https://doi.org/10.48048/tis.2022.5762

Antioxidant Activity of Cocoa Flavored Sterilized Milk Fortified with Whey Protein Hydrolysate Derived from Gastrointestinal Proteinases Digestion

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Received: 3 May 2021, Revised: 6 July 2021, Accepted: 16 July 2021

Abstract

The objectives of this work were to study the change in α-amino content and antioxidant activity of whey protein hydrolysate (WPH) using gastrointestinal proteinases (pepsin and pancreatin). In addition, whey protein hydrolysate powder (WPHP) with high antioxidant activity were produced by simulated GI digestion using an *in vitro* pepsin - pancreatin hydrolysis for supplementation in flavored milk. Whey protein was hydrolyzed with pepsin for 2 h followed by pancreatin for 3 h. WPH was collected to determine the α-amino content and antioxidant activity. The results showed that digested WPH contained higher α-amino content and antioxidant activity than undigested whey. To study optimum temperature, WPH was dried using Spray Dryer at 130, 140, and 150 °C. Results showed that WPHP at 150 °C exhibited the highest antioxidant activity with a major area peak of molecular weight around >12,000 - 990 Da (43.4 %) and 990 - 336 Da (23.7 %). WPHP at 150 °C was selected to supplement in Cocoa flavored sterilized milk (CFSM_WPHP). α-Amino content, antioxidant activity, and microbiological of CFSM_WPHP were investigated. CFSM_WPHP contained higher α-amino content and antioxidant activity than Control (without addition of WPHP). Total plate count, fecal coliform, and *E. coli* of CFSM_WPHP were under Notification of the Ministry of Public Health of Flavored milk. These results suggested that WPHP has the potential as nutritive and antioxidative supplementation for Cocoa flavored sterilized milk.

Keywords: Flavored milk, Whey protein hydrolysate, Antioxidant activity, Spray dryer, Molecular weight distribution

Introduction

Recently, consumers in Thailand are increasingly looking for healthy products. Drinking milk products is evolving to capitalize on this trend. Sterilized milk is milk packed in containers and heated to temperatures over 100 °C and can be kept at room temperature for a long period of time [1]. However, the severe heat treatment of the milk results in a loss of nutritive value [2]. In addition, it causes extensive Maillard reactions, leading to browning, formation of a sterilized milk flavor, and some loss of available lysine [1]. Therefore, the addition of nutritional and functional ingredients that have antioxidant properties in sterilized milk could improve its nutritional value and health benefits of the products.

Whey protein is a beneficial food product with substantial nutritional and functional properties which contribute to a smooth mouthfeel and a mild dairy flavor that also blends well with popular flavors like vanilla, chocolate (cocoa), and strawberry [3]. Due to their nutritional benefits and excellent digestibility, whey protein hydrolysates (WPHs) are also part of protein-based beverage formulas [4]. Additionally, several reports suggested that WPHs prepared from various proteinase enzymes (Alcalase, Flavourzyme, Neutrase and Protamex) can act as antioxidant substances [5,6]. However, the antioxidant activity of WPHs prepared by gastrointestinal proteinases (pepsin and pancreatin) supplemented in sterilized milk has not been reported. In addition, stability of antioxidant peptides during gastrointestinal (GI) digestion is an important parameter governing bioactivity of a peptide *in vivo*. When peptides pass through the GI tract, they are modified through GI proteinases, which could consequently alter antioxidant properties [7]. The use of gastrointestinal proteinases to generate antioxidant peptides, has the benefit that the peptides formed will retain after physiological digestion in the GI tract [8]. Pepsin, is a powerful enzyme in gastric juice that breaks down dietary proteins reaching the stomach into peptides and amino acids. Pancreatin contains many enzymes, including trypsin, α-chymotrypsin, and elastase and exopeptidases of carboxypeptidases A and B, with trypsin and chymotrypsin predominantly responsible for the majority of digestion in the duodenum [9]. Therefore, we hypothesized that WPHs prepared by simulated GI digestion using an *in vitro* pepsin - pancreatin hydrolysis might produce antioxidant peptides which are resistant to the physiological digestion.

