Antioxidant Activity of Casein Hydrolysate

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Abstract

Casein hydrolysate, obtained by the hydrolysis of casein under suitable conditions, contains bioactive casein peptides which have positive effects on health. This study prepared high antioxidant activity casein hydrolysate using alcalase and trypsin. Antioxidant activity of the casein hydrolysate samples was evaluated through DPPH and ABTS radical scavenging activity. The effect of hydrolysis time on the degree of hydrolysis was also investigated. Free amino acid levels increased with hydrolysis time with 8.18 g/100g at the beginning and 60.92 g/100g of casein hydrolysate at 180 min.Casein hydrolysate obtained from trypsin hydrolysis at 180 min indicated highest antioxidant activity using DPPH radical scavenging activity, expressed as ascorbic acid (VC) equivalent and Trolox equivalent with the activity of 33.7 mg VC equivalent/ml and 38.8 mg Trolox equivalent/ml, respectively. Casein hydrolysate obtained from alcalase hydrolysis indicated lower activity than from trypsin at the same hydrolysis time. Antioxidant activity using ABTS scavenging assay indicated that casein hydrolysate obtained from trypsin hydrolysis at 180 min had the strongest antioxidant activity at 38.9 mg VC equivalent/ml and 38.8 mg Trolox equivalent/ml. Results demonstrated that hydrolysis time influenced antioxidant activity, with longer hydrolysis time producing significantly higher antioxidant activity.

Keywords: Casein, casein hydrolysate, bioactive peptide, antioxidant, degree of hydrolysis

Introduction

Introduction

Milk is a good source of well-balanced nutrients and demonstrates a wide range of biological activities that influence the physiological systems of the human body. These biological activities are mainly due to the peptides and proteins in milk. Milk contains approximately 3.5% protein with casein and whey proteins as the two major sources. Casein comprises about 80% of the total protein content in cow milk and 20% from whey protein. Recent research has shown that milk proteins yield bioactive peptides which defines as specific protein fragments, that have a positive impact on physiology of human or animals, thereby improving their health [1]. Milk-derived bioactive peptides have been identified as potential ingredients of health promoting foods. Beneficial health effects are classified as antimicrobial, antioxidative, antithrombotic, antihypertensive, antimicrobial or immunomodulatory [2, 3]. However, some of the biological activity of milk protein components is latent, and is released only upon proteolytic action. Bioactive peptides are inactive within the sequence of the parent protein and can be released in three ways: (a) enzymatic hydrolysis by digestive enzymes, (b) food processing, and (c) proteolysis by enzymes derived from microorganisms or plants [3]. Milk-derived bioactive peptides are usually comprised of 2–20 amino acids and become active after release from the precursor protein where they are encrypted either by digestion or proteolysis [4]. Many of the identified bioactive peptides were produced *in vitro* using gastrointestinal enzymes such as pepsin and trypsin. Other enzymes were applied to generate bioactive peptides from various protein sources including alcalase, chymotrypsin, pancreatin, and thermolysin, enzymes from bacterial and fungal sources. However, few studies have investigated the effect of enzymes on the antioxidant activity of casein hydrolysate. Therefore, this research was conducted to find the optimum conditions to yield high levels of bioactive peptides.

Materials and methods

Materials

Raw milk was obtained from Kokko cow milk cooperation, Maha Sarakham, Thailand. Skimmed milk powder was purchased from Fonterra Co. Ltd., New Zealand. Trypsin (pig stomach, 10,000 U/mg) and alcalase (*Bacillus licheniformis*,10,000 U/mg) were purchased from *Sigma* Chemical *Co.* (St. Louis, MO, USA). 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) and

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^+$) were purchased from Fluka Chemical Co. (Fluka AG, Buchs, CH-9470, Switzerland) and sodium tetraborate was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of casein and casein hydrolysate

Casein was prepared from skim milk powder (0% fat).The casein was precipitated by adjusting the pH to 4.6 using 2M lactic acid. The precipitated casein was then separated by filtration through cheesecloth. The whey protein was discarded and the casein was adjusted to pH 7 with sodium hydroxide and used to prepare casein hydrolysate.

