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Original research

Effect of alcalase-mediated hydrolysis on the free radical scavenging activity and reducing power of whey protein isolate

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ABSTRACT

In the present study, the effect of enzymatic hydrolysis on the antioxidant properties of whey protein isolate (WPI) was studied. Therefore, the whey protein solution was hydrolyzed for 5 h using alcalase enzyme. The antioxidant properties were measured using different methods including reducing power assay, DPPH radical scavenging test, and ABTS radical scavenging activity. The results showed that the enzymatic hydrolysis significantly ($p < 0.05$) increased the antioxidant activity of WPI and the resulting hydrolysates had better reducing power and free radical scavenging activity compared to the non-hydrolyzed counterpart. The results also showed that the highest level of antioxidant activity was related to the samples that were hydrolyzed for 5 h using alcalase enzyme and the lowest level of antioxidant activity was related to the native WPI. Therefore, the results of the present study suggested that the hydrolysates of WPI produced by action of the alcalase enzyme can be used as a natural antioxidant to replace synthetic antioxidants that have harmful effects on health, in various food formulations. The use of these antioxidant hydrolysates in food products can improve their health-related properties.

Keywords: Whey proteins; Bioactive peptides; Enzymatic hydrolysis; Antioxidant activity

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1. Introduction

The role of proteins as physiologically active compounds is widely investigated. Many proteins that are naturally present in food, exhibit their physiological properties directly or through the enzymatic hydrolysis (Dullius et al., 2018). In the recent years, some proteins have been shown to be a rich source of biologically active or bioactive peptides. These peptides are normally inactive in the parent protein sequence but can be released by different methods the most common of which are as following: 1- Hydrolysis by digestive enzymes such as pepsin and pancreatin, 2- Hydrolysis by proteolytic microorganisms, and 3- Hydrolysis by proteolytic enzymes derived from the microorganisms or plants (Lorenzo et al., 2018; Youssef et al., 2019). Bioactive peptides are actually specific protein fragments that have positive effects on body function and condition and can ultimately affect human health (Korhonen & Pihlanto, 2006). Bioactive peptides, due to their amino acid sequence, can have important effects on body systems such as the cardiovascular, digestive, immune, and nervous systems. These positive effects on human health can be attributed to the peptide sequences with antimicrobial, antioxidant, anticoagulant, anticancer, antihypertensive, etc. properties (Bagheri et al., 2014). For antioxidant peptides and hydrolysates formed by enzyme-mediated hydrolysis, it was suggested that these bioactive peptides are able to reduce the formation of oxidative products along with the induction of antioxidant enzymes *in vivo* (Lorenzo et al., 2018; Alavi et al., 2019).

Nowadays, milk proteins are recognized as the most important source of bioactive peptides, and a large number of bioactive peptides have been identified in hydrolyzed milk proteins and fermented dairy products (Madureira et al., 2010). Enzymatic hydrolysis is the most common method for producing bioactive peptides from native milk proteins. The most widely used enzymes for the production of bioactive peptides from milk proteins are pepsin, alcalase, trypsin, and chymotrypsin (Mohanty et al., 2016). Whey proteins, one of the two major protein types of milk, are rich sources of essential amino acids and their enzymatic hydrolysates have been examined extensively for their excellent bioactive and functional properties (Vavrusova et al., 2015; Nooshkam &

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Madadlou, 2016). Whey protein hydrolysates have displayed a wide range of *in vitro* and/or *in vivo* biological activities including ACE-inhibitory, antioxidant, antihypertensive, anti-diabetic, antimicrobial, and opioid properties (Goudarzi & Madadlou, 2013; Dullius et al., 2018). Peng et al. (2009) investigated the reducing power and radical scavenging activity of hydrolysates derived from whey protein hydrolysis and observed that the hydrolysates had higher antioxidant activity than native whey protein at all time of hydrolysis. In fact, the higher antioxidant activity of hydrolysates may be due to the increased availability of hydrogen ions due to the peptide hydrolysis (Kong et al., 2013). In addition, enzymatic hydrolysis increases the concentration of carboxyl groups in the environment and increases the antioxidant activity of proteins by inhibiting pro-oxidants such as metal ions (Lin et al., 2012).