Therefore, the objectives of this study were to produce whey protein hydrolysate with a high antioxidant activity using gastrointestinal proteinases, namely pepsin and pancreatin. In addition, the drying temperature might influence the antioxidant activity of WPHP. Thus, the optimum drying temperature to produce WPH powder with high antioxidant activity was determined. Furthermore, antioxidant activity and microbiological of cocoa-flavored sterilized milk supplemented with WPH powder were also investigated.

Materials and methods

Materials

Whey powder (13 - 14 % protein) imported from France was purchased from Sunisa Bakery Shop Korat (Nakhon Ratchasima, Thailand). Trinitrobenzenesulfonic acid (TNBS), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), 2, 4, 6-93 tripyridyl-s-triazine (TPTZ) and 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), pepsin obtained from porcine gastric mucosa and pancreatin obtained from porcine pancreas were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents were of analytical grade, except for HCl and NaOH which were food grade.

Changes in α-amino content and antioxidant activity of whey protein hydrolysate during simulated GI digestion using an *in vitro* pepsin–pancreatin hydrolysis

Preparation of whey protein hydrolysate (WPH)

Preparation of whey protein hydrolysate were performed by using the enzyme from gastrointestinal tract (pepsin and pancreatin) as described by Wiriyaphan *et al.* [7] with some modification. Whey powder (50 g) was mixed with 450 mL of distilled water (10 % w/v) in flask. The mixture was adjusted to a pH of 2.0 with 6 N HCl and then the volume was adjusted to 500 mL. Consequently, pepsin was added at an enzyme-to-substrate ratio of 0.5: 100 (w/w) and incubated at 37 °C for 2 h. The sample was heated at 90 °C for 10 min to terminate the reaction. The suspension (1 mL) was kept and centrifuged at 10,000 g for 10 min and the supernatant was used for α-amino content and antioxidant activity determination compared to undigested whey (0 h). The remaining suspension in flask was then adjusted to pH 7.4 with 6 N NaOH and further digested with 1 % (w/w) porcine pancreatin for 3 h. The mixture was heated at 90 °C for 10 min to terminate the reaction. The suspension (1 mL) was kept and centrifuged at 10,000 g for 10 min and the supernatant was used for the α-amino content and antioxidant activity determination. The remaining suspension in flask was kept at 4 °C for the production of whey protein hydrolysate powder.

Determination of α-amino content

α-Amino content of WPHs derived from undigested whey (0 h), pepsin digestion (2 h), and pepsin (2 h) followed by pancreatin digestion (3 h) was investigated using Trinitrobenzene Sulfonic acid (TNBS) according to Adler-Nissen [10]. In brief, 50 µL of diluted sample was mixed with 500 µL of 0.2125 M phosphate buffer pH 8.2. Five hundred microliter of TNBS was added in the mixture and incubated at 50 °C in a water bath for 1 h. One mL of 0.1 N HCl was added to stop the reaction then left at room temperature for 30 min. Absorbance was measured at 420 nm. α-Amino content was expressed as mg Leucine eq./mL sample.

ABTS radical scavenging activity assay

ABTS radical scavenging activity of WPHs derived from undigested whey (0 h), pepsin digestion (2 h), and pepsin (2 h) followed by pancreatin digestion (3 h) was done according to the procedure described by Wiriyaphan *et al.* [11]. In brief, 20 µL of diluted sample was mixed with 1,980 µL of ABTS working solution and left at room temperature in the dark for 5 min. Subsequently, absorbance of the mixture was measured at 734 nm. ABTS radical scavenging activity was expressed as mg Trolox eq./mL sample.

Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power of WPHs derived from undigested whey (0 h), pepsin digestion (2 h), and pepsin (2 h) followed by pancreatin digestion (3 h) was done according to the procedure described by Wiriyaphan *et al.* [11]. In brief, 100 µL of diluted sample was mixed with 1,000 µL of FRAP working solution and incubated at 37 °C in a water bath for 15 min. Subsequently, the absorbance of the mixture was measured at 593 nm. FRAP value was expressed as mg Trolox eq./mL sample.