To prepare casein hydrolysate using trypsin, 3 ml of trypsin solution (concentration 10,000 units/mg) was added to 15ml of crude casein (casein concentration 11 mg/ml) and incubated at 37°C for 30, 60, 90, 120, 150, 180, and 240 min. After incubation, trypsin activity was inhibited by boiling in water for 10 min, and then cooling down to 20°C. A sample from each time period was then added with distilled water at the ratio (10: 2 g). The pH was adjusted to 4 by 1M HCl, incubated at 45°C for 10 min and then the pH was adjusted to 7 with NaOH. The sample was centrifuged at 9000 rpm for 10 min to remove the water and the residue was stored at -20 °C until required for use.

To prepare casein hydrolysate using alcalase, alcalase enzyme was added to casein solution in the ratio of 0.1: 100w/v. Each sample was hydrolyzed for 0, 30, 60, 90, 120, 150, and 180 min, respectively. Each hydrolysis time was stopped by boiling in water for 10 min before cooling to room temperature and following the same procedure as for trypsin hydrolysis.

Degree of hydrolysis (DH)

The degree of hydrolysis of casein hydrolysate was determined using the *o*-phthaldialdehyde (OPA) method [5]. The measurement was performed by mixing the casein hydrolysate sample with OPA reagent (50 µl : 1 ml), mixed and kept for 2 min at 25°C. The mixed sample was then measured at 340 nm using a spectrophotometer (Shimadzu, Japan). The degree of hydrolysis (DH) was calculated using the following equation:

$$\mathrm{DH}\ (\%) = \frac{[P_t - P_o]}{[P_{max} - P_o]} \times 100$$

where P_t is the absorbance of the sample hydrolysate, P_o is the absorbance of the undigested sample (control), and P_{max} is the absorbance of the sample digested with acid.

Chemical compositions of casein hydrolysate

Casein hydrolysate was analyzed for protein, ash, and moisture content according to AOAC [6] official method.

Analysis of free amino acid

Free amino acid was determined following the Ninhydrin method described by AOAC [6] official method. The samples were measured at 570 nm by a spectrophotometer (Shimadzu, Japan).The leucine standard was used at different concentrations. Free amino acid was calculated as:

$$Free\ amino\ acid\ (\%) = \frac{[L_t - L_o]}{[L_{max} - Lo]} \times\ 100$$

where L_t is the amino acid content of the sample, $L_o$is the undigested amino acid content of the sample, and L_{max} is the amino acid content of the digested sample.

DPPH radical scavenging assay

The free radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was evaluated as described by AOAC official method [6]. Stock solution was prepared by mixing 0.004g of DPPH in 100 ml of methanol. The mixed sample was measured at 517 nm using a spectrophotometer (Shimadzu, Japan). The percentage of DPPH scavenging activity was calculated and the inhibition rates were expressed as vitamin C (VC) and Trolox equivalent.

$$DPPH\ Inhibition\ (\%) = [\frac{(A_o - A_1)}{A_o}]\ \mathrm{x}\ 100$$

where A_0 is the absorbance without extract, and A_1 is the absorbance with extract.

$ABTS^+$radical scavenging capacity assay

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^+$) radical scavenging capacity was determined following the method of Alyaqoubi et al. [7]. The mixed sample was measured for absorbance at 734 nm using a spectrophotometer (Shimadzu, Japan). Ascorbic acid (VC) and Trolox were used as standards at different concentrations. The inhibition of $ABTS^+$ radical was calculated from the equation:

$$ABTS\ Inhibition\ (\%) = [\frac{(A_o - A_1)}{A_o}] \text{ x } 100$$

where A_o is the absorbance without extract,and A_1 is the absorbance with extract.

Results and discussion

Degree of casein hydrolysis

The degree of hydrolysis of casein for 30, 60, 90, 120, 150, 180, and 240 min using trypsin is shown in Figure 1. The degree of hydrolysis (%) increased as the hydrolysis time increased. The degree of hydrolysis was 47.32, 54.07, 59.69, 64.20, 69.60, 72.18, and 89.94% at 30, 60, 90, 120, 150, 180, and 240 min, respectively. These results were similar to Okoh et al. [8] who determined that the degree of hydrolysis increased with hydrolysis time from 73.3% at 0.5 h to 100% after 4 h.