According to the importance of bioactive peptides and hydrolysates for human health, the present study was carried out to produce antioxidant hydrolysates from a milk protein. In this regard, whey protein isolate (WPI) was hydrolyzed by alcalase enzyme to release bioactive hydrolysates. After that, the antioxidant properties of the hydrolysates were measured by reducing power assay and free radical scavenging activity tests.

2. Material and Methods

2.1. Materials

WPI with more than 90% protein was purchased from Arla Food Ingredients (Viby J, Denmark). 2,2′-azinobis-(3-ethylbanzthiazo-line-6-sulfonate) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were procured from Sigma-Aldrich (Sa. Louis, MO, USA). The food-grade enzyme alcalase 2.4L (≥2.4 U/g) was also obtained from Sigma-Aldrich. Other chemicals which used in this study were of analytical grade and obtained from Merck and Sigma-Aldrich.

2.2. Enzymatic hydrolysis of WPI

WPI solution with a concentration of 20 mg/mL was prepared by dissolving an appropriate amount of WPI powder in distilled water. Sodium azide (0.1 mg/mL) was added to the solution as an antimicrobial agent. The solution was then stirred for 2 h at 20°C and kept overnight at 4°C for the complete hydration. After that, the hydrolysis of WPI solution was performed at 65°C and pH 8.5 over a period of 5 h by using the alcalase enzyme in the ratio of enzyme to substrate 1:100, while the solution was constantly stirring (suggested by the manufacturer as optimal conditions for enzyme activity). After the hydrolysis process, the enzyme was inactivated by heating at 85° C for 10 min. After that, the solution was cooled and then was stored a 20°C for the subsequent uses.

2.3. Degree of hydrolysis (DH)

The DH value was determined by pH-stat method using the following equation (Adler-Nissen, 1986):

$$\mathrm{DH}\ (\%) = \frac{\mathrm{V} \times \mathrm{N_V}}{\alpha \times \mathrm{M_P} \times \mathrm{h_{tot}}} \qquad (1)$$

where V is the amount of NaOH added to a substrate to keep the pH constant during the hydrolysis; N_V is the normality of NaOH; M_P is the mass of protein (in grams); h_{tot} is the total number of the peptide bonds in WPI which is assumed to be 8.6 meq g (Goudarzi et al., 2012); and α is the average degree of dissociation of the α-NH_2 groups liberating during hydrolysis which is determined using the following formula:

$$\alpha = \frac{10^{(\mathrm{pH}-\mathrm{pK})}}{1 + 10^{(\mathrm{pH}-\mathrm{pK})}} \qquad (2)$$

where pK is the average dissociation value for the α-amino groups releasing during the enzymatic hydrolysis which is dependent on temperature, peptide chain length, and nature of the terminal amino acid.

2.4. Reducing power

The reducing power of hydrolyzed whey protein solution at different times (0, 1, 2, 3, 4 and 5 h) was evaluated by Peng et al. (2010) method with a slight modification. Briefly, 1.0 mL of native or hydrolyzed WPI was mixed with 2.5 mL of 0.2 M phosphate buffer (pH value of 6.6) and 2.5 mL of 1.0% potassium ferric cyanide. The mixture was incubated for 20 min at 50°C and then 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 6000 g for 10 min. 2.5 mL of the resulting supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. Then, the absorption of these mixtures was read at 700 nm after 10 min storage at room temperature by a UV-Vis spectrophotometer. Higher absorption indicates more reducing power representing higher antioxidant activity.

2.5. DPPH radical scavenging activity

The effect of enzymatic hydrolysis on the DPPH radical scavenging activity of WPI was determined based on the method described by Lin et al. (2012) with some modifications. For this purpose, the ethanolic solution of DPPH with a concentration of 0.1 mM was prepared. After that, 1.0 mL of DPPH solution was added to 1.0 mL of hydrolyzed/non-hydrolyzed whey protein sample or distilled water as control and mixed very well. The resulting mixtures were stirred vigorously and kept in the dark at 25°C for 30 min. The adsorption of these mixtures was then read at 517 nm and finally, the percentage of DPPH free radical scavenging activity of hydrolysate resulting from the enzymatic hydrolysis of whey proteins was calculated by the following formula:

$$\text{DPPH scavenging activity}\ (\%) = \frac{\mathrm{A_C} - \mathrm{A_S}}{\mathrm{A_C}} \times 100 \qquad (3)$$

where A_C is the absorbance of the control and A_S is the absorbance of sample at 517 nm.