Metal chelating activity assay

Metal chelating activity of WPHs derived from undigested whey (0 h), pepsin digestion (2 h), and pepsin (2 h) followed by pancreatin digestion (3 h) was determined according to the method described by Decker and Welch [12]. In brief, 100 µL of diluted sample was mixed with 2,400 µL of deionized water, 50 µL of 2 mM $FeCl_2$ and 100 µL of 5 mM 3-(2-pyridyl)-5,6-bis (4-phenyl-sulphonic acid)-1,2,4-triazine (ferrozine). After incubation at room temperature in the dark for 20 min, the absorbance of the mixture was measured at 562 nm. Metal chelating activity was expressed as mg EDTA eq./mL sample.

Production of whey protein hydrolysate powder (WPHP)

The remaining suspension of whey protein hydrolysate (the mixture of remaining undigested whey protein, peptides and amino acids) prepared from pepsin for 2 h followed by pancreatin for 3 h was used for production of WPHP. The remaining suspension (220 mL) was mixed with 20 % w/v of maltodextrin. The mixture was dried using Spray Dryer (SD-06 Spray Dryer, UK) at 130, 140 and 150 °C. The drying conditions were varied by choosing different inlet air temperatures, 130, 140 or 150 °C. Outlet temperature ranged between 70 - 80 °C. WPH powder was kept in aluminum foil bags at 4 °C for further experiments.

Measurement of moisture content and water activity

The moisture content of WPHP at various temperatures was determined following AOAC [13]. The water activity was assessed using the dew point water activity meter, AQUA LAB 4TE.

Determination of α-amino content and antioxidant activity

WPHP (0.1 g) at various temperatures was re-solubilized in 1 mL of deionized water, vortexed at room temperature for 1 min, and then centrifuged at 10,000 g for 10 min. The supernatant was used to determine α-amino content and antioxidant activity as aforementioned.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern

SDS–PAGE pattern of whey protein and WPHP was performed according to a method described by Laemmli [14]. Whey protein and WPHP were solubilized in 50 mM Tris-Cl pH 8.2 and 2 % SDS solution at a final protein concentration of 1 µg/mL. The sample (10 µg of protein) was mixed with 10 mL of 2x sample buffer and incubated at 95 °C for 5 min. The mixture (10 µg) was loaded onto 15 % acrylamide gel. Gels were run at a constant voltage at 110 V, stained with 0.1 % Colloidal coommassie blue G250 and destained with deionized water. The gel was photographed with Chromatein™ Prestained Protein Ladder.

Size exclusion chromatography (SEC)

The molecular weight (MW) distribution of peptide in WPHP was determined using a size exclusion chromatography technique according to a method described by Khongla *et al.* [15] with some modification of sample concentration loading (420 mg solid/mL). Peptides were monitored at the absorbance of 215 nm. Cytochrome C (12,000 Da), Aprotinin (6,512 Da), AGNQVLNLQADLPK (1,480.6 Da), MILLLFR (1,095 Da), PLL (341 Da), LL (244 Da) and tyrosine (181 Da) were used to make a standard curve. Blue dextran (2,000 kDa) was used to measure the void volume of the column. The molecular weight of peptides was calculated by the elution volume.

Cocoa flavored sterilized milk supplemented with WPHP

Cocoa flavored sterilized milk supplemented with WPHP (CFSM_WPHP) and control (addition of maltodextrin instead of WPHP) were prepared following **Table 1**. Water was heated to a temperature of

50 °C, then dry ingredients and 2 % w/w of WPHP were added. Subsequently, cow milk was added to the mixture and homogenized. The mixture was pasteurized in stainless steel pot at 80 °C for 10 min, filled in glass bottles (100 mL), and then sterilized using autoclave (Hirayama, Hiclave HV-85, Saitama, Japan) at 108 °C for 15 min. The control and CFSM_WPHP were cooled at room temperature, and kept for further analysis.