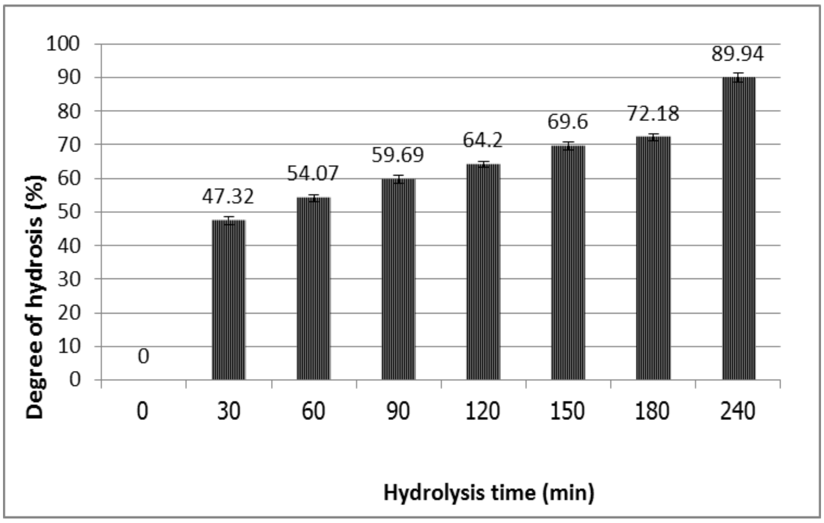

Figure1. Degree of casein hydrolysis (%) using trypsin enzyme at different times

Chemical compositions of casein and casein hydrolysate

The chemical compositions of casein contained crude casein (7.87%), higher than that of casein hydrolysate hydrolyzed for 180 min (3.58%). However, moisture and ash contents of casein hydrolysate were higher than crude casein at 7.96% and 21.54% and 6.45% and 17.37%, respectively.

Change in free amino acids during enzymatic hydrolysis of casein protein

The total free amino acid contents of the casein hydrolysate with different degrees of hydrolysis using trypsin are shown in Figure 2. The hydrolysis time significantly affected the concentration of amino acid. The content of free amino acid was lowest in casein without hydrolysis (8.18 g/100g), whereas the highest level was observed in casein hydrolyzed for 240 min (90.23 g/100g). The increase in the free amino acids in the hydrolysate may be due to enzymatic hydrolysis of peptides from casein. These results were similar to Wang et al. [9] who studied the characterization of casein hydrolysate using enzyme hydrolysis. They reported that total free amino acid increased with increasing hydrolysis time, which indicated that increasing hydrolysis time affected peptide hydrolysis to obtain free amino acid.

