} - \text{soluble N in sample}}{\text{Total N in sample}} \times 100$$

Antioxidant Activity Determination

For this purpose, two antioxidant indices including radical 2,2-diphenyl-1-picryhydrazyl radical scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP) were used.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

TSPH solution (1 ml) was added to 1.5 ml of 0.2 mM DPPH in 99.5% ethanol. The mixture was incubated at room temperature in the dark for 30 min prior to monitoring absorbance at 517 nm. The control sample was prepared using the same method, except that distilled water was used instead of a sample. Finally, the power of hydrolyzed proteins was calculated for DPPH radical control using the following formula (Yen and Wu 1999).

$$\text{Radical scavenging assay DPPH} = \frac{\text{Absorption sample}}{\text{Absorption control}} \times 100.$$

Ferric Reducing Antioxidant Power (FRAP)

TSPH solution (1 ml) was added to 2.5 ml of phosphate buffer 0.2 M (pH 6.6), mixed with 2.5 ml of a 1% (w/v) potassium ferricyanide solution. The solution was incubated for 30 min at 50 °C. After this, 2.5 ml of distilled water and 0.5 ml of ferric chloride were added 0.1% and incubated for 10 min at ambient temperature. Then absorption was read with spectrophotometer at 700 nm (Zhang et al. 2014; Ketnawa and Liceaga 2017). Higher absorption shows the higher reducing power of hydrolyzed proteins (Oyaizu 1986).

Preparation of Cell Culture

The colon cancer cells (HCT-116) were purchased from Iran Pasteur Institute's Cellular Bank and transmitted to the lab. Then colon cancer cells were cultured in DMEM/F12 (Gibco-American) medium (enriched with growth factors and non-serum) in a 96-well plate. Cells were kept on with 5% of CO_2/air at 37 °C in incubator. After filling about 70–80%, the cells were dissected from the plate surface by adding trypsin and then cultured by passaging once a week (Mokhtari et al. 2008).

Anticancer Activity of Rainbow Trout Protein Hydrolysate (RTPH)

HCT-116 cells were cultured in a 96-well plate with 1×10^4 cells/well. After 24 h, the cells were washed with phosphate buffer, and exposed to different concentrations of fractions (F-I, F-II and F-III), to cell-plates with three replicates and incubated at 37 °C and 5% CO_2 for 24 h. After 3 h, the solution on the cells was discarded, next, after washed with PBS, 100 µl of dimethyl sulfoxide was added, and the absorbance value was measured at the absorption wavelength of 570 nm using the ELISA reader (Anthos 2020, America) (Sheu et al. 2008). Cells without treatment by RPH solution were used as blank and cisplatin (strong anti-cancer) substance at a concentration of 28 M/ml was used. Percent cell viability was calculated using the equation as follows:

$$\text{Viability (\%)} = \frac{\text{Optical absorption of cells treated with hydrolyzed protein in each well}}{\text{Average optical absorption of control cells}} \times 100.$$

Table 2 Chemical composition of rainbow trout skin and TSPH

Treatments	Moisture (%)	Fat (%)	Ash (%)	Protein (%)
Trout skin	71.58 ± 0.62	9.21 ± 0.72	1.18 ± 0.42	17.51 ± 0.31
HA	8.62 ± 0/58[a]	20.12 ± 0.05[a]	5.27 ± 0.87[a]	73.46 ± 0.58[a]
HF	7.43 ± 0.35[a]	24.32 ± 0.06[b]	6.87 ± 0.35[a]	69.48 ± 0.7[a]

The same letters above the numbers indicate that there is no significant difference ($p < 0.05$). Values are given as mean ± SD from triplicate determinations ($n = 3$). Trout skin based on the wet matter, HA and HF based on the dry matter