Table 1 Ingredients of cocoa-flavored sterilized milk supplemented with WPHP (CFSM_WPHP) and Control.

Ingredients	Content (g)	
	Control	**CFSM_WPHP**
Cow milk	600	600
Skim milk powder	14.4	14.4
Whey powder	14.4	14.4
Whey protein hydrolysate powder	0	24
Maltodextrin	24	0
Sugar	36	36
Cocoa powder	6	6
Water	505.2	505.2
Total	1,200	1,200

Microbiological quality test

Cocoa flavored sterilized milk supplemented with WPHP (CFSM_WPHP) was tested for microbiological qualities (Total plate count, Fecal Coliform and *E. coli*) following the United States Food and Drug Administration (U.S. FDA) Biological Analytical Manual (BAM) [16,17].

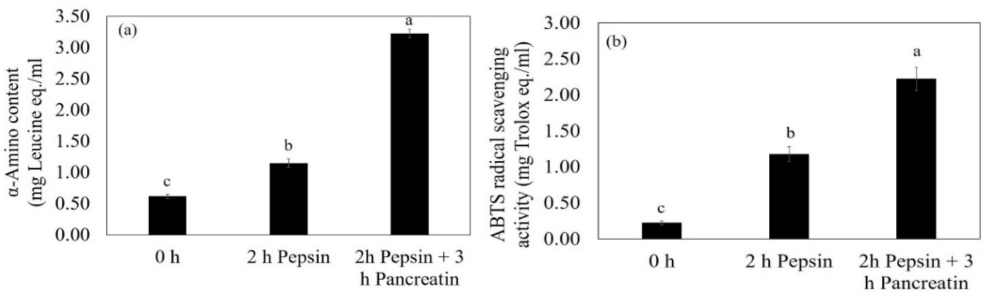
Statistical analyses

Data were expressed as means ± standard deviations. An independent-sample t-test was used for the comparison of the means of 2 independent group samples. Data was analyzed using variance analysis (ANOVA) and the comparison of means was made using the multiple range test of Duncan (DMRT) for more than 2 groups of samples, at 95 % confidence level using a PASW Statistics 18 Release 18.0.0 software.

Results and discussion

Changes in α-amino content and antioxidant activity of whey protein hydrolysate during simulated GI digestion using an *in vitro* pepsin–pancreatin hydrolysis

α-Amino content of whey protein hydrolysate (WPH) increased after pepsin digestion, and pepsin-pancreatin digestion when compared to undigested whey (0 h) (**Figure 1(a)**). After pepsin-pancreatin digestion, WPH exhibited the highest α-amino content (3.23±0.07 mg Leucine eq./mL of the sample) (**Figure 1(a)**). High α-amino content indicated the high presence of small peptides. Pepsin is an endopeptidase that hydrolyzes proteins in the stomach into polypeptides, smaller peptides, and amino acids.



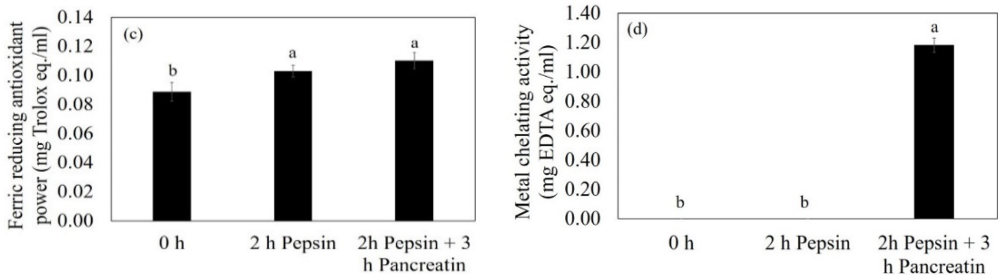


Figure 1 (a) α-Amino content; (b) ABTS radical scavenging activity; (c) Ferric reducing antioxidant power; (d) Metal chelating activity of whey protein hydrolysate derived from undigested whey (0 h), pepsin digestion (2 h), and pepsin (2 h) followed by pancreatin digestion (3 h). Values are expressed as means ± S.D. (n = 4). Bars with different letters indicate means with significant differences ($p < 0.05$).