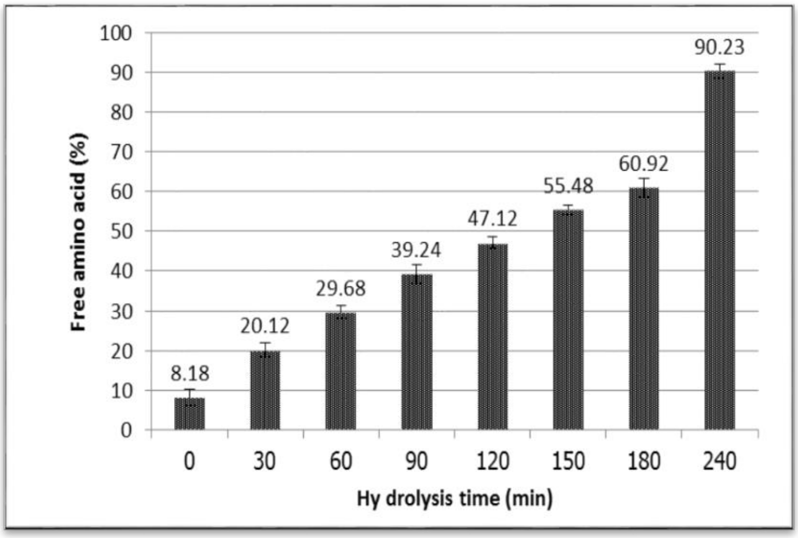

Figure 2. Total amino acid in casein hydrolysate hydrolyzed at different times

Effect of hydrolysis time on antioxidant activity of casein hydrolysate

Results of antioxidant activity assay using DPPH radical scavenging expressed as DPPH radical inhibition rate (%) are shown in Figure 3A. The highest antioxidant activity was observed in casein hydrolysate hydrolyzed using trypsin enzyme for 180 min (53.43%) and remained constant at 240 min. The lowest activity was found in casein without hydrolysis (24.00%). These results agreed with Mao et al. [10] who found that DPPH radical scavenging increased from the start to 7h hydrolysis time and then decreased.

DPPH radical scavenging of casein hydrolysate was also expressed as VC and Trolox equivalent (Table 1). Antioxidant activity was significantly different ($p<0.05$) for different hydrolysis times. The highest DPPH radical scavenging was found in hydrolysate obtained from 180 and 240 min. The ascorbic acid (VC) equivalent was 33.70 µg /ml and 33.30 µg /ml, and Trolox equivalent was 38.80 µg / ml and 38.40 µg / ml for 180 and 240 min, respectively. The lowest radical scavenging was found in casein without hydrolysis. These results were similar to Kumar et al. [11] who studied casein hydrolysate from camel milk using enzymatic and antioxidant activity. Their results exhibited that DPPH radical scavenging significantly increased ($p<0.05$) with increasing hydrolysis time and degree of hydrolysis.

Antioxidant activity using $ABTS^{+}$ radical scavenging capacity assay (expressed as ABTS radical inhibition rate (%)) is shown in Figure 3B and Table 1. The highest $ABTS^{+}$ radical inhibition was observed at hydrolysis time of 180 min (50.61 %) and the lowest inhibition in casein without hydrolysis (8.69%).Hydrolysis time significantly influenced $ABTS^{+}$ radical scavenging capacity of casein hydrolysate ($p<0.05$). The highest radical scavenging was found at hydrolysis time of 180 min (Table 1). The highest activity was found in hydrolysate hydrolyzed at 180 and 240 (38.90 mgVC equivalent /ml and 32.90 mg Trolox equivalent / ml, respectively). From these results, casein hydrolysate for the next experiments was set at 180 min.

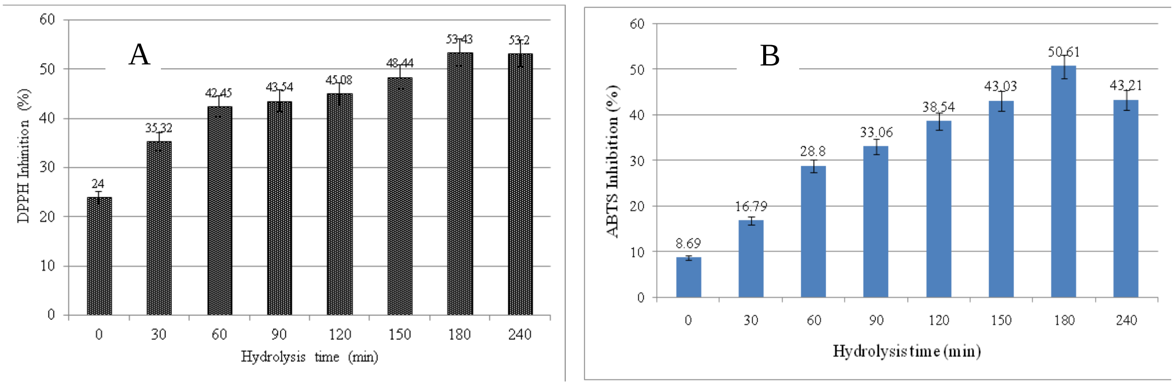

Figure 3. DPPH (A) and ABTS (B) Inhibition (%) of casein hydrolysate at different hydrolysis times