HA hydrolyzed using Alcalase, *HF* hydrolyzed using Flavourzyme

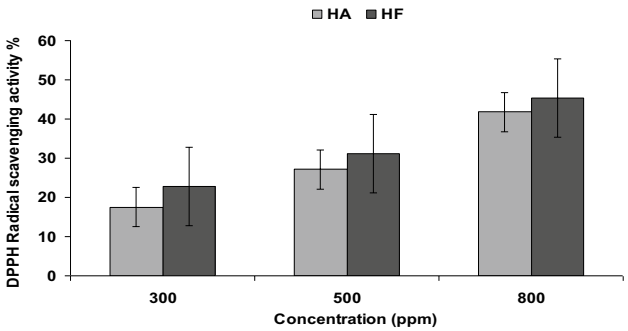


Fig. 1 DPPH free radical scavenging power of HA (hydrolyzed using Alcalase) and HF (hydrolyzed using Flavourzyme). The data are mean ± SD

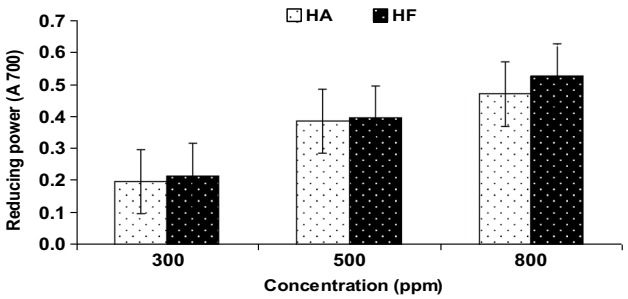


Fig. 2 Reducing power Hydrolyzed skin of hydrolyzed using Alcalase (HA) and hydrolyzed using Flavourzyme (HF). The data are mean ± SD

Statistical Analysis

All analyses of rainbow trout protein hydrolysate (RTPH) were conducted in triplicate. The data was presented as mean ± SD. One-way ANOVA was carried out with level of significance at ($p < 0.05$). For drawing graphs used Prism version 5.

Results

Physicochemical Properties

The degree of hydrolysis of the HF was higher than (43.83%) the HA (28.38%) ($p < 0.05$). The results of chemical composition in tout skin and TSPH are shown in Table 2. Data were determined based on wet skin weight. Accordingly, the approximate compounds of wet raw material were fat, protein, ash and moisture, respectively. Rainbow trout skin moisture was the largest combination with the highest rate of 71.58%. Hydrolyzed proteins HA and HF showed higher levels of protein with 73.46% and 69.48%, respectively.

Antioxidant Properties of TSPH

Radical Scavenging Power of TSPH (Non-fractionated)

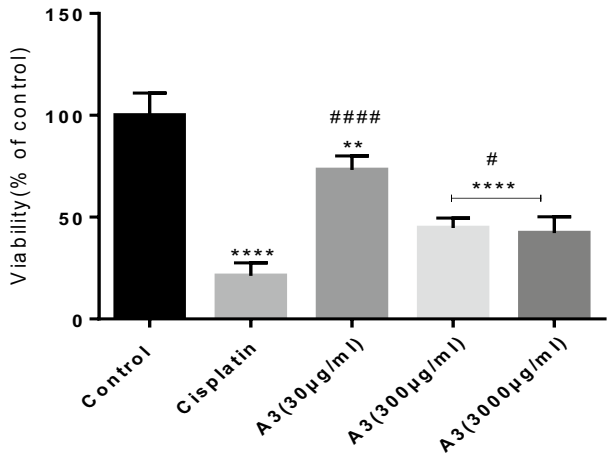
One of the important indicators for measuring the antioxidant activity of proteins is their ability to inhibit DPPH. The DPPH free radical scavenging power is shown in Fig. 1. TSPH exhibited significant antioxidant activity against the DPPH radical removal test ($p < 0.05$). The HF in concentration of 300, 500 and 800 ppm has higher DPPH radical inhibitory power than HA ($p < 0.05$).

The Reducing Power of Rainbow Trout Hydrolyzed Proteins

The reducing power of the hydrolyzed proteins is shown in Fig. 2. Hydrolyzed proteins with Flavourzyme at three concentrations of 300, 500 and 800 ppm showed the higher reduction power compared to the Alcalase hydrolyzed protein. The reducing power of the hydrolyzed protein increased with the concentration of Flavourzyme and Alcalase ($p < 0.05$).