Many enzymes, including trypsin, amylase, and lipase, are found in pancreatin. After pepsin digestion, trypsin in pancreatin further hydrolyzed polypeptides into smaller peptides and amino acids resulting in an increased α-amino content (**Figure 1(a)**). Embiriekah *et al.* [6] reported that WPHs derived from pepsin digestion were composed of large peptides, while WPHs derived from trypsin digestion contained a mixture of 4 to 7 amino acid residues and large peptides.

When compared to undigested whey, 2,2′-Azino-bis(3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) radical scavenging activity of WPH increased after pepsin and pepsin-pancreatin digestion (**Figure 1(b)**). Similar results were found by Castro and Sato [18] and Mann *et al.* [3] who reported an increase in the antioxidant activity of WPHs compared to the intact proteins. This could be attributed to the exposure of reactive groups during hydrolysis. Whey protein hydrolysate treated with pepsin-pancreatin had the highest ABTS radical scavenging activity (2.22±0.16 mg Trolox eq./mL of the sample). This result correlated well with α-amino content (**Figure 1(a)**) which indicated that ABTS radical scavenging activity increased with an increase in the α-amino content. In general, antioxidant activity increased with an increase in the α-amino content and degree of hydrolysis (DH) [19,20]; since high α-amino content results in high small peptides, increasing peptide solubility. Therefore, the increase of ABTS radical scavenging activity of WPH with higher small peptides may be attributed to the increasing hydrogen donating properties of the active peptides, which could react with free radicals. Pepsin is the most efficient in hydrolyzing peptide bonds between hydrophobic and preferably aromatic amino acids (Phe, Trp, and Try) and acidic amino acids (Glu and Asp) at the carboxyl side of a peptide bond [21]. Thus, the hydrolysis of whey protein with pepsin might generate peptides containing Tyr, Phe, Trp, Leu, Glu, or Asp in the sequences. It has been reported that Trp and Tyr have the indolic and phenolic groups, respectively, which act as hydrogen donors [22]. Trypsin is specific for Lys and Arg at the carboxyl side of a peptide bond, resulting in numbers of peptide chains with independent Lys and Arg at the C-terminal [23]. Embiriekah *et al.* [6] suggested that WPH derived from peptic hydrolysis is mainly composed of peptides with Tyr amino acid residues, while the hydrolysate derived from tryptic hydrolysis is mainly composed of peptides with Arg and Lys amino acid residues that contain positively charge. Therefore, hydrolysis of whey protein using pepsin followed by pancreatin would generate more small peptides with positively charged amino acid residues, increasing peptide solubility leading to increased ABTS radical scavenging activity. Cys contains a thiol group that can donate sulfur hydrogen which can directly interact with radicals [22]. Whey protein is a cysteine-rich protein [24]. Therefore, Cys in WPH might contribute to ABTS radical scavenging activity.

The ferric-reducing antioxidant power of WPH increased after pepsin digestion and pepsin-pancreatin digestion when compared to undigested whey (**Figure 1(c)**). However, the FRAP (Ferric reducing antioxidant power) value of WPH obtained from only pepsin and pepsin-pancreatin digestion was comparable ($p > 0.05$). This result did not correlate well with the α-amino content and ABTS radical scavenging activity. Wiriyaphan *et al.* [25] suggested that the amino sequence and peptide size significantly affected the antioxidant property of peptides. Tyr or Trp residue located at one of the extremities of the peptides sequence exhibited the most efficient radical scavenging activity [26]. Compared to the antioxidant activity of the amino acids on their own, the peptide structures can lead to synergistic and antagonistic effects [22]. Our results indicated that not only α-amino or peptide content had an effect on antioxidant activity, but also amino sequence might be an important factor influencing the antioxidant activity of peptides