Table 1. Comparison of antioxidant activity of casein hydrolysate at different hydrolysis times by DPPH and ABTS radical scavenging assay

Time (min)	**DPPH**		**ABTS**	
	Vitamin C Equivalent (µg/ml)	**Trolox Equivalent (µg/ml)**	**Vitamin C Equivalent (µg/ml)**	**Trolox Equivalent (µg/ml)**
0	8.00 ± 0.2^{f}	9.70 ± 0.1^{f}	3.50 ± 0.4^{g}	3.74 ± 0.5^{g}
30	13.90 ± 0.1^{e}	16.50 ± 0.2^{e}	9.00 ± 1.1^{f}	9.50 ± 1.0^{f}
60	19.70 ± 0.1^{d}	23.10 ± 0.9^{d}	15.10 ± 0.4^{e}	14.80 ± 0.3^{e}
90	20.80 ± 0.6^{d}	24.40 ± 0.7^{d}	18.20 ± 0.6^{d}	17.30 ± 0.5^{d}
120	22.40 ± 0.8^{c}	26.20 ± 0.9^{c}	23.30 ± 0.4^{c}	21.30 ± 0.4^{c}
150	26.40 ± 0.7^{b}	30.70 ± 0.7^{b}	28.00 ± 0.8^{b}	24.90 ± 0.6^{b}
180	33.70 ± 1.0^{a}	38.80 ± 1.8^{a}	38.90 ± 1.2^{a}	32.90 ± 5.2^{a}
240	33.30 ± 0.7^{a}	38.40 ± 0.8^{a}	28.30 ± 0.8^{b}	25.10 ± 0.6^{b}

Means within a row with different superscript letters (a, b, c,…) are different($p<0.05$) (n=3)

Effect of enzymes (alcalase and trypsin) on antioxidant activity of casein hydrolysate

The comparison of DPPH radical scavenging of casein hydrolysate using alcalase and trypsin enzymes at different hydrolysis times is shown in Figure 4. Results indicated that the highest radical scavenging was for hydrolysis time at 180 min. The highest inhibitions were 54.04% and 51.29% by trypsin and alcalase hydrolysis, respectively. Non-hydrolyzed casein had the lowest inhibition at 24.0% and 24.16% by trypsin and alcalase hydrolysis, respectively. Types of enzymes affected the antioxidant activity of casein hydrolysis significantly ($p<0.05$). Casein hydrolysate from alcalase hydrolysis showed higher antioxidant activity than trypsin hydrolysis from 30 to 150 min, while trypsin was better at 180 min.

