Systematical characterization of functional and antioxidative properties of heat-induced polymerized whey proteins

Feng Gao¹ · Xuefei Zhang¹ · Jiaqi Wang¹ · Xiaomeng Sun¹ · Cuina Wang¹

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Abstract Effects of pH (6–8), protein concentration (6–11%, w/v), heating temperature (70–95 °C) and time (5–30 min) on functional and antioxidative properties of heat-induced polymerized whey protein were systematically investigated. All samples were determined for solubility at pH 4.6, emulsion capacity and stability, and antioxidative properties involving 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis(2-ethylbenzothiazoline-6-sulfonate) (ABTS) scavenging abilities. Heating resulted in significant loss in solubility, emulsion capacity and stability for whey protein, $p < 0.05$. Heating decreased DPPH but enhanced ABTS scavenging ability for whey protein significantly, $p < 0.05$. Changes caused by pH variation were much stronger than those observed for other factors. Both protein concentration and heating time had negative effects while heating temperature had positive effect on emulsion capacity of whey protein. Data indicates that functional and antioxidative properties of whey protein could be altered by factors including pH, protein concentration, heating temperature and time.

Keywords Whey protein · Heating · Solubility · Emulsion properties · Antioxidative properties

Introduction

Whey proteins are produced as byproducts from cheese manufacture and are mixtures of globular proteins (Ikeda and Morris, 2002). β -Lactoglobulin generally accounts for half of the total whey protein. This protein is thermo-sensitive and undergoes reversible conformation modifications at temperatures lower than 70 °C while high temperatures can promote its irreversible denaturation and polymerization (Gauche et al., 2010). Upon heating, thiol/disulphide interchange reactions are enhanced in β -lactoglobulin which lead to high molecular weight polymer formation. Upon cooling, the polymerized whey protein (PWP) remains soluble depending on conditions (Alting et al., 2000). Denaturation and aggregation of whey protein involve many physical forces such as van der Waals, hydrophobic, electrostatic, and hydrogen bonding interactions and covalent disulfide bonds. Each force is a function of solution pH, protein concentration and the extent of thermal treatment (Liu and Zhong, 2013).

Proteins form interfacial films and interact to form networks associated with gels. The ability of proteins to provide these properties is called functionality. The functionality includes hydration-related and surface-related properties which influence the appearance, texture, and flavor retention of food products. Hydration-related functional properties include solubility while surface-related properties include emulsification at oil–water interfaces (Morr and Ha, 1993).

Solubility, practical measure of protein denaturation and aggregation, is a good index of protein functionality (Jambrak et al., 2008). Whey protein solubility is often defined as those proteins soluble at pH 4.6 after a centrifugation (Ryan et al., 2013). Native whey protein is soluble at pH from 2 to 9, indicating a large proportion of

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✉ Cuina Wang
wangcuina@jlu.edu.cn

¹ Department of Food Science, College of Food Science and Engineering, Jilin University, Xi'an Road 5333#, Changchun 130062, Jilin, China

surface hydrophilic residues that encourages protein–water interactions over protein–protein hydrophobic interactions (Ryan et al., 2012). When subjected to denaturation, such as heating, previously buried hydrophobic groups become exposed, causing changes in solubility. The changes are determined by environmental factors such as protein concentration, pH, and heating load (Dissanayake and Vasiljevic, 2009).

Despite the hydrophilic nature of protein molecular surface, a significant number of hydrophobic amino acid groups are exposed at the molecular surface. The amphiphilic properties cause good emulsifying properties for whey protein (Yamauchi et al., 1980). In the emulsions, whey proteins adsorbed on fat–water interfaces to stabilize emulsion droplets against aggregation and coalescence (Khtar and Dickinson, 2003). Many factors including protein concentration, pH of the medium (Pearce and Kinsella, 1978), hydrophobic properties (Shimizui et al., 1981), the number of sulfhydryl groups, and molecular flexibility (Dissanayake and Vasiljevic, 2009) determine the emulsifying properties.

Whey proteins have been reported to act as antioxidants. β -Lactoglobulin plays a key antioxidant role in whey protein and Cys-121 plays an essential role (Liu et al., 2007). There are also evidences for significant antioxidant activities of α -lactalbumin (Kerasioti et al., 2014) and serum albumins (Klajnert and Bryszewska, 2002). Amino acids in whey protein can act as antioxidants mainly either by the reducing action of their sulfhydryl groups (cysteine and methionine) or proton donation of aromatic residues to electron deficient radicals (tryptophan, tyrosine and phenylalanine) (Zilic et al., 2012).

This study aims to conduct a systematical characterization for functional and antioxidative properties of heat-induced polymerized whey proteins, covering a wide range of pH from 6 to 8, protein concentration of 6–11% (w/v), heating temperature of 70–95 °C and time of 5–30 min.

