Journal of Food Science and Agricultural Technology

International peer-reviewed scientific online journal

Published online: http://rs.mfu.ac.th/ojs/index.php/jfat

## Original Research Article

# Effect of protein hydrolysis on physical properties and antioxidant activities of cow's milk

***Sasiprapa Vanitcharoen[1], Nattapol Tangsuphoom[1*], Uthaiwan Suthisansanee[2], Chalat Santivarangkana[3]***

*[1]Food Science Unit, Institute of Nutrition, Mahidol University, Salaya, Nakhon Pathom 73170, Thailand*
*[2]Human Nutrition Unit, Institute of Nutrition, Mahidol University, Salaya, Nakhon Pathom 73170, Thailand*
*[3]Center of Innovation and Reference on Food for Nutrition, Institute of Nutrition, Mahidol University, Salaya, Nakhon Pathom 73170, Thailand*

**ARTICLE INFO**

*Article history:*
Received 31 July 2018
Received in revised form 30 September 2018
Accepted 02 October 2018

*Keywords:*
Antioxidant activity
Enzymatic hydrolysis
Hydrolyzed milk protein
Milk
Protease

**ABSTRACT**

Milk is an excellent source of nutrients, especially protein. Milk protein hydrolysates and peptides are known for their health benefits, particularly antioxidative effects. The aim of this study is to determine the physical properties and antioxidant activities of cow's milk subjected to enzymatic hydrolysis. Pasteurized skim milk (3.5% protein) was hydrolyzed by adding Alcalase 2.4L, Protamex 1.5MG, Flavourzyme 1000L or porcine trypsin at the enzyme-to-substrate ratios of 0.1-1% (w/w). Hydrolysis was conducted for ≤8 h at refrigerated (4ºC), and enzyme's optimal temperatures (50ºC, except 37ºC for trypsin) prior to heating at 72ºC for 3 min and immediate cooling to inactivate the enzyme. Progress of hydrolysis was monitored by determining the degree of hydrolysis (DH). Color values (L*, a*, b*), total color difference against non-hydrolyzed milk (ΔE*), pH, and physical stability of the hydrolyzed samples were measured. Antioxidant activities were determined as ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC). DH of hydrolyzed milk increased with enzyme concentration, hydrolysis temperature, and time. The highest DH of 25% was observed in samples hydrolyzed with Alcalase, followed by Flavourzyme, Protamex and trypsin. L*, a* and pH values of milk were unaffected by hydrolysis. Protein hydrolysis of milk led to the increase in b* and the samples prepared with trypsin showed the highest ΔE*. Hydrolysis with Alcalase or trypsin resulted in phase separation of the milk, especially at high DH. Only Protamex- and Flavourzyme-hydrolyzed milk with 10-20% DH, in which separation was absent, were analyzed for antioxidant activities. FRAP and ORAC of non-hydrolyzed milk were 17.71 and 177.02 μmol TE/ml, respectively and the values increased with DH. At any similar DH, Protamex-hydrolyzed milk showed higher FRAP than those hydrolyzed with Flavourzyme, which had higher ORAC value. The highest FRAP (25.23 μmol TE/ml) and ORAC (262.65 μmol TE/ml) were obtained by hydrolysis with 0.5% Protamex at 50ºC for 30 min and 1% Flavourzyme at 50ºC for 90 min, respectively. Our findings indicated that enzymatic hydrolysis of protein under suitable condition could slightly improve the antioxidant activities of cow's milk, while causing minimal changes in physico-chemical characteristics as compared with non-hydrolyzed milk.



* *Corresponding author*: Tel.: +66-2800-2380 Ext. 413; Fax: +66-2441-9344.
E-mail address: nattapol.tng@mahidol.ac.th

Published by School of Agro-Industry, Mae Fah Luang University

ISSN: 2408-1736

## INTRODUCTION

Milk, especially from cow, is generally consumed by billions of people around the world as a food source that is rich in essential nutrients. Milk is considered to be an excellent source of essential amino acids for human nutrition, growth and development (Kanwar et al., 2009). Whole milk contains approximately 3.5 g protein in 100 g of which 80% is casein and another 20% is whey proteins. Other significant nutrients in whole milk include fat, lactose and calcium, which account for 3.3 g, 4.7 g and 119 mg/100 g, respectively (FAO, 2013).