2.6. ABTS radical scavenging activity

The ability of whey protein or its enzymatic hydrolysis for scavenging of ABTS radicals was determined according to the method described by Dryakova et al. (2010) with some modifications. For this purpose, at first, the free radicals of ABTS were produced. In this regard, the $ABTS^+$ solution was produced by adding ABTS stock solution (7.4 mM) to potassium persulfate (2.6 mM) in phosphate buffered saline (PBS, pH 7.4). Then, the absorbance of $ABTS^+$ solution was adjusted to 0.7 ± 0.02 at 734 nm by diluting with distilled water. After that, 200 µL of samples (or 200 µL of distilled water as control) was mixed with 1000 µL of

$ABTS^+$ solution and the resulting mixture was stored in a dark place at room temperature for 10 min and finally, the absorbance was measured at 734 nm using a UV-Vis spectrophotometer. The $ABTS^+$ radical scavenging activity was calculated by using the following equation:

$$\text{ABTS scavenging activity (\%)} = \frac{A_C - A_S}{A_C} \times 100 \quad (4)$$

where A_C is the absorbance of the control and A_S is the absorbance of sample at 734 nm.

2.7. Statistical analysis

Data analysis and evaluation was performed using SPSS software version 16 at 5% probability level. Also, one-way ANOVA was used to determine the significance of the difference between the means (at least three replications) by Duncan's method.

3. Results and Discussion

3.1. DH results

The degree of WPI hydrolysis during 5 h enzymatic hydrolysis by alcalase enzyme is shown in Fig. 1. The DH is time dependent and has increased over time. The results showed that in the first hour, the DH increased rapidly, but in other hours the hydrolysis increased more slowly. These results were in accordance with those of Peng et al. (2010). They hydrolyzed whey protein with a protease, stating that the DH was time-dependent. In fact, it can be said that in the first hour, there is more substrate for the enzyme, so the DH increases rapidly, but in the following hours the amount of substrate decreases and therefore the degree of hydrolysis increases with a slower slope. In accordance with our findings, Mohammadian and Madadlou (2016) also reported that the DH of WPI was increased by increasing hydrolysis time during the hydrolysis by Corolase N enzyme as a protease. In another study, Peng et al. (2009) studied the enzyme-mediated hydrolysis of WPI. They studied the DH during the hydrolysis and reported that the DH of the WPI samples increased almost linearly with increasing hydrolysis time in the first 3 h, and reached a plateau at 35–36% after 5 h.

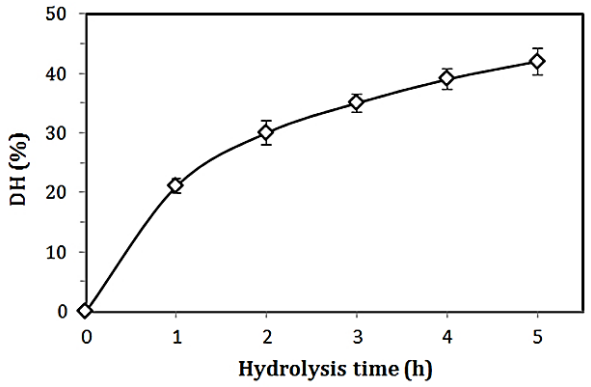

Fig. 1. Degree of hydrolysis (DH) of whey protein solution during enzymatic hydrolysis.