Materials and methods

Materials

Whey protein isolate (WPI, 92% on dry weight basis) was purchased from Fonterra Co-operative Group (Auckland, New Zealand). The protein was composed of 69.2% (w/w) β -lactoglobulin, 14.2% α -lactalbumin, 3.3% bovine serum albumin, and 2.1% immunoglobulin G. Other components are as follows (w/w): 0.36% fat, 1.6% ash, 0.7% lactose monohydrate, and 0.07% calcium. All reagents used for antioxidative properties analysis were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Beyotime

Biotechnology (Shanghai, China). Deionized water (resistivity is 18.2 M Ω) was obtained using a Milli-Q deionization reversed osmosis system (Millipore Corp., Bedford, MA, USA).

Polymerization of whey protein

Whey protein stock solution (20%, w/v, calculated on protein basis) was prepared by dissolving whey protein powder slowly into deionized water at room temperature (25 ± 1 °C) with the help of magnetic stirring (700 rpm) for 2 h. The stock solution was stored at 4 °C until usage. A series of solutions were prepared as listed in Table 1. Stock solution was restored to room temperature (25 ± 1 °C), diluted to required concentration, and then adjusted to certain pH using sodium hydroxide (1 M) or hydrochloric acid (1 M). Whey protein solutions in glass tubes (1.9 cm internal diameter $\times$ 18 cm long) coated with silicon were heated in a water bath (Mettler, IKA Ared, Pedrollo, Italy) without agitating at certain temperature for an appropriate time. After heating, all samples were rapidly cooled in ice bath to room temperature (25 ± 1 °C) in about 10 min to avoid further denaturation and aggregation (De Wit, 2009).

Solubility determination

Solubility was determined according to the method of previous study with little modification (Dissanayake and Vasiljevic, 2009). All samples were adjusted to pH 4.6 ± 0.1 using 2 N hydrochloric acid, and then centrifuged at $6000 \times g$ for 20 min at 25 °C. Protein concentration was determined using bicinchoninic acid (BCA) protein assay kit. The absorbance ($A_{562 \text{ nm}}$) of samples was measured using microplate reader (Synergy HT, BioTek, USA) and the solubility was calculated as the following equation:

$$\text{Solubility (\%)} = P_s/P_t \times 100 \quad (1)$$

where P_s and P_t represent the protein concentration in supernatant and original solution, respectively.

Emulsion capacity and stability measurement

Emulsion properties were determined according to the method reported by Garcia-Moreno et al. (2017). All samples (30 mL) diluted at 1% were added with 10 mL sunflower oil and then homogenized at 13,000 rpm for 2 min using an ultra-Turrax (T25 basic; IKA, Staufen, Germany). The coarse emulsion (50 μ L) was drawn and stabilized by 5 mL sodium dodecyl sulfate (SDS, 1%). After 10 min, another 50 μ L was drawn and then stabilized by SDS. The emulsifying properties were calculated by

Table 1 Conditions for preparing heat-induced polymerized whey proteins

Constant parameters	Variable	Levels
10%, 30 min, 85 °C	pH	6, 7, 8
pH 7, 30 min, 85 °C	Protein concentration (% w/v)	6, 7, 8, 9, 10, 11
10%, pH 7, 30 min	Heating temperature (°C)	70, 75, 80, 85, 90, 95
10%, pH 7, 85 °C	Heating time (min)	5, 10, 15, 20, 25, 30

recording the absorbance at 500 nm using a UV–VIS Spectrophotometry (Shimadzu, Tokyo, Japan). The emulsion capacity (EC) and stability (ES) were calculated as the following equations:

$$EC (m^2 g \text{ protein}^{-1}) = (2 \times 2.303 \times A_0) / 0.25 \times m_p \quad (2)$$

$$ES (\text{min}) = (A_0 \times t) / (A_0 - A_{10}) \quad (3)$$

where A_0 and A_{10} are the absorbance at 500 nm at 0 min and 10 min, m_p is the mass of protein and t is the time interval between both aliquots were drawn.

Antioxidative properties assay

All samples were diluted to concentrations of 1, 2.5, 5, and 10 mg/mL for analysis. 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.2 mM) was prepared by dissolving the powder in ethanol (99%) slowly. A portion of 150 μL sample was mixed with DPPH solution (150 μL) under agitating for 10 s, and then kept for 30 min at room temperature. The absorbance of all solutions was read at 517 nm by a microplate reader (Synergy HT, BioTek, USA).

For 2,2'-azinobis(2-ethylbenzothiazoline-6-sulfonate) (ABTS) scavenging ability analysis, stock solution containing ABTS (7 mM) and potassium persulfate (2.45 mM) was prepared and stored in dark at 4 °C for 12–16 h. The stock solution was then diluted with distilled water until the absorbance achieved 0.7 ± 0.02 at 734 nm. Subsequently, the diluted ABTS solution (100 μL) was mixed with 50 μL sample and kept resting for 1 h at room temperature. The absorbance of all samples was recorded at 734 nm by a microplate reader (Synergy HT, BioTek, USA).