MTT Assay

Figures 3, 4 and 5 showed viability (%) of HCT-116 cancer cell line treated with different concentrations of HA and HF. In investigate viability (%), the effect of concentrations (30, 300 and 300 µg/ml) of fish skin protein hydrolyzed F3 and



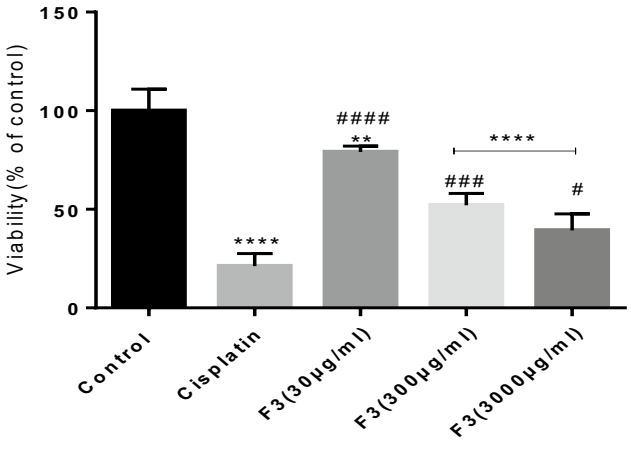
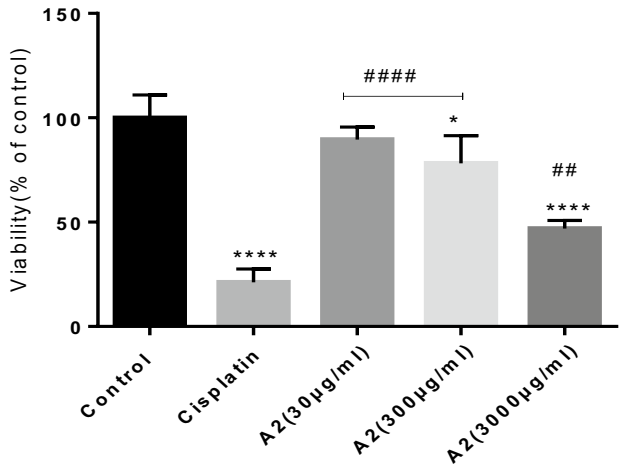


Fig. 3 The viability (%) of HCT-116 cells against different concentrations of F3 and A3 protein hydrolyzed proteins (< 3 kDa) over a period of 48 h (results are reported as survival percentages compared to the untreated sample). * Significantly, compared to the control group, $p < 0.05$ and # was significantly greater than that of the cisplatin or positive control group



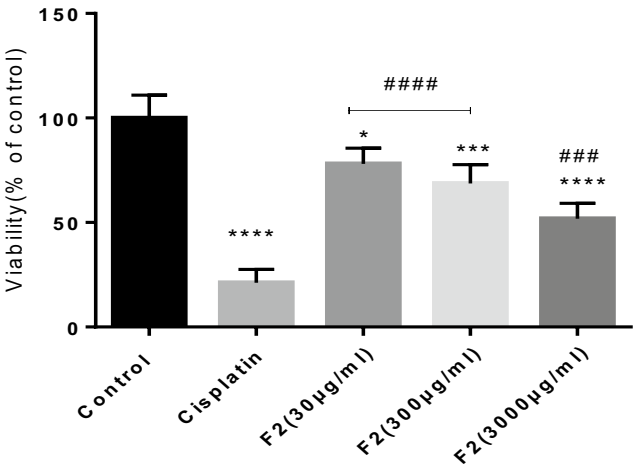
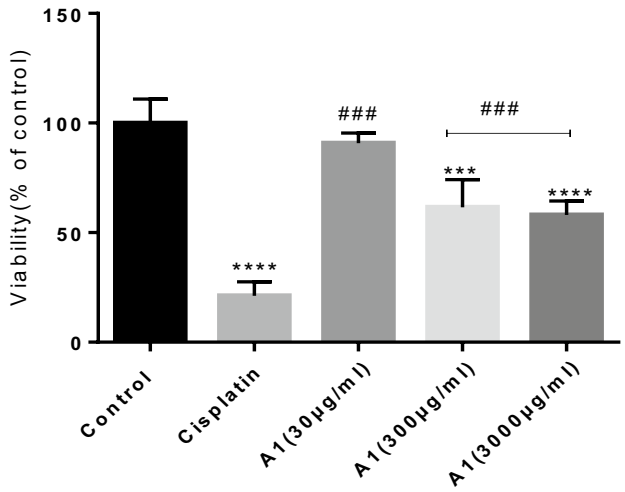