Hydrolysis of protein is known to increase the nutritional value, e.g., digestibility and bioavailability, as well as enhance the functional properties of protein, e.g., solubility, foaming and emulsifying properties, mainly in heat-denatured proteins (Yongqing et al., 2017). Protein hydrolysates have been reported to have numerous health benefits, including antioxidative, antihypertensive, antibacterial, mineral-binding, antithrombotic, and anti-gastric activities (Gowda et al., 2006 and Prieto et al., 2014). The functional properties and effects on health of hydrolysates and peptides depend largely on their molecular weight (MW), as well as the inherent amino acid composition and sequence (Deeslie and Cheryan, 1992). In general, hydrolysates with lower MW ranges had better antioxidant activity and solubility than the native proteins (Li et al., 2013). Enzymatic hydrolysis is commonly used in the modification of protein structure by which the peptide bonds of protein are cleaved, resulting in small peptides and amino acids with improved chemical, physico-chemical, and biochemical properties, comparing with non-hydrolyzed proteins (Klompong et al., 2007). In such production of protein hydrolysates, appropriate type and amount proteolytic enzymes and hydrolysis conditions, in particular pH, temperature and time, are needed to be optimized in order to achieve the hydrolysates with desired properties and activities.

Milk peptides are derived from milk proteins by enzymatic breakdown using digestive enzymes or proteinase enzymes produced by *Lactobacilli* during the fermentation of milk (Jauhiainen and Korpela, 2007). Hydrolyzed milk proteins have functional characteristics that are potentiated and modulated by hydrolysis, which gives them advantages for use in various products in the food industry. Hydrolysis also results in a reduction in allergenic potential of milk proteins by the destruction of allergenic epitopes. Peptides containing 2-20 amino acids has been reported to be antihypertension and immunogenicity (Korhonen and Marnila, 2006). The fractions with MW between 3-5 kDa have also been reported to exhibit better antioxidant activity than other MW ranges. Some studies reported that casein hydrolysates of <1 kDa hydrolyzed by Alcalase had higher antioxidant activity than that produced by trypsin. In addition, fractionation of hydrolysates with MW <1 kDa could increase the antioxidant properties, especially that consists of hydrophobic and specific hydrophilic amino acids (Ahemd et al., 2017). The most biologically potent peptides are commonly short in length, comprising of 2-9 amino acids, and possessing MW <3 kDa (Hayes, 2013). So, milk protein hydrolysates have a variety of applications as functional food ingredients (Chalamaiah et al., 2012). However, bitterness is a negative attribute associated with most food protein hydrolysates and has been classically associated with the release of peptides containing hydrophobic amino acid residues (Ney, 1971). It has been reported that the MW of hydrolysates should be smaller than 1 kDa in order to avoid bitterness (Gonzalez et al., 1994). Although there are previous studies on the production of milk protein hydrolysates and their applications in food and beverage, the enzymatic hydrolysis was performed in the solution of milk protein concentrate/isolate rather than in situ by directly adding the enzyme into milk.

Therefore, this study aimed to determine the physical properties and antioxidant activities of cow's milk subjected to protein hydrolysis using commercial protease enzymes. The obtained product is expected to have superior nutritional value and biochemical properties than native cow's milk; and thus may be an alternative way to utilize substandard raw milk.

## MATERIALS AND METHODS

### Materials

Pasteurized non-fat milk (3.5% w/w protein) was obtained from Dairy Plus (Bangkok, Thailand) and kept refrigerated at 4±2°C until being used. Protease enzymes, namely Alcalase 2.4L, Protamex 1.5 MG and Flavourzyme 1000L were donated from Brenntag Ingredients (Bangkok, Thailand). Trypsin from porcine pancreas, and all chemicals, unless stated otherwise, were obtained from Sigma-Aldrich (St. Louis, Missouri, U.S.A.).

### Hydrolysis of cow's milk by protease enzyme

Appropriate amount of Alcalase 2.4L, Protamex 1.5MG, Flavourzyme 1000L or porcine trypsin was added to milk at the enzyme-to-substrate (E:S) ratios of 0.1-1% w/w. Hydrolysis was conducted at native pH of milk (6.6-6.8) for up to 8 h at enzyme's optimal temperatures (50ºC, except 37ºC for trypsin) in a water bath or at 4±2°C in a refrigerator. Hydrolysate fractions were taken at different hydrolysis time intervals, prior to heating at 72ºC for 3 min and immediate cooling to inactivate the enzyme. The hydrolyzed milk samples were transferred to glass vials and properly kept, either refrigerated at 4±2°C or frozen at -20°C, for further analyses.