Both DPPH and ABTS radical scavenging ability (SA) of all samples was calculated using the following equation:

$$SA (\%) = 1 - (A_S - A_B) / A_R \times 100 \quad (4)$$

where A_S , A_B , and A_R represent the absorbance of samples with DPPH/ABTS, blank, and DPPH/ABTS solutions, respectively.

Half inhibition concentration (IC_{50} , mg/mL) is the value required to scavenge 50% of radicals. All samples were calculated for IC_{50} by using regression equation plotted with percent scavenging abilities at four different whey protein concentrations.

Data analysis

All measurements were conducted at least in triplicates for three trials. All data obtained from analysis were expressed as mean $\pm$ standard deviation (S.D.). The significant differences were calculated using Version SPSS 21 (SPSS Inc. Chicago, IL). The significance level was set at $p < 0.05$. Data were checked for homogeneity by Levene's test. When the data were homogeneous, one-way analysis of variance (ANOVA) and then a least squared differences (LSD) model was used. All the figures were drawn by Origin 8.0 (OriginLab Corporation, Northampton, USA).

Results and discussion

Changes in solubility of heat-induced polymerized whey proteins

Solubility is an important characteristic for functional application of proteins in food systems. Solubility of proteins relates to surface hydrophobic (protein–protein) and hydrophilic (protein–solvent) interaction (Pelegrine and Gomes, 2012); in current case, such solvent is the water. Whey protein solubility is mainly determined by structure of β -lactoglobulin (Stanciu et al., 2012). After heating treatment, no obvious optical precipitation in the soluble whey protein aggregate was observed although more hydrophobic groups have been exposed (Palatnik et al., 2015). This behavior can be attributed to the fact that whey protein surface contains a certain number of electrostatic groups creating a repulsive force sufficient to prevent precipitation.

The aggregated protein was precipitated by adjusting pH to a value close to the isoelectric point. After precipitation at pH 4.6, the solubility in term of the native protein after centrifugation is shown in Table 2. Solubility of native samples was $93.20 \pm 3.46\%$ while those of heated samples were 1.88 ± 0.10 to $4.75 \pm 0.04\%$. Denaturation and aggregation significantly decreased whey protein solubility ($p < 0.05$) in comparison with native protein and was related to all factors. Similar results were reported by others (Pelegrine and Gomes, 2012). Exposure of hydrophobic patches due to unfolding and aggregation may be responsible for the decreased solubility (O'Loughlin

Table 2 Changes in solubility of heat-induced polymerized whey proteins

pH	Non-heated	6	7	8			
Solubility (%)	93.20 ± 3.46 ^a	1.91 ± 0.03 ^b		1.90 ± 0.09 ^b		2.38 ± 0.03 ^c	
Pro. con. (%)	Non-heated	6	7	8	9	10	11
Solubility (%)	93.20 ± 3.46 ^a	1.96 ± 0.20 ^b	2.01 ± 0.07 ^b	1.95 ± 0.18 ^b	1.88 ± 0.10 ^b	1.90 ± 0.13 ^b	2.10 ± 0.06 ^b
Temperature (°C)	Non-heated	70	75	80	85	90	95
Solubility (%)	93.20 ± 3.46 ^a	4.75 ± 0.04 ^b	2.39 ± 0.04 ^c	1.95 ± 0.07 ^d	1.89 ± 0.08 ^d	1.98 ± 0.09 ^d	1.95 ± 0.05 ^d
Time (min)	Non-heated	5	10	15	20	25	30
Solubility (%)	93.20 ± 3.46 ^a	2.06 ± 0.10 ^b	2.02 ± 0.05 ^b	2.07 ± 0.11 ^b	2.08 ± 0.08 ^b	1.99 ± 0.03 ^b	1.89 ± 0.08 ^c

Different lower case letters denote significant difference between samples within a row at $p < 0.05$

et al., 2012). Solubility decrease resulted from heating has also been reported for caprine whey protein (Sanmartin et al., 2013).

Samples at pH 8 showed significantly higher solubility than those of samples at pH 6 and pH 7 ($p < 0.05$) and there was no significant difference between samples at pH 6 and pH 7. It was reported that large aggregates forming rate decreased with pH increased from 6 and 8 (Nicolai et al., 2011). Faster large aggregates formation rate may cause more proteins shifted to denaturation at pH 6 and 7. No significant difference was observed among samples ranging from 6 to 11%, indicating that the denaturation of whey protein was independent of protein concentration. Exposure to temperatures above 60 °C can irreversibly affect the solubility of whey proteins and change the relative hydrophobicity at the protein surface. The solubility significantly decreased from 4.75 ± 0.04 to $1.95 \pm 0.07\%$ ($p < 0.05$) with heating temperature increased from 70 to 80 °C. No significant difference was found between samples heated from 80 to 95 °C. It was reported that whey protein solution (10%, pH 6.8) heated at 80 °C for 10 min may result in almost complete denaturation (Haque et al., 2013). Samples heated from 5 to 25 min showed no significant difference ($p > 0.05$) in term of solubility. Prolonged heating time up to 30