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Physico-Chemical Properties of Milk Powders Treated With Proteolytic Enzymes

Thanut Amatayakul

ABSTRACT

The use of thermolysin, flavourzyme or trypsin (at their optimal pH and temperature) was studied for the production of milk powder with angiotensin converting enzyme (ACE) inhibitory activity. The treated milk samples were evaluated for non-protein nitrogen (NPN) and ACE inhibitory activity. Milk powders were prepared from each protease, spray dried and evaluated for color, median size, size distribution and bulk density. The reconstituted milk samples were then re-evaluated for ACE inhibition. There was an increase in milk protein digestion with increasing digestion time. A steep increase in ACE inhibition during the first 3 hr (from 0 to 30%) was observed in the samples treated with thermolysin and flavourzyme. From 3–24 hr, fluctuation in the ACE inhibition was observed between 30 and 50%. Therefore, a digestion time of 3 hr was selected for milk powder production. There was no significant ($P > 0.05$) difference in the bulk density and ACE inhibition among samples. Differences in the particle size and distribution among samples were found. However, the ACE inhibitory activity of reconstituted milk decreased by 10 times compared with samples prior to the drying process. This corresponded with an increase in yellowness (b*) as a result of a Maillard reaction during milk powder production.

Keywords: milk powder, protease, ACE inhibitory activity

INTRODUCTION

Hypertension is a global, epidemic issue (Chockalingam *et al*., 2006). It is the condition in which patients have a blood pressure greater than 140/90 mmHg (Fox, 2011). Hypertension is a potential risk factor for arteriosclerosis, stroke, myocardial infarct and end-stage renal diseases (Alexander, 1995; Iseki *et al*., 2000). An increase in blood pressure is partly due to the conversion of angiotensin I to agniotensin II (a vasoconstrictor) and inactivation of bradykinin (a vasodepressor) by the angiotensin converting enzyme (ACE) (Sears and Casadei, 2002). Currently, antihypertensive peptides of food origin are gaining attention as an alternative to synthetic drugs and researchers have reported that peptides resulting from enzymatic hydrolysis inhibited ACE and, therefore, reduced blood pressure (Chen *et al*., 2013; Fernández-Musoles *et al*., 2013). The experiments have been carried out both *in vivo* and *in vitro*, with the oral administration of Ala-Val-Phe peptide (half maximal inhibitory concentration, $IC_{50} = 320$ $\mu g.mL^{-1}$) purified from insect protein (*Spodoptera littoralis*; Lepidoptera) at 5 $mg.kg^{-1}$ body weight to spontaneously hypertensive rat (SHR) significantly decreasing their blood pressure by approximately 12 mmHg after 5 hr (Vercruysse *et al.*, 2010). A comparable result between oral administration of captopril (an antihypertensive drug) at 2 $mg.kg^{-1}$

Faculty of Agricultural Product Innovation and Technology, Srinakharinwirot University, Nakhon Nayok 26120, Thailand.
E-mail: tanut@swu.ac.th

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and a fractionate of the pepsin digested oyster protein (Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe) with an IC_{50} value of 66 μmol.L^{-1} at 20 mg.kg^{-1} to SHR was reported (Wang *et al.*, 2008). The hydrolysis of skimmed milk with cell-free extract of *Lactobacillus helveticus* JCM1004 resulted in the highest percentage of ACE inhibitory activity (approximately 90%) at 8 hr of digestion (Pan *et al.*, 2005). In their experiment, the two potent, antihypertensive peptides were Val-Pro-Pro and Ile-Pro-Pro. A single gastric incubation of the peptides in SHR also showed a significant reduction in blood pressure during a 12 hr period. These results illustrated that antihypertensive peptide from food origins can be a good alternative to synthetic drugs.