Undigested whey (0 h) and WPH after pepsin digestion (2 h) did not exhibit any metal chelating activity (**Figure 1(d)**). Metal chelating activity increased after pepsin-pancreatin digestion (3 h). It has been reported that the ability to chelate metal ions is greatly affected by peptide structures and amino acid side chain residues in their sequences [27]. Peptides containing acidic (Asp and Glu) and basic (His, Lys, and Arg) amino acids in their side chains contribute to metal chelating activity [28]. Pepsin is preferential to acidic amino acids (Glu and Asp) of a peptide bond. Thus, hydrolyzing whey protein with pepsin might generate peptides containing Glu and Asp in the sequences which might contribute to metal chelating activity. However, WPH after pepsin digestion did not exhibit any metal chelating activity because, at acidic conditions (pH 2.0), peptides carry a net positive charge, therefore, they cannot chelate with metals (Fe^{2+}), which contain the same charge. While, at neutral conditions (pancreatin digestion, pH 7.4), peptides carry a net negative charge, therefore, they can chelate with metals (Fe^{2+}). It has been reported that casein has a metal chelating activity because of the presence of negatively charged phosphopeptides [29,30]. In addition, pancreatin contains trypsin which is specific for Lys and Arg at the carboxyl side of a peptide bond. Therefore, hydrolysis with pancreatin (trypsin) might generate peptides containing Lys and Arg in the sequences which contribute to the metal chelating activity.

The optimal temperature of whey protein hydrolysate powder (WPHP) production

Moisture content and water activity

Water activity (a_w) and moisture content (MC) are crucial factors determining the quality, preservation, and shelf life of foodstuffs. In addition, moisture content and a_w are used to predict microbial growth in food products [31]. Moisture content and a_w of WPHP decreased with an increase in drying temperature (**Table 2**). At 150 ºC, moisture content of WPHP was 5.65±0.70 % w.b. In general, the moisture content of whey powder prepared by using Spray Dryer ranges between 3.5 - 5 % [32]. WPHP contained slightly higher moisture content than previously reported. This could be due to the difference in drying temperature. At 150 °C, the a_w of WPHP was 0.39±0.01 (**Table 2**) which is classified as powdered foods with low moisture (LMF, a_w <0.6) [33].

Table 2 Moisture content, water activity, α-amino content, and antioxidant activity of whey protein hydrolysate powder at various temperatures.

Temperature (°C)	Moisture content (% w.b.)	Water activity (a_w)	α-Amino content (mg Leucine eq./g) ^{ns}	ABTS radical scavenging activity (mg Trolox eq./g)	Ferric reducing antioxidant power (mg Trolox eq./g)	Metal chelating activity (mg EDTA eq./g)
130	$8.42±0.17^{c}$	$0.51±0.01^{c}$	09.86±0.49	$3.00±0.06^{c}$	$0.12±0.00^{b}$	$0.94±0.05^{b}$
140	$6.96±0.28^{b}$	$0.43±0.00^{b}$	10.06±0.20	$3.27±0.04^{b}$	$0.12±0.01^{b}$	$1.02±0.00^{b}$
150	$5.65±0.70^{a}$	$0.39±0.01^{a}$	10.15±0.28	$3.63±0.03^{a}$	$0.21±0.00^{a}$	$1.36±0.08^{a}$

Different superscripts letters a, b, c, within a column indicate significant differences ($p < 0.05$). Values are expressed as means ± S.D. (n = 3).

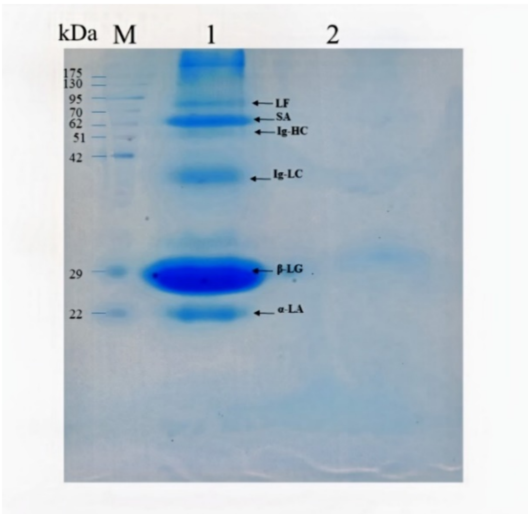
α-Amino content and antioxidant activity