### Analyses

#### *Degree of hydrolysis (DH)*

DH of hydrolyzed milk was determined by TNBS method (McKellar, 1981). Prior to analysis, the milk sample was clarified according to the method described by Chove et al. (2013) with modifications. Milk samples was heated at 100°C for 10 min to denature the whey proteins. Then, 50 ml warm water (40°C) was added to 5 ml milk. The pH of the mixture was adjusted to be 4.6 using 0.5 ml of 10% (w/v) acetic acid in order to precipitate the proteins. After standing for 10 min, 0.5 ml of 1 M sodium acetate was added and the sample was placed in ice-cold water for 10 min before removing the precipitate by filtration through filter paper (Whatman No. 41). The precipitate was washed by deionized water and the volume of filtrate was made up to 100 ml. The obtained clear extract was further filtered by 0.2 µm Millipore filter before being analyzed using TNBS method.

For TNBS method, triplicate samples of pH 4.6 soluble extracts (0.2 ml) was mixed with 2 ml of 1 M potassium borate buffer pH 9.2 and 0.8 ml of 5 mM 2,4,6-trinitrobenzene sulfonic acid. After 30 min incubation at 25°C, 0.8 ml of 2 M $Na_2PO_4$ solution containing 18 mM $Na_2SO_3$, and 5 ml deionized water was added, then the absorbance at 420 nm was measured by using a microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, Vermont, U.S.A.). DH was calculated by the following equation:

$$DH\ (\%) = [(L_t - L_0) / (L_{max} - L_0)] \times 100$$

where $L_t$ is the absorbance of hydrolyzed milk sample, $L_0$ is the absorbance of non-hydrolyzed milk, and Lmax is the absorbance of milk hydrolyzed with 6 M HCl for 24 h at 95°C.

***pH and color***

The pH value of each fraction was measured using a pH meter (P25, Istek Inc., Korea) at 25°C. Color values (L*, a*, b*) of the hydrolyzed milk was measured using a spectrocolorimeter (ColorFlex EZ, Hunter Associates Laboratory, Reston, Virginia, U.S.A.). For this color system, L* represents lightness from 0-100, +a* is redness, –a* is greenness, +b* is yellowness and –b* is blueness. Total color difference (ΔE*) between each hydrolyzed sample and the control sample, i.e., non-hydrolyzed milk, was calculated as:

$$\Delta E^* = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2}$$

where L*0, a*0, and b*0 are color values of the control sample.

***Physical stability***

Physical stability was determined by observing the occurrence of cream separation and/or precipitation of hydrolyzed milk. The total height of the sample and the height of the serum layer or sediment was measured immediately after sample preparation, and calculated followed the equation:

$$Separation\ (\%) = \frac{Height\ of\ serum\ layer\ or\ sediment}{Total\ height\ of\ sample} \times 100$$

***Ferric reducing antioxidant power (FRAP)***

FRAP assay was performed by modifying the method of Benzie and Strain (1996). The FRAP reagent contains 300 mM acetate buffer pH 3.6, 10 mM 2,4,6-tripyridyl-strazine in 20 mM HCl and FeCl3•6H2O at the volume ratio of 10:1:1. Sample (20 µl) was mixed with 150 µl FRAP reagent in 96-well microplate and then incubated in the dark at room temperature for 8 min. The reaction was monitored using a microplate reader at 600 nm. The FRAP values was observed according to the standard curve of Trolox solution (7.8-250 µM) and calculated as FRAP value (µmol TE/ml sample).

***Oxygen radical absorbance capacity (ORAC)***

ORAC assay was performed by modifying the method of Ou et al. (2001). Aliquot (150 µl) of 3 nM fluorescein working solution and 25 µl of sample or 1,000 µM Trolox stock solution (3-100 µM) was dissolved in 75 mM phosphate buffer pH 7.4. The mixture was transferred into a 96-well microplate and incubated at 37°C for 15 min in every well. Then, 25 µl of 150 mM 2,2-azobis (2-amidinopropane) dihydrochloride was added to the microplate to start the reaction. The fluorescence intensity was monitored for 90 min using a microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The result was calculated by assessing the area under the fluorescence decay curve (AUC) in the unit of µmol Trolox equivalent (TE)/g sample. The AUC was calculated as:

$$AUC = 1 + (f_1/f_0) + (f_2/f_0) + (f_3/f_0) + (f_4/f_0) + \ldots + (f_{34}/f_0) + (f_{35}/f_0)$$

where $f_0$ is the initial fluorescence reading at 0 min and fi is the fluorescence reading at time i.

The data was analyzed by applying the above equation in a spreadsheet (Microsoft Office Excel 2013, Microsoft, Redmond, Washington, U.S.A.) to calculate the AUC. The net AUC was obtained by subtracting the AUC of the blank from that of the sample. The relative ORAC value was calculated as:

$$Relative\ ORAC\ value = \left[\frac{(AUC_{Sample} - AUC_{Blank})}{(AUC_{Trolox} - AUC_{Blank})}\right] \times \left(\frac{Molarity\ of\ Trolox}{Molarity\ of\ sample}\right)$$

**Statistical analysis**

Experiment was conducted in Completely Randomized Design of triplicate samples prepared from 3 separate batches. One-way Analysis of Variance was used to evaluate the significant difference ($p \leq 0.05$) and the difference between means was compared using Duncan's Multiple Range test. Data analysis was conducted using a statistical software (IBM SPSS Statistics 19 for Windows, IBM, Armonk, New York, U.S.A.).

## RESULTS AND DISCUSSION

**Degree of hydrolysis (DH)**

DH can be used as an indicator for the comparison of different proteolytic processes (Panyam and Kilara, 1996). DH of the milk hydrolyzed with Alcalase 2.4L at E:S ratio of 0.1% w/w under refrigeration for 6 h gradually increased with hydrolysis time from 1.46 to 8.45% (Fig 1). When the higher concentration of enzyme, i.e., 0.5% E:S ratio, was used, the DH of hydrolyzed milk increased to reach its maximum of 19.09 % at 120 min of hydrolysis. Further extension of hydrolysis time up to 6 h did not increase the DH of the milk. Hoyle and Merritt (1994) revealed that alkaline protease like Alcalase exhibited higher activities than neutral and acid enzymes.

For Flavourzyme-treated milk samples, DH values slightly increased with hydrolysis time until 120 min (Fig. 2). Similar to hydrolyzed milk obtained from other enzymes, increasing enzyme concentration and hydrolysis temperature tended to increase the DH of obtained hydrolyzed milk. The highest DH of 16.35 % was obtained from hydrolysis of milk using 1% w/w Flavourzyme at 50ºC for 120 min.

The DH of hydrolyzed milk treated with Protamex 1.5MG at E:S ratio of 0.1% w/w at 4ºC slightly increased with hydrolysis time to 10.34% at 120 min and did not show any obvious increments at longer hydrolysis period (Fig. 3). Hydrolysis at optimum temperature of enzyme yielded hydrolyzed milk with higher DH at any similar hydrolysis time. The maximum DH of 15.45% was reached at 90 min of hydrolysis and decreased when the reaction was extended. An increase in E:S ratio to 0.5% w/w raised the DH of hydrolyzed milk at any similar hydrolysis time and temperature. Surprisingly, the DH of milk hydrolyzed with 0.5% w/w Protamex at 4 and 50ºC were somewhat similar.

Milks hydrolyzed with trypsin at E:S ratio of 0.1% w/w up to 8 h at both temperatures showed DH <10% (Fig. 4). Hydrolysis using trypsin at 4ºC did not change the DH of milk at any length of reaction. At 37ºC, which is the optimal temperature for trypsin, the DH increased with hydrolysis time until the highest DH of 7.43 % was reached at 60 min and remained unchanged when the hydrolysis was prolonged to 8 h.

In summary, DH of hydrolyzed milk increased with enzyme concentration, hydrolysis temperature, and time. The highest DH of 20% was observed in samples hydrolyzed with Alcalase, followed by Protamex, Flavourzyme and trypsin. Similar effect of hydrolysis condition on DH was also observed in previous study that the DH of protein hydrolysates reached its highest within 2 h of hydrolysis, and slightly decreased at longer time (Li-Chan and Nakai, 1990).

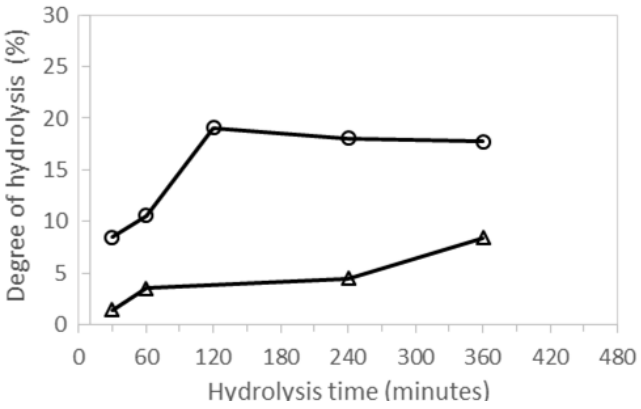


**Figure 1.** Degree of hydrolysis of milk hydrolyzed with Alcalase 2.4L at the enzyme-to-substrate ratios of (△) 0.1 and (○) 0.5% w/w at 4ºC

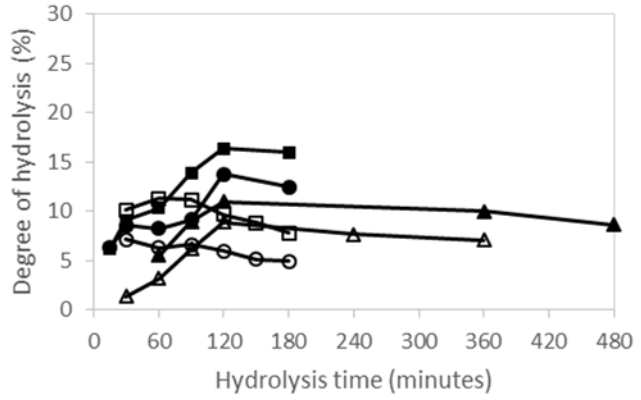


**Figure 2**. Degree of hydrolysis of milk hydrolyzed with Flavourzyme 1000L at the enzyme-to-substrate ratios of (△,▲) 0.1, (○,●) 0.5 and (□,■) 1% w/w at (△,○,□) 4 and (▲,●,■ *) 50ºC*

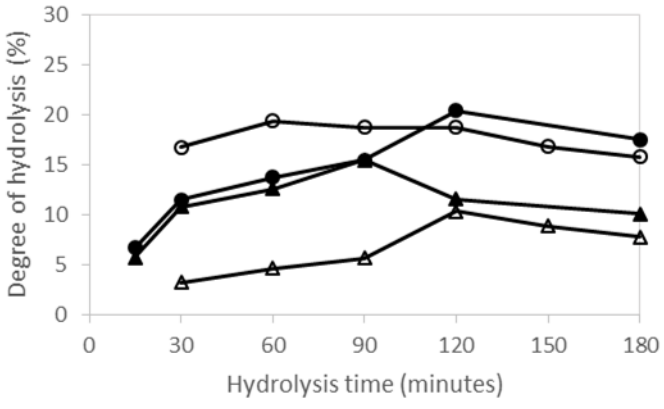


**Figure 3.** Degree of hydrolysis of milk hydrolyzed with Protamex 1.5 MG at the enzyme-to-substrate ratios of (△,▲) 0.1 and (○,●) 0.5% w/w at (△,○) 4 and (▲,●) 50ºC

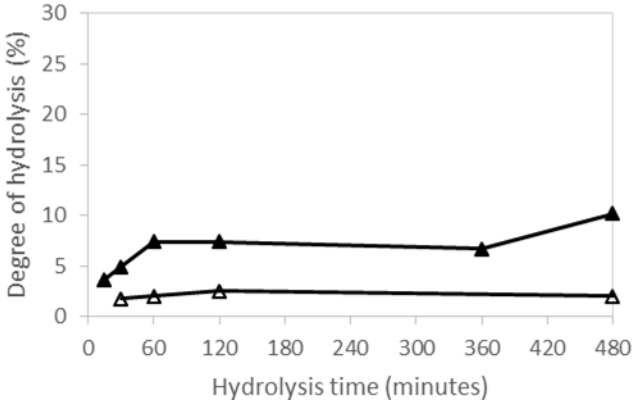


**Figure 4.** Degree of hydrolysis of milk hydrolyzed with trypsin at the enzyme-to-substrate ratios of 0.1% w/w at (△) 4 and (▲) 37ºC

**pH and color**

Hydrolysis by protease did not change the pH of hydrolyzed milk. The pH value of all samples ranged from 6.5 to 6.65, which were similar to the native pH of cow's milk. It was suggesting that even though the hydrolysis was prolonged to 6-8 h at temperature up to 50ºC, spoilage of milk did not take place.

The L*, a* and b* values of non-hydrolyzed milk were 51.98, -2.92 and 1.32, respectively. Depending on the type of protease, color values of the hydrolyzed milk samples were affected, to different extents, by enzyme concentration and hydrolysis time. Hydrolysis of protein in milk using Alcalase at any condition did not significantly change L* and a* values but significantly decreased the b* value of the milk, when comparing with non-hydrolyzed milk (Data not presented, available as Supplement Tables).

The color values of hydrolyzed milk prepared with Flavourzyme at any condition were similar to the non-hydrolyzed milk. For Protamex-hydrolyzed milk, L* and b* values did not change significantly from the non-hydrolyzed milk, regardless of the E:S ratio and hydrolysis time and temperature. On the other hand, a* value of samples hydrolyzed with any concentration of Protamex at 50ºC was significantly lower than that of non-hydrolyzed milk but the value increased with hydrolysis time. Hydrolysis using trypsin at E:S ratio of 0.1% w/w at 4 and 37ºC did not significantly affect L* and a* values but significantly increased b* value of the milk. This suggested that the hydrolyzed milk became yellower, especially when the length of hydrolysis was extended.

In summary, protein hydrolysis under all conditions in this study did not change lightness of the milk but tended to affect the yellowness, particularly those prepared with Alcalase and trypsin. Such change in color might arise from the change in microstructure of milk emulsion since the emulsifying properties of milk proteins, which is natural emulsifier in milk, was deteriorated by hydrolysis. When total color difference against non-hydrolyzed milk (ΔE*) was considered, it was found that the values ranged between 1.60 and 5.62. Milk samples treated with Flavourzyme had the lowest ΔE* of 1.60-4.66; while the highest value of 3.63-5.62 was observed in the milk hydrolyzed with trypsin. In addition, ΔE* value of Alcalase- and Protamex-hydrolyzed samples was 2.35-5.01 and 2.71-4.77, respectively. The ΔE* value of >3.0 is suggested to be detectable by human's eye (Cserhalmi et al., 2006).

**Physical stability**

Phase separation was observed to various extents in hydrolyzed milk samples. Hydrolysis using Flavourzyme resulted in milk with the greatest physical stability, since there was no observable phase separation. Hydrolyzed milk samples prepared using Alcalase Protamex and trypsin separated into 2 layers of cream and serum,

of which separation of 11-22%, 4-8% and 8-17% were observed, respectively. The degree of separation tended to increase with the hydrolysis time, as well as temperature. The extent of phase separation observed in hydrolyzed milk samples was consistent with their DH. Samples with higher DH seemed to be less stable and more separated into layers of curd and serum after being heated to inactivate the enzyme. However, separation in Protamex-hydrolyzed milk with 10-20% DH was neglectible (<8%) and could obtain homogeneity after subjecting to homogenization.

Extensive hydrolysis of milk proteins might lead to aggregation and curd formation. Calcium ions released from the destructive casein micelles could induce intermolecular interactions between proteins to become larger molecules that were less soluble. Thus the aggregation of protein was taken place. Hydrolysates obtained from different enzymes were also different in their amino acid sequences, especially those of hydrophobic amino acids. The difference in amino acid sequence consequently resulted in the different molecular arrangement and solubility among hydrolyzed proteins (Creusot and Gruppen, 2008). It should be noted that milk samples hydrolyzed with Alcalase and trypsin not only separated into layers, but also formed curd or gel, especially those obtained from prolonged hydrolysis time and those with high DH. The observed separation was consistent with the extensive change in color of Alcalase- and trypsin-hydrolyzed milk.

**Antioxidant activity**

Based on DH and physical properties, certain hydrolyzed milk prepared using Protamex at E:S ratios of 0.1 and 0.5% w/w or Flavourzyme at E:S ratios of 0.5 and 1% w/w at 4 or 50ºC for 30-120 min were selected to determine their antioxidant activities Such hydrolyzed milk had 10-15% DH with the absence of phase separation and ΔE* <4. Our preliminary results indicated that the MW of proteins in such hydrolyzed milk samples, determined using SDS-PAGE, was in a similar range between 1,000 and 15,000 Da with the greatest proportion at 3,000-4,000 Da.

FRAP and ORAC of non-hydrolyzed milk were 17 and 177 μmol TE/ml, respectively (Table 1). Both values increased when milk was treated with protease enzyme and also increased with DH of the hydrolyzed milk. At any similar DH, Protamex-hydrolyzed milk showed higher FRAP but lower ORAC than those hydrolyzed with Flavourzyme. The highest FRAP and ORAC of 25 and 262 μmol TE/ml were obtained by hydrolysis with 0.5% Protamex at 50ºC for 30 min and 1% Flavourzyme at 50ºC for 90 min, respectively.

The improved antioxidant activities of hydrolyzed milk could be explained by the greater exposure of the antioxidative amino acid residues due to peptide bonds cleavage. Additionally, it has been reported that during hydrolysis, the protein chains unfolded and resulted in the development of hydrophobicity. The balance between hydrophilic and hydrophobic forces of peptides has a crucial influence on the solubility of protein hydrolysate, as well as its antioxidant activity (Sarmadi and Ismail, 2010). The antioxidant activity of protein hydrolysates is also influenced by characteristics and sequences of amino acid in the derived peptides, which arise from the specificity of protease enzyme used for hydrolysis (Wu et al., 2006). Different proteases produce peptides with different amounts, sizes, compositions and sequences of amino acid.

**Table 1.** Antioxidant activity of milk hydrolyzed with Flavourzyme 1000L and Protamex 1.5MG at different hydrolysis conditions

| Protease enzyme | Hydrolysis condition | | | DH | FRAP | ORAC |
|---|---|---|---|---|---|---|
| | E:S ratio (% w/w) | Temperature (°C) | Time (min) | (%)[1] | (μmol TE/ml)[1] | (μmol TE/ml)[1] |
| Flavourzyme 1000L | 0.5 | 50 | 90 | 9.16±6.64$^{d}$ | 18.12±1.08$^{c}$ | 186.58±4.48$^{f}$ |
| | | | 120 | 13.78±5.44$^{b}$ | 18.52±0.43$^{bc}$ | 243.74±0.34$^{bc}$ |
| | 1 | 4 | 120 | 9.65±1.17$^{cd}$ | 18.85±0.75$^{bc}$ | 248.49±16.16$^{b}$ |
| | | 50 | 30 | 9.08±3.24$^{d}$ | 18.70±1.13$^{bc}$ | 216.84±3.32$^{d}$ |
| | | | 60 | 10.30±1.96$^{cd}$ | 18.92±0.41$^{bc}$ | 233.15±2.70$^{c}$ |
| | | | 90 | 13.91±1.96$^{b}$ | 18.56±0.56$^{bc}$ | 262.65±8.22$^{a}$ |
| Protamex 1.5MG | 0.1 | 4 | 120 | 10.33±0.87$^{cd}$ | 24.12±0.81$^{a}$ | 199.93±10.85$^{e}$ |
| | | 50 | 30 | 10.78±4.69$^{c}$ | 18.60±0.30$^{bc}$ | 183.33±12.77$^{f}$ |
| | | | 60 | 11.63±8.37$^{c}$ | 21.92±3.56$^{ab}$ | 153.67±4.92$^{g}$ |
| | | | 90 | 15.45±0.60$^{a}$ | 25.08±0.65$^{a}$ | 134.85±5.90$^{h}$ |
| | 0.5 | 50 | 30 | 11.48±3.45$^{c}$ | 25.23±0.45$^{a}$ | 153.89±6.37$^{g}$ |
| | | | 90 | 15.48±3.11$^{a}$ | 22.48±4.76$^{a}$ | 138.58±3.75$^{h}$ |
| Non-hydrolyzed milk | | | | 5.42±0.34$^{e}$ | 117.03±3.01$^{i}$ | 17.71±1.76$^{c}$ |

[1]Means±standard deviations of triplicate samples

$^{a,b,c,d,e}$ Means within the same column with different superscripts are significantly different (p≤0.05).

## CONCLUSIONS

Enzymatic hydrolysis of protein in cow's milk resulted in the changes in color, less physical stability and improved antioxidant activities of the hydrolyzed milk. The extent of effect largely depended on the type and concentration of proteolytic enzyme, as well as temperature and duration of hydrolysis reaction, which is the determinant of DH of the obtained protein hydrolysates. Hydrolysis with Protamex 1.5MG at E:S ratios of 0.1-0.5% w/w or Flavourzyme 1000L at E:S ratios of 0.5-1% w/w at 50ºC for 30-90 min was found to improve the antioxidant activities, particularly ORAC and FRAP values, of cow's milk while causing minimal changes in physicochemical characteristics as compared with non-hydrolyzed milk.

## ACKNOWLEDGEMENTS

The authors are thankful to the Agricultural Research Development Agency (Public Organization) for financial support (CRP5905020890).

## REFERENCES

Ahmed, B., Zhiyong, H., Mahmoud, A.-A., Maomao, Z., Shuang, Z., Fang, Q., & Jie, C. (2017). Fractionation and identification of novel antioxidant peptides from buffalo and bovine casein hydrolysates. Food Chemistry, 232, 753-762

Benzie, I., & Strain, J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical Biochemistry, 239(1), 70-76

Chalamaiah, M., Kumar, B., Hemalatha, R., & Jyothirmayi, T. (2012). Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. Food Chemistry, 135, 3020-3023.

Chove, L. M., Issa-Zacharia, A., Grandison, A. S., & Lewis, M. J.

(2013). Proteolysis of milk heated at high temperatures by native enzymes analysed by trinitrobenzene sulphonic acid (TNBS) method. African Journal of Food Science, 7(8), 232-237.

Creusot, N., & Gruppen, H. (2008). Hydrolysis of whey protein isolate with *Bacillus licheniformis* protease: Aggregating capacities of peptide fractions. Journal of Agricultural and Food Chemistry, 56, 10332-10339.

Cserhalmi, Z., Sass-Kiss, Á., Tóth-Markus, M., & Lechner, N. (2006). Study of pulsed electric field treated citrus juices. Innovative Food Science and Emerging Technologies, 7(1), 49-54.

Deeslie, D., & Cheryan, M. (1992). Fractionation of soy protein hydrolysates using ultrafiltration membranes. Journal of Food Science, 57(2), 411-413.

FAO. (2013). Milk and dairy products in human nutrition. Rome: Food and Agriculture Organization of the United Nations.

Gonzalez-T, F., Camacho, E., Jurado, M., & Paez, E. G. (1994). Enzymatic hydrolysis of whey proteins II. Molecular weight range. Biotechnology and Bioengineering, 44(4), 529-532.

Gowda, L., Rao, A., & Prakash, V. (2006). Process for the preparation of angiotensin converting enzyme (ACE) inhibitors and its use. U.S. Patent, US7125702B2.

Hayes, M. (2013). Biological activities of proteins and marine-derived peptides from byproducts and seaweeds. In: Kim, S.-K., editor. Marine proteins and peptides. Biological activities and applications. Hoboken: John Wiley & Sons, 139-165.

Hoyle, N., & Merritt, J. (1994). Quality of fish protein hydrolysates from herring (*Clupea harengus*). Journal of Food Science, 59(1), 76-79.

Jauhiainen, T., & Korpela, R. (2007). Milk peptides and blood pressure. Journal of Nutrition, 137, 825-829.

Kanwar, J., Kanwar, R., Sun, X., Punj, V., Matta, H., Morley, S., & Sehgal, R. (2009). Molecular and biotechnological advances in milk proteins in relation to human health. Current Protein Peptide Science, 10(4), 308-338.

Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chemistry, 102(4), 1317-1327.

Korhonen, H., & Marnila, P. (2006). Bovine milk immunoglobulins against microbial human disease. Dairy-derived ingredients. food and nutraceutical uses. Oxford: Woodhead, 269-289.

Li-Chan E., & Nakai S. (1990). Importance of hydrophobicity of proteins in food emulsions. In: El-Nokaly M., & Cornell D., editors. Microemulsions and emulsions in foods. Washington, D.C.: ACS Publications, 193–212.

Li, Z., Wang, B., Chi, C., Gong, Y., Luo, H., & Ding, G. (2013). Influence of average molecular weight on antioxidant and functional properties of cartilage collagen hydrolysates from *Sphyrna lewini*, *Dasyatis akjei* and *Raja porosa*. Food Research International, 51(1), 283-293.

McKellar, R. (1981). Development of off-flavor in ultra-high temperature and pasteurised milk as a function of proteolysis. Journal of Dairy Science, 64, 2138-2145.

Ney, K. (1971). Prediction of bitterness of peptides from their amino acid composition. Z Lebensm-Unters Forsch, 147, 64-68.

Ou, B., Hampsch-Woodill, M., & Prior, R. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. Journal of Agricultural and Food Chemistry, 49(10), 4619-4626.

Panyam D, & Kilara A. (1996). Enhancing the functionality of food proteins by enzymatic modification. Trends in Food Science and Technology, 7, 120-125.

Patel, K., & Borchardt, R. T. (1990). Chemical pathway of peptide degradation II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. Pharmaceutical Research, 7, 703-711.

Prieto, C., Guadix, E., & Guadix, A. (2014). Recent patents on whey protein hydrolysates manufactured by proteolysis coupled to membrane ultrafiltration. Recent Patents on Chemical Engineering, 3, 115-128.

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: a review. Peptides. 31(10), 1949-1956.

Tavano, O. L. (2013). Protein hydrolysis using proteases: An important tool for food biotechnology. Journal of Molecular Catalysis B: Enzymatic, 90, 1-11.

Wu, H.-C., Chen, H.-M., & Shiau, C.-Y. (2006). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). Food Research International, 36(9-10), 947-957.

Yongqing, H., Zhenlong, W., Zhaolai, D., Genhu, W., & Guoyao, W. (2017). Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance. Journal of Animal Science and Biotechnology, 8, 24.