A number of proteolytic enzymes have been used to produce ACE inhibitory peptide from various sources of proteins. For example, Alcalase, flavourzyme, Neutrase, Pepsin, Protamax and trypsin were used under their optimal conditions to hydrolyze salmon byproduct protein (Ahn *et al.*, 2012), with the hydrolyzate from the Alcalase treatment giving the highest ACE inhibition compared with other enzyme treatments. Hydrolysis of tilapia byproduct using Cryotin-F and flavourzyme yielded hydrolyzate with 60–70% ACE inhibition (Raghavan and Kristinsson, 2009). Flavourzyme addition increased the ACE inhibition of fermented milk during 5 hr of lactic acid fermentation (Tsai *et al.*, 2008). Very potent ACE inhibitory peptides (IC_{50} between 1 and 5 μM) were produced from the hydrolysis of α-lactalbumin and β-casein by thermolysin (Otte *et al.*, 2007a). Thermolysin and protenase K treatment of ovine and caprine β-lactoglobulin were reported to give better ACE inhibitory activity than those treated with trypsin and chymotrypsin (Hernández-Ledesma *et al.*, 2002). In another experiment, thermolysin gave hydrolyzate of milk protein that possessed the highest ACE inhibition compared with other enzymes (Otte *et al.*, 2007b).

With an increase in consumer consciousness and a preference for natural products, foods with antihypertensive properties can be an alternative to synthetic drugs. Milk is a good source of dietary protein which contains active peptide sequences with a very potent ACE inhibition (FitzGerald *et al.*, 2004). Although many researchers (Pan *et al.*, 2005; Otte *et al.*, 2007b; Tsai *et al.*, 2008) have successfully enriched both milk proteins and fermented milks with ACE inhibitory peptides by the action of protease or microorganisms, the production of milk powder with ACE inhibitory property has not yet been explored. The current study examined the physico-chemical properties of milk powder treated with flavourzyme, thermolysin or trypsin.

MATERIALS AND METHODS

Materials

Pasteurized whole milk (CP-Meiji Co, Ltd, Saraburi, Thailand) was purchased from a local supermarket. Angiotensin-I-converting enzyme (ACE; EC 3.4.15.1, from rabbit lung, 0.25 units.mL^{-1}), Hippuryl-L-histidyl-L-leucine (HHL), thermolysin from *Bacillus thermoproteolyticus rokko* (50–100 units per milligram protein), flavourzyme or protease from *Aspergillus oryzae* (≥ 500 units.g^{-1}), trypsin from porcine pancreas (1,000–2,000 BAEE units per milligram solid) were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Milk protein hydrolysis

Pasteurized whole milk was mixed with thermolysin, flavourzyme or trypsin to 80 micro units.mL^{-1} under their optimal conditions as shown in Table 1 over 24 hr. Sodium azide was added to the prepared milk at 0.1% (weight per volume) to prevent microbial spoilage. Samples were taken at 3 hr intervals and were heated at 90 °C for 10 min to inactivate enzymes. Then, the protein in the milk was precipitated using trichloroacetic acid and the filtrate was used for determination of non-protein nitrogen (NPN), free amino acid and ACE inhibition.

Table 1 Temperature and pH used during hydrolysis of milk protein treated with thermolysin, flavourzyme or trypsin.

Enzyme	Temperature (°C)	pH
Thermolysin	70	8.0
Flavourzyme	50	7.0
Trypsin	37	8.0

Determination of non-protein nitrogen

To assess the level of enzymatic hydrolysis, NPN of filtrates was determined according to the method described by Barbano *et al*. (1991).

Determination of angiotensin converting enzyme inhibitory activity

The ACE inhibitory activity of whole milk and milk powder (after reconstitution) was determined according to the method of Jimsheena and Gowda (2010) with some modification. The filtrates (20 µL) were mixed with 150 µL of 100 mM sodium borate buffer pH 8.3, 20 µL of 5 mM HHL (prepared in sodium borate buffer pH 8.3) and 20 mL of ACE at 12.5 micro units.mL^{-1} (prepared in sodium borate buffer pH 8.3). The mixture was incubated at 37 °C for 3 hr. Then, 30 µL of 1 M HCl was added to inactivate the reaction. The amount of hippuric acid released from the reaction was measured using high performance liquid chromatography (1100 series; Agilent Technologies. Santa Clara, CA, USA) equipped with Vertisep USP C18 (4.6 × 250 mm). A mobile phase (50% methanol containing 0.1% trifluoroacetic acid) was run under isocratic elution at 1 mL.min^{-1}. The detecting wavelength was at 228 nm. The percentage of ACE inhibitory activity was calculated using Equation 1:

$$\%\text{ACE inhibitory activity} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (1)$$

where $A_{control}$ and A_{sample} are the absorbance of hippuric acid in the control (sample at time 0 hr) and sample, respectively.