α-Amino content of WPHP at various drying temperatures had no significant difference ($p > 0.05$). This indicated that drying temperatures at 130, 140 and 150 °C had no effect on α-amino content. Because heat will not effectively break the peptide bonds between peptides or protein structure of WPHP. The highest antioxidant activity was found in WPH at 150 °C with ABTS radical scavenging activity, ferric reducing antioxidant power, and metal chelating activity of 3.63±0.03 mg Trolox eq./g, 0.21±0.00 mg Trolox eq./g and 1.36±0.08 mg EDTA eq./g sample, respectively (**Table 2**). The higher antioxidant activity of WPH powder at 150 °C than that of WPH powder at 130 and 140 °C might be due to lower moisture content in WPH powder at 150 °C (**Table 2**). Based on moisture content, a_w, α-amino content, and antioxidant activity, WPH dried at 150 °C was selected to add in sterilized flavored milk for the development of functional beverage.

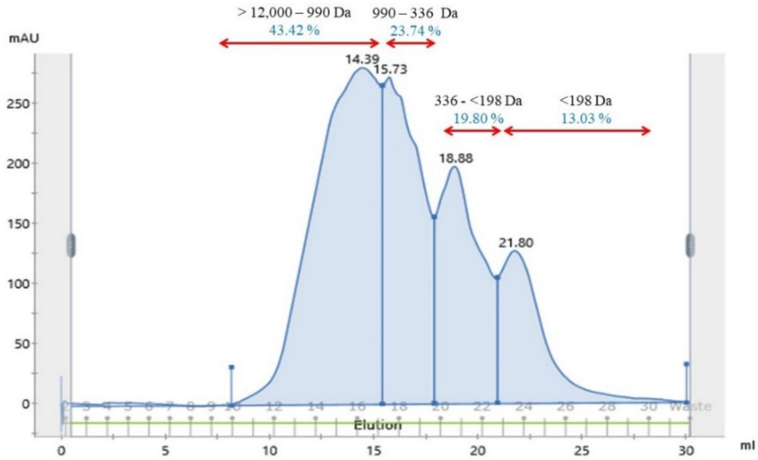
SDS-PAGE pattern and molecular weight distribution

SDS-PAGE patterns of whey protein and whey protein hydrolysate are shown in **Figure 2(a**). Whey protein (lane 1) showed major component of β-lactoglobulin (β-LG) and α-Lactalbumin (α-LA), and minor proteins lactoferrin (LF), serum albumin (SA), immunoglobulin heavy chains (Ig-HC), and Ig light chains (Ig-LC). After digestion of whey protein with pepsin followed by pancreatin, all molecular weight bands were no longer visible in the gel (lane 2, **Figure 2(a)**). This indicated that whey protein was degraded into small peptides and amino acids (lane 2) where short peptides and amino acids in the hydrolysates would elute out of the gel.

Figure 2(b) shows the molecular weight distribution of peptides in whey protein hydrolysate. WPH showed the major peak of MW around >12,000 - 990 Da (43.42 %), followed by area peak of MW around 990 - 336 Da (23.74 %), 336 - <198 (19.80 %) and <198 Da (13.03 %), respectively. These indicated that WPH contained a mixture of different peptide sizes and amino acids. Li-jun *et al.* [34] reported that the molecular weight distribution of WPH hydrolyzed by Alcalase for 7 h ranged from 300 to 1,400 Da, and most of the whey peptide was under 1,000 Da. Peptides with molecular weight <3,000 Da exhibited the most potent antioxidant peptides [35]. Low molecular-weight peptides exhibiting high antioxidant activities might contribute to them having easy reactions with free radicals and thereby eliminating free radicals [35,36]. Therefore, low molecular weight peptides in WPH prepared by pepsin-pancreatin might easily react with free radicals, and contribute to antioxidant activity.