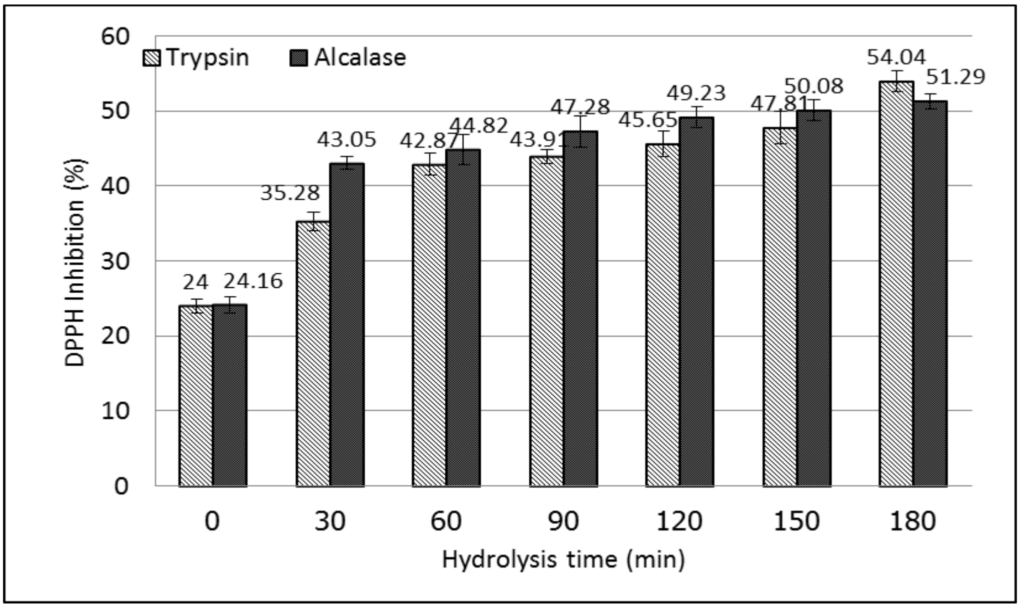

Figure 4. Antioxidant activity of casein hydrolysate by alcalase and trypsin hydrolysis at different hydrolysis times using DPPH radical scavenging assay

The antioxidant activity by DPPH radical scavenging assay indicated that DPPH inhibition of casein hydrolysate from both enzymes increased with increasing hydrolysis time (Figure 4).The highest inhibition was found at hydrolysis time of 180 min. However, casein hydrolysate by alcalase enzyme showed higher antioxidant activity than trypsin enzyme at lower hydrolysis times.

For analysis of antioxidant activity using ABTS, the antioxidant activity of casein hydrolysate obtained from the two enzymes with different hydrolysis times was compared. The antioxidant activity is shown in Table 2 and Figure 5. Results revealed that casein hydrolysate obtained from both trypsin and alcalase hydrolysis at 180 min indicated the highest antioxidant activity with inhibition rates of 50.26% and 49.69%, respectively. On the other hand, casein hydrolysate obtained from trypsin and alcalase hydrolysis for casein without enzyme hydrolysis indicated the lowest antioxidant activity at 8.45% and 10.22%, respectively.

Furthermore, casein hydrolysate obtained from alcalase hydrolysis showed better antioxidant activity than the hydrolysate from trypsin hydrolysis for 30-150 min, but after 180 min the antioxidant activities were similar.

Table 2. Comparison of antioxidant activity of casein hydrolysate by alcalase and trypsin (VC equivalent (µg/ml)

Hydrolysis time	**DPPH**		**ABTS**	
(min)	**Alcalase**	**Trypsin**	**Alcalase**	**Trypsin**
0	1.0±0.4^{fA}	0.7±0.6^{fA}	0.70±0.04^{fA}	0.8±0.06^{gA}
30	12.8±0.2^{eB}	13.9±0.1^{eA}	20.5±0.5^{eA}	9.0±1.1^{fB}
60	23.4±0.4^{dA}	19.7±0.1^{dB}	22.4±0.5^{deA}	15.1±0.4^{eB}
90	27.1±0.5^{dA}	20.8±0.6^{dA}	24.9±0.4^{dA}	18.2±0.6^{dA}
120	29.1±0.3^{cA}	22.4±0.8^{cB}	27.7±0.3^{cA}	23.3±0.4^{cB}
150	33.0±0.3^{bA}	26.4±0.7^{bB}	28.9±0.3^{bA}	28.0±0.8^{bA}
180	34.7±0.1^{aA}	37±1.0^{aB}	31.1±0.7^{aB}	38.9±1.2^{aA}

Means within a row with different superscript letters ($^{A, B}$) are different ($p<0.05$) (n=3)

Means within a column with different superscript letters ($^{a, b, c,\ldots}$) are different ($p<0.05$) (n=3)