Fig. 4 The viability (%) of HCT-116 cells against different concentrations of F2 and A2 hydrolyzed proteins (3–30 kDa) over a period of 48 h (results were reported as survival percentages compared to the untreated sample). * Significantly, compared to the control group, $p < 0.05$ and # was significantly greater than that of the cisplatin or positive control group

A3 with a molecular weight less than 3 kDa on HCT-116 cells were shown that the concentration of 3000 µg/ml of HF and HA had the highest viability (%) of 0.39 ± 0.04 and 0.06 ± 0.03 which showed significant difference ($p < 0.001$). IC50 F3 and A3 were 727.4 and 249.5 µg/ml, respectively (Table 3).

The cytotoxic effects of HF and HA with the molecular weight of 3–30 kDa were shown in Fig. 4. In viability (%), the effects of concentrations (30, 300 and 3000 µg/ml) of F2 and A2 fish skin hydrolyzed proteins with a molecular weight between 3 and 30 kDa on HCT-116 cells were measured, which showed a concentration of 3000 µg/ml of HA in viability (%) of 0.49 ± 0.02 that was statistically significant ($p < 0.0001$), and IC50 A2 was 2738.6 (Table 3). However, HF did not show inhibitory cell proliferation.

HCT-116 colon cancer cell line was used as cell culture model to study the activity of cytotoxic hydrolyzed protein in trout skins. The F1 and A1 fractions have a molecular weight greater than 30 kDa. F1 fraction had more activity than A1, which was statistically significant ($p < 0.001$). HF showed a cytotoxic effect of IC50 with 1446.0 µg/ml in HCT-116 cancer cells (Table 3). HA did not show inhibitory cell proliferation (Fig. 5) ($p > 0.05$).



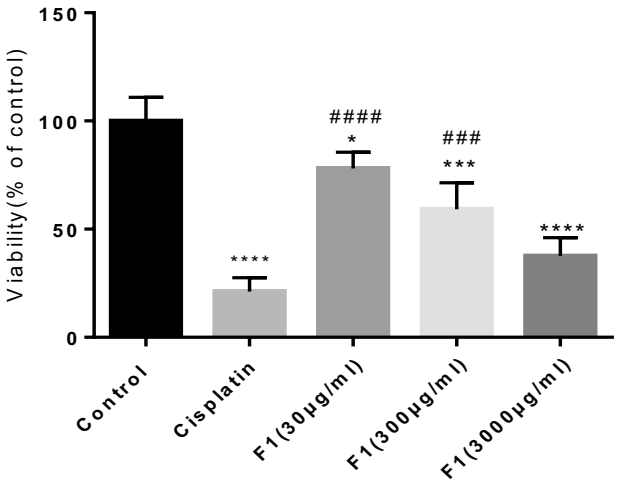


Fig. 5 The viability (%) of HCT-116 cells against different concentrations of F1 and A1 hydrolyzed proteins (> 30 kDa) over a 48-hour period (results are reported as survival percentages compared to the untreated sample). * Significantly, compared to the control group, $p < 0.05$ and # was significantly greater than that of the cisplatin or positive control group

Table 3 Anticancer activity (IC50, µg/ml) F1 and A1 (> 30 kDa), F2 and A2 (3-30 kDa), F3 and A3 (< 3 kDa)

Enzyme	Molecular weight of the protein	IC50 (µg/ml)
Alcalase	A3	249.5
	A2	2738.6
	A1	–
Flavourzyme	F3	727.4
	F2	–
	F1	1446.0