3.2. Reducing power

The results of reducing power measurement for non-hydrolyzed and hydrolyzed samples are shown in Fig. 2. The results showed that the hydrolysis of WPI increased its reducing power, which means that the antioxidant activity of whey protein was increased. Moreover, the results indicated that the increasing of the hydrolysis time has also significantly increased the reducing capacity of the resulting hydrolysates ($p < 0.05$). Therefore, the highest reducing power was related to the hydrolysate sample prepared by 5 h hydrolysis using alcalase. The reducing power method is the most effective method for determining the ability of antioxidants to donate electrons. The higher reducing power of hydrolysates compared to the non-hydrolyzed proteins may be due to the fact that enzymatic hydrolysis produces peptides that have more available hydrogen ions, which can increase their antioxidant activity (Kong & Xiong, 2006). These results were consistent with the results of Cumby et al. (2008) who reported that the reducing power of canola protein was significantly ($p < 0.05$) increased by enzymatic hydrolysis. In accordance with our findings, Moghadam et al. (2020) reported that the reducing power of walnut proteins was significantly improved through the trypsin-mediated hydrolysis. This higher reducing power was attributed to the increased usability of hydrogen ions obtained using smaller peptides as compared to the non-hydrolyzed protein.

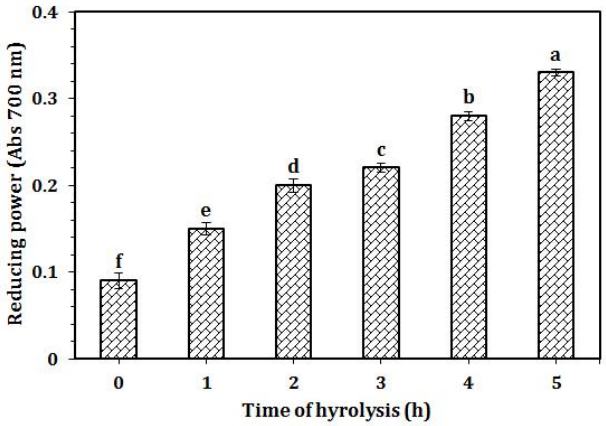

Fig. 2. Effect of hydrolysis on reducing power of WPI. Means followed by different letters are significantly different ($p < 0.05$).

3.3. DPPH free radical scavenging activity

The DPPH radical scavenging activity of WPI and its enzymatic hydrolysis is shown in Fig. 3. The results showed that the alcalase-mediated hydrolysis significantly increased the DPPH radical scavenging activity of WPI ($p < 0.05$). Moreover, the results indicated that the hydrolysates prepared at higher hydrolysis time had a higher ability to scavenge the free radicals of DPPH. The highest DPPH radical scavenging activity was related to the hydrolysates formed at 5 h hydrolysis time. This result suggested that the smaller peptides had a higher ability to scavenge the DPPH free radicals which makes them very interesting for being used as natural antioxidant in food formulations. In accordance with our findings, Chang et al. (2007) also hydrolyzed the porcine hemoglobin with different enzymes and reported that the highest

DPPH radical scavenging activity was related to the hydrolysates released by using alcalase enzyme. Mohammadian and Madadlou (2016) also hydrolyzed the WPI with Corolase N. They used the DPPH radical scavenging activity test to follow up the antioxidant activity of whey protein solution during hydrolysis reaction. Their results showed that the whey protein hydrolysates had higher DPPH radical scavenging activity than native WPI at any time of the hydrolysis reaction. The increased radical-scavenging activity of hydrolyzed WPI was likely to result from structural changes in the protein, leading to a greater radical quenching ability (Peng et al., 2010).

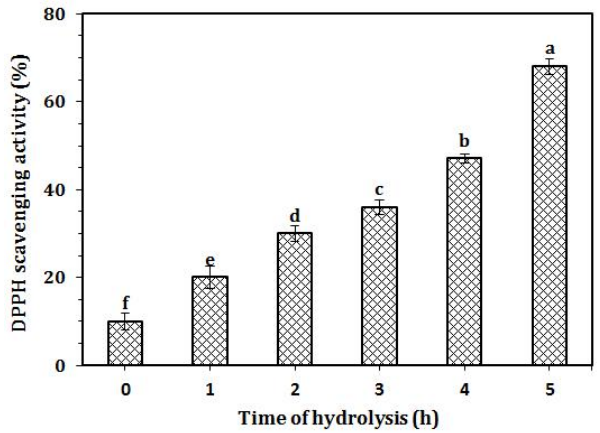

Fig. 3. Effect of hydrolysis on DPPH radical scavenging activity of WPI. Means followed by different letters are significantly different ($p < 0.05$).