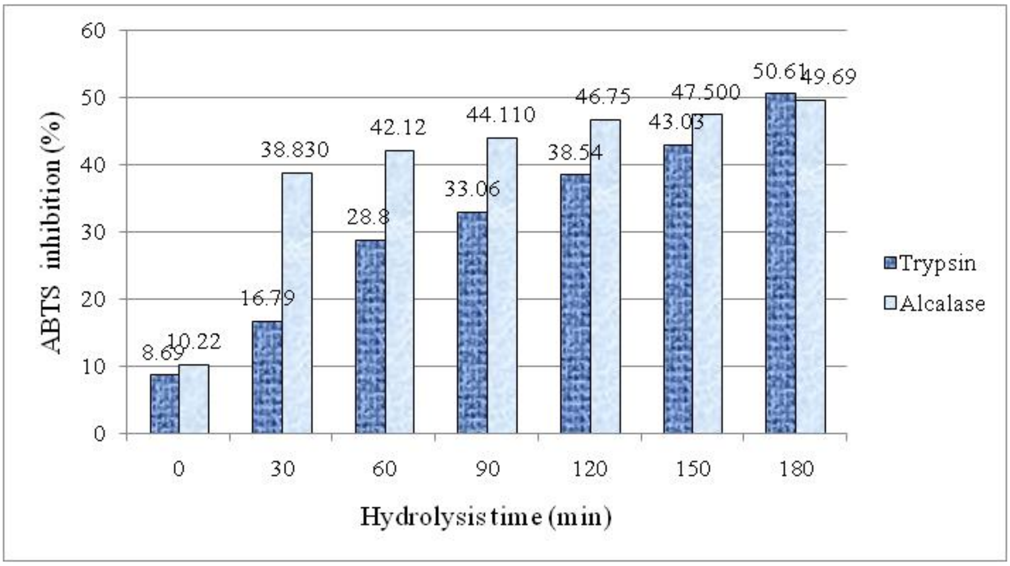

Figure 5. Comparison of antioxidant activities (ABTS radical assay) of casein hydrolysate obtained from alcalase and trypsin hydrolysis at different hydrolysis times

Conclusions

Hydrolysis time influenced the degree of hydrolysis of casein hydrolysate. Hydrolysis significantly increased with time with the highest at 90.23% at 240 min. Results demonstrated that the type of enzyme and duration of hydrolysis affected the antioxidant activity of casein hydrolysis. The highest antioxidant activity was found in hydrolysate obtained from trypsin at 180 min. The total free amino acid content of casein hydrolysate also increased with hydrolysis time. However, other assays for antioxidant activity evaluation and the bioactive peptides derived from casein during hydrolysis are needed for further investigation.

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References

[1] H. Korhonen, and A. Pihlanto. Bioactive peptides: production and functionality. *Int Dairy J*. 16 (2006) 945–60.

[2] R.J. FitzGerald, and H. Meisel. Milk protein hydrolysates and bioactive peptides. *Adv. Dairy Chem*. 3 (2003) 675–698.

[3] H. Korhonen, and A. Pihlanto. Food-derived bioactive peptides opportunities for designing future foods. *Curr. Pharm. Des*. 9 (2003) 1297–1308.

[4] H. Meisel, and R.J. FitzGerald. Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Curr. Pharm. Des*. 9 (2003) 1289–1295.

[5] I. Irshad , A. Kanekanian , A. Peters , and T. Masud. Antioxidant activity of bioactive peptides derived from bovine casein hydrolysate fractions. *J Food Sci Technol*. 52 (2015) 231–239.

[6] AOAC. Official method of analysis association of AOAC international.16th ed. Maryland: USA. (2000).

[7] S. Alyaqoubi, A. Abdullah, M. Samudi, N. Abdullah, Z.R. Addai, and M. Al-ghazali. Effect of different factors on goat milk antioxidant activity. *Int J Chem Tech Res* 6 (2014) 3091-3196.

[8] S.O. Okoh, O.T. Asekun , O.B. Familoni, and A.J. Afolayan. Antioxidant and free radical scavenging capacity of seed and shell essential oils extracted from *Abrus Precatorius* (L). *Antioxidants*. 3 (2014) 278-287.

[9] J. Wang , Y. Su , F. Jia , and H. Jin. Characterization of casein hydrolysates derived from enzymatic hydrolysis. *Chem Cent J*. 7 (2013) 1-8.

[10] X.Y. Mao, X. Cheng, X. Wang, and S.J. Wu. Free-radical-scavenging and anti-inflammatory effect of yak milk casein before and after enzymatic hydrolysis. *Food Chem*. 126 (2011) 484–490.

[11] D. Kumar, M.K. Chatli, R. Singh, N. Mehta, and P. Kumar. Enzymatic hydrolysis of camel milk casein and its antioxidant properties. *Dairy Sci. & Technol*. 96 (2016) 391–404.