(a)



(b)

Figure 2 (a) SDS–PAGE pattern of whey protein and whey protein hydrolysate powder. (M) Marker; (lane 1) whey protein; (lane 2) whey protein hydrolysate; (b) Molecular weight distribution of whey protein hydrolysate powder treated with pepsin for 2 h followed by pancreatin for 3 h.

Cocoa flavored sterilized milk supplemented with WPH powder

α-Amino content and antioxidant activity

The α-Amino content and antioxidant activities based on ABTS radical scavenging activity, ferric reducing antioxidant power, and metal chelating activity of cocoa-flavored sterilized milk supplemented with WPH powder (CFSM_WPHP) increased up to 6.9, 76.9, 12.4 and 62.4 %, respectively, when compared with their respective controls (**Table 3**). These indicated that adding WPH can improve the antioxidant activity of cocoa-flavored sterilized milk. Our results also indicated that antioxidant peptides in WPH derived from pepsin-pancreatin digestion had good heat stability due to the severe heat treatment in sterilized milk. High antioxidant activity in the control could be due to polyphenol content in cocoa. The ABTS radical scavenging activity of pasteurized chocolate-flavored milk after the addition of 2 % WPC, flavourzyme, alcalase, and corolase WPHs increased up to 10.0, 14.4, 43.3 and 42.7 %, respectively, when compared with their respective controls [3].

Table 3 α-Amino content and antioxidant activity of control and cocoa-flavored sterilized milk supplemented with WPH powder (CFSM_WPHP).

Formula	**α-Amino content (mg Leucine eq./100 mL)**	**ABTS radical scavenging activity (mg Trolox eq./100 mL)**	**Ferric reducing antioxidant power (mg Trolox eq. /100 mL)**	**Metal chelating activity (mg EDTA eq. /100 mL)**
Control	93.68±1.07	53.85±6.30	17.82±0.06	16.34±0.15
CFSM_WPH	100.12±0.77*	95.28±4.41*	20.03±0.23*	26.53±0.10*

* Indicate significant differences ($p < 0.05$) within a column.

Microbiological qualities

Total plate count, Coliform, Fecal Coliform, and *E. coli* were not detected in 0.1 mL of cocoa-flavored sterilized milk supplemented with WPH powder (**Table 4**). Microbiological qualities of flavored milk were under the Notification of the Ministry of Public Health (No.351) B.E.2556 (2013) Re: Flavored Milk [37].

Table 4 Microbiological qualities of cocoa-flavored sterilized milk supplemented with WPH powder.

Microbiological Test	**Microbiological qualities**	
	Cocoa flavored sterilized milk supplemented with WPHP	**Notification of the Ministry of Public Health (No.351) B.E.2556 (2013) Re: Flavored Milk.**
Total Plate Count (CFU/0.1 mL of sample)	<1 CFU/0.1 mL	Not detected in 0.1 mL
E .coli (MPN/0.1 mL of sample)	Not detected in 0.1 mL	Not detected in 0.1 mL

Conclusions

Whey protein hydrolysate prepared by using the enzymes from gastrointestinal tract (pepsin and pancreatin) had higher α-amino content and antioxidant activity than that of undigested whey. In this study, 150 °C was the optimum temperature for whey protein hydrolysate powder (WPHP) production with high α-amino content and antioxidant activity. WPHP showed a major area peak of MW around >12,000 - 990 Da and 990 - 336 Da. The addition of WPHP in cocoa-flavored sterilized milk could improve antioxidant activity. Total plate count, Coliform Fecal Coliform, and *E. coli* of CFSM_WPHP were under Notification of the Ministry of Public Health of Flavored Milk. These suggested that WPHP exhibited the potential of being used as a food additive and/or functional peptides due to the antioxidant properties.

Acknowledgments

The authors would like to thank the Rajamangala University of Technology Isan, Thailand for its facilities support. We would also like to thank Ms. Martha Maloi Eromine for her support in editing the English language in this manuscript.

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