3.4. *ABTS scavenging activity*

The results of ABTS free radical scavenging activity are shown in Fig. 4. ABTS de-colorization assay is a good method to measure the *in vitro* antioxidant activity. In the ABTS radical scavenging assay (an electron transfer-based assay), the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$), which has a dark blue color, is reduced by an antioxidant into colorless ABTS, which can be monitored spectrophotometrically. In this assay, the water soluble ABTS radical can react easily with antioxidants by transferring protons (Re et al., 1999; Ilyasov et al., 2020). The results showed that the scavenging activity of whey protein was significantly increased by alcalase-mediated hydrolysis ($p < 0.05$). The results also showed that the highest ABTS free radical scavenging activity was related to hydrolysates formed after 5 h of enzymatic hydrolysis and with increasing of hydrolysis time, ABTS free radical scavenging activity was also increased. In fact, the results indicated that the peptides produced by enzymatic hydrolysis had a higher ability to scavenge ABTS radicals compared to the native non-hydrolyzed WPI. Increased ability to inhibit ABS free radicals by hydrolysis can be due to the structural changes in whey protein that can increase its ability to scavenge radicals. Degradation of whey protein structure by enzymatic hydrolysis can unfold the protein structure and expose amino acids that have a greater ability to donate electrons and can react with ABTS free radicals and convert them to more stable products and terminate the free radical chain reaction (Peng et al., 2010). In accordance with our findings, Moghadam et al. (2020) reported that the ABTS radical scavenging activity of walnut proteins was significantly improved through the enzymatic hydrolysis using trypsin. In another study conducted by Ee et al. (2019) also it was reported that the enzymatic hydrolysis of protein from roasted butterfly pea seeds using bromelain and trypsin, significantly improved the ABTS free radical scavenging activity. Generally, these results showed that the WPI hydrolysates formed by alcalase-mediated hydrolysis can be considered as promising natural antioxidant agents owing to their high reducing power as well as their excellent ability to scavenge the free radicals of ABTS and DPPH.

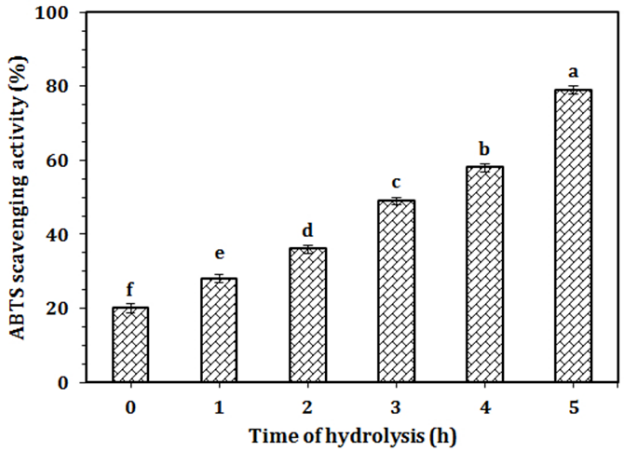

Fig. 4. Effect of hydrolysis on ABTS radical scavenging activity of WPI. Means followed by different letters are significantly different ($p < 0.05$).

4. Conclusion

In this study, alcalase enzyme was used to hydrolyze WPI to release antioxidant hydrolysates. The results generally showed that the enzymatic hydrolysis using alcalase increased the antioxidant activity of WPI as measured by different methods including reducing power assay, DPPH radical scavenging activity test, and ABTS free radical scavenging activity. Experiments have also suggested that the antioxidant activity of the hydrolysates produced may be due to various mechanisms such as radical stabilization as well as hydrogen donation. Therefore, our study showed that the whey protein antioxidant hydrolysates formed by enzymatic hydrolysis can be used as natural antioxidant agents in different food formulation to produce functional foods with health-promoting attributes. For example, whey protein hydrolysates also seems to provide a practical solution to a production of, e.g., processed meat with less risk for oxidative damage in the product and during digestion, since whey protein antioxidant hydrolysates have the ability to reduce radical formation by several mechanisms. However, it seems that more comprehensive studies are still needed to examine the amino acid sequence of the peptides produced in this study. Also, the effects of using these antioxidant compounds on the sensory and physical properties of food products should be thoroughly investigated in order to be desirable from the consumer's point of view.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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