Preparation of milk powder

After the selection of an appropriate hydrolysis time, each milk sample was treated with each enzyme (under its optimal conditions and with the addition of sodium azide at 0.1%) using the selected time. The hydrolyzates were heated at 90 °C for 10 min to inactivate enzymes. Before evaporation, the pH of the hydrolyzates were adjusted back to 6.40. Then, the hydrolyzates were adjusted to 50% (weight per weight, w/w) using a rotary evaporator (Model R124; Buchi; Flawil, Switzerland). The concentrated milk samples were dried using a spray dryer (Model B-191 mini spray dryer; Buchi; Flawil, Switzerland) setting the inlet temperature and aspirator to 140°C and 100%, respectively. The resultant milk powders were stored in sealed aluminum foil bags at -20 °C until further analysis.

Determination of milk powder particle size and distribution

The median size and size distribution of particles of milk powder were determined using image analysis of the micrograph with some modification from the method described by Liang and Hartel (2004). Milk powder was dusted over a clear sticky tape. The tape was then attached to a glass slide. The imaging system (Nikon; Tokyo, Japan) used to take photomicrographs consisted of an Eclipse E-200 light microscope equipped with a DS-Fi2 camera, a Ds-U3 controller and NIS-Elements imaging software. ImageJ software (version 1.47; National Institute of Health; Bethesda, MD, USA) was used to analyze the diameter (assuming a circular shape) of approximately 800–1000 particles over 20 fields.

Determination of bulk density

The milk powder sample was filled up to 100 mL in a measuring cylinder by pouring action. The bulk density was determined from the weight and volume and was regarded as the loose bulk density (Sharma *et al.*, 2012).

Measuring color parameters of milk powder

The color of the milk powder was measured using a ColorFlex EZ spectrophotometer (Hunter Associates Laboratory Inc.; Reston, VA, USA), where L*, a* and b* represent white (+100) to black (-100), red (+) to green (-) and yellow (+) to blue (-), respectively.

Statistical analysis

Experiments were done in triplicate. Statistical analysis was performed using the R software package (R Core Team, 2014). Analysis of variance and, when it was applicable, Tukey's honestly significant difference were applied at $P < 0.05$.

RESULTS AND DISCUSSION

Non-protein nitrogen

NPN of milk samples treated with thermolysin, flavourzyme or trypsin at 80 micro units.mL^{-1} is presented in Figure 1. All samples showed a rapidly increasing trend in NPN generation during 0-3 hr. The hydrolysis continued gradually throughout the 24 hr period. However, the thermolysin treatment gave the highest percentage of NPN at 24 hr (approximately 0.7%). A similar trend was observed for the sample treated with flavourzyme, although the production of NPN was lower. The lowest percentage of NPN was observed in the samples treated with trypsin. All these results suggested that milk protein was rapidly degraded during the first 3 hr, even though it was slowly degraded to shorter peptides afterward. In general, liquid milk contains approximately 0.1–0.2% (weight by weight, w/w) NPN of milk or about 5% of the total protein in milk (Walstra *et al.*, 2006). At most, the hydrolysis of milk protein in this study accounts for only about 20% of the protein (0.6% from 3% milk protein). This should not affect any physical characteristics of the milk powder.