Discussion

The degree of hydrolysis of the HF was higher than the HA. Flavourzyme is an endogenous and exopeptidase enzyme, and in addition to breaking the peptide bond from inside, it eliminates of terminal peptide, C terminal and N terminal peptides or smaller portions (Le Gouic et al. 2019). The HF in different concentration showed higher DPPH radical inhibitory power than HA. Klompong et al. (2008) reported a difference in free radical inhibitory of DPPH between Flavourzyme and Alcalase probably in relation to the size of proteins or peptides. DPPH free radical inhibitory power is widely used to measure the antioxidant capacity of the peptide, phenolic matter and food (Karadag et al. 2009). The results of DPPH radical inhibitory antioxidant test showed that by increasing the concentration of hydrolyzed protein, the inhibitory content of the radicals increased. Among the concentrations used in this study, the concentration of 800 ppm showed the highest percentage of inhibitory activity, that showed the similar finding to those reported earlier (Klompong et al. 2008; Jemil et al. 2014; Sripokar et al. 2019), reported that DPPH radical scavenging increases with increasing concentrations. 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 (Hamzeh et al. 2017). The reducing power of the hydrolyzed protein increased with the concentration of Flavourzyme and Alcalase. The increasing trend of reducing power is also seen by increasing of the concentration proteins which achieved the same results of this study (Halim et al. 2018; Sripokar et al. 2019). The reduction power or the ability to reduce the ionic ferric hydrolyzed proteins is the greater absorption that represents the further reduction. The highest reducing power was related to the Flavourzyme hydrolyzed protein (Fig. 2) at 800 ppm concentration. The regenerative capacity of the protein hydrolyzed with Alcalase and Flavourzyme was in the range of 0.06–0.6 mg/ml, might be due to the low protein content in the solution. In a similar study, the regenerative capacity of the seaweed protein (*Selaroides leptolepis*) hydrolyzed with Alcalase and Flavourzyme recorded in the range of 0–1 mg/ml (Klompong et al. 2008). Increasing or decreasing the hydrolyzed Reducing power skin protein may be associated with the exposing of the electron density in the amino acid side chain groups, which acts like polar or charge portions during hydrolysis. On the other hand, indole and phenolic tyrosine groups play an important role in hydrogen donation in an oxidation system. In general, the antioxidant activity of the fish protein hydrolysate is usually associated with the composition, sequence, and hydrophobicity of amino acids (Pezeshk et al. 2018).

Although the viability (%) of Flavourzyme was higher, but it has less than IC50 in compare with Alcalase, while the IC50 was 45.53 µM/ml for cisplatin. Hydrolyzed proteins (AH) and (FH) showed dose-dependent cytotoxic effects on HT-29 cells, so that the A3 and F3 fractions with molecular weights less than 3 kDa have lower viability (%), and as cytotoxic enhancers, and fractions of 3–30 and > 30 kDa exhibited higher viability (%) with less cytotoxic activity (Figs. 3, 4, 5). Zhang and Mu (2018) expressed < 3 kDa having a stronger antiproliferative effect on the HT-29 colon cancer cell line that showed the similar results of this research. Kim (2011) reported peptide fractions with less molecular weight has higher molecular weight release and mobility, and more interaction with the components in the cancerous cell, resulting more anticancer activity. Therefore, the above experiments may provide preliminary information for further study on cytotoxicity (AH) and (FH) properties through nutrition.

Conclusion

Hydrolysates prepared from RTPH could be a source of bioactive peptide with antioxidant and anticancer activities. The hydrolyzed protein of rainbow trout showed the antioxidant and anti-cancer potentials by Flavourzyme and Alcalase enzymes. The fish hydrolyzed protein showed antioxidant activity, concentration-dependent activity. The Flavourzyme enzyme showed a higher activity in the DPPH-free radical control than in the Alcalase activity. In addition, anticancer activity in both hydrolysis, fractions of less than 3 kDa (A3 and F3) had the highest cellular inhibition in HCT-116 colon cancer cells compared with fractions greater than 3 kDa. Therefore, rainbow trout peptides can be a source of biologically active peptides with anticancer effects on HCT-116 colon cancer cells in vitro, that can be used as a natural antioxidant in food.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest relevant to this study.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and this article does not contain any studies with human participants performed by any of the authors.

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