Angiotensin converting enzyme inhibitory activity of liquid milk

The ACE inhibitory activity of liquid milk treated with thermolysin, flavourzyme or trypsin was monitored over 24 hr. The results expressed as ACE inhibition (%) are presented

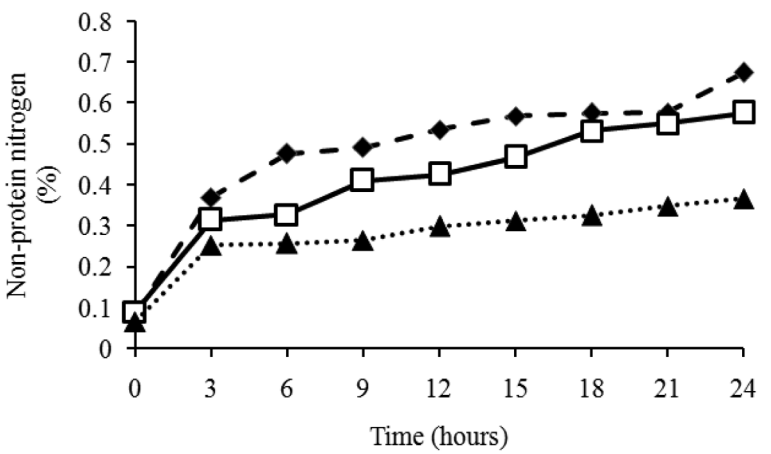

Figure 1 Non-protein nitrogen of liquid milk treated with thermolysin (◆), flavourzyme (□) or trypsin (▲) during 24 hr digestion (n = 3).

in Figure 2. During the first 3 hr, an abrupt increase to 30 and 40% in the percentage of ACE inhibition was found in the liquid milk treated with thermolysin and flavourzyme, respectively. Only a gentle increase in the ACE inhibition from 0 to 15% was observed in the samples treated with trypsin during the same period. Subsequently, there was a fluctuation in the ACE inhibition. In all samples, the value dropped after 21 hr of hydrolysis. This was likely due to the fact that the ACE inhibitory peptides produced at 3 hr as well as the intact protein precursor were further hydrolyzed, as seen from Figure 1. This led to both active and non-active peptides. Eventually, the non active proportion was greater at the end of hydrolysis. A similar observation was reported by Rui *et al.* (2012) where flavourzyme (an exopeptidase) further degraded ACE inhibitory peptide from beans resulting in a decrease in the ACE inhibitory activity. In the current study, the ACE inhibitory activity at 3 hr was relatively high (approximately 30–40%), while, from 3 to 24 hr, a fluctuation in the ACE inhibition was observed between 30 and 50%. Therefore, a hydrolysis time of 3 hr was selected for further production of milk powder.

Median size and size distribution of milk powder particle

The median size of the particles in the control milk powder prepared from liquid milk hydrolyzed using thermolysin, flavourzyme or trypsin is presented in Table 2. The distribution in particle size of all milk powders is shown in Figure 3. The results showed that the median particle size of all milk powders ranged between 30 and 50 μm and the distribution was between 20 and 80 μm (Figure 3). Thermolysin hydrolyzed milk powder had the lowest median size of milk particle at 34 μm. The largest particle size was observed in the control milk powder. Interestingly, a reverse relationship between the NPN value and the particle size of milk powder was observed.

Bulk density

The bulk density analysis of the control milk powder (untreated variation) and of samples treated with thermolysin, flavourzyme or trypsin is shown in Table 2. The bulk density of all milk powder samples was in the range 0.26 to 0.28 $g.mL^{-1}$. There was no significant difference among samples although the samples which had a smaller particle size (thermolysin and flavourzyme) showed a higher mean value.

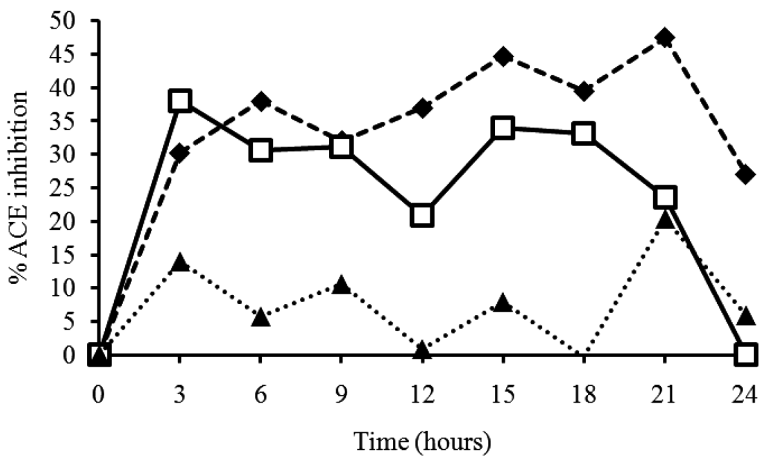

Figure 2 Angiotensin converting enzyme (ACE) inhibitory activity of liquid milk treated with thermolysin (◆), flavourzyme (□) or trypsin (▲) during 24 hr digestion. The ACE inhibitory activity was expressed as percentage of ACE inhibition (n = 3).

Table 2 Median size, color parameters (L*, a*, b*), bulk density, and angiotensin converting enzyme (ACE) inhibition[e] of control milk powder and milk powder treated with thermolysin, flavourzyme or trypsin for 3 hr.

Sample	Median size[g] (μm)	Bulk density[d] ($g.mL^{-1}$)	L*[f]	a*[f]	b*[f]	ACE inhibition[d] (%)
Control milk powder	50	0.26±0.02[a]	94.37±1.11[a]	-1.33±0.07[c]	8.83±0.32[b]	0.59±0.84[b]
Thermolysin hydrolyzed milk powder	34	0.28±0.01[a]	94.74±0.96[a]	-1.18±0.06[b]	9.77±0.38[a]	3.79±0.08[a,b]
Flavourzyme hydrolyzed milk powder	42	0.28±0.02[a]	94.56±0.04[a]	-0.73±0.03[a]	10.01±0.07[a]	4.17±1.38[a]
Trypsin hydrolyzed milk powder	44	0.27±0.01[a]	95.32±0.46[a]	-1.18±0.03[b]	8.58±0.11[b]	3.32±1.94[a,b]

[a,b,c] = Mean values with the same lowercase superscript letter within the same column are not significantly different ($P > 0.05$).
[d] = Number in sample = 3.
[e] = ACE inhibition is the value of reconstituted samples (10% weight per volume).
[f] = Number in sample = 6.
[g] = Results were obtained from image analysis of approximately 80–1000 particles over 20 fields (n = 1).

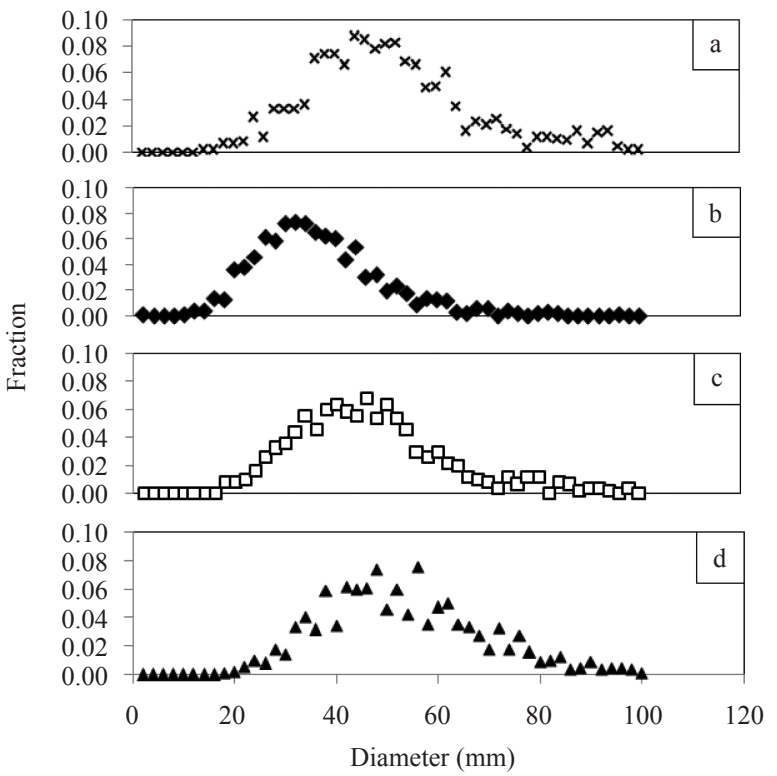

Figure 3 Size distribution of milk powder particles of: (a) Control milk powder. Size distribution of milk powder particles of milk powder prepared from liquid milk hydrolyzed for 3 hr using: (b) Thermolysin; (c), Flavourzyme; and (d) Trypsin. Results were obtained from image analysis of approximately 80–1000 particles over 20 fields (n = 1).

Color parameters of milk powders

Color parameters of milk powders consisting of L* (white to black), a* (red to green) and b* (yellow to blue) values were measured and the results are shown in Table 2. There was no significant difference in lightness (L*) among samples. The L* values were in the range 94.37–95.32 indicating that all samples were very white. The a* values were in the range -1.33 to -0.73, and the b* parameters for all samples ranged from 8.58 to 10.01. The flavourzyme-treated milk powder showed the highest value of a* which was significantly different from the others. While there was no significant difference between the thermolysin- and trypsin-treated milk powders, their values were greater than the control milk sample. There was no statistical difference for parameter b* between the thermolysin- and flavourzyme-treated samples with both showing higher b* values than the other samples. When the values of a* and b* approach 0, the color become gray. Therefore, from the results, the thermolysin- and flavourzyme-hydrolyzed milk powder became more yellow than the control and the trypsin hydrolyzed milk powder. It is interesting to note that there appeared to be a correlation between the NPN value and an increase in b* values probably due to the Maillard browning reaction between free amino acids and lactose during the evaporation and spray drying of the milk (Jaeger *et al.*, 2010).

Angiotensin converting enzyme inhibitory activity of reconstituted milk

The ACE inhibitory activity of reconstituted milk was expressed as a percentage of ACE inhibition (Table 2). As a result of the milk powder production, the ACE inhibition of the thermolysin- and flavourzyme-treated samples dropped substantially from about 30–40% down to 3–4%. For the samples treated with trypsin, the activity only decreased from about 15 down to 3%. The ACE inhibition of the control milk sample and of those treated with thermolysin, flavourzyme and trypsin was 0.59, 3.79, 4.17 and 3.32, respectively. Only the sample treated with flavourzyme was significantly higher than the control sample. However, if the values of ACE inhibition in Table 2 are based on the powder form, the treated milk powders still possess 30 to 40% of ACE inhibitory activity. A decrease in ACE inhibition in the reconstituted samples was likely to be due to the Maillard reaction as previously discussed. Jiang *et al.* (2013a) reported that a Maillard reaction between the IPP peptide and ribose decreased its ACE inhibitory activity as a result of heat treatment. In another study, a loss in the ACE inhibitory activity of bovine casein peptides through a Maillard reaction with ribose sugars as affected by heating time was reported (Jiang *et al.*, 2013b). With regard to the long evaporation process in the current study, a similar explanation could be used to explain the result of ACE inhibition.

CONCLUSION

Flavourzyme and thermolysin were suitable to use to produce liquid milk with ACE inhibitory activity. Treatment with these two enzymes yielded milk with a high ACE inhibitory activity (approximately 30–40%), while the sample treated with pepsin gave a lower level of ACE inhibitory activity (approximately 10–20%, during the first 3 hr. Although extending hydrolysis of the milk protein seemed to increase the ACE inhibitory activity, digestion for too long a period may result in degradation of active peptide and decreased ACE inhibition. Therefore, a hydrolysis time of 3 hr was selected for further production of milk powder. The results showed that hydrolysis of milk proteins affected the size of milk particles. Heat applied during milk powder production including that from evaporation and drying was believed to cause a Maillard reaction which decreased significantly the ACE inhibitory activity of reconstituted milk. It also increased the yellowness of the milk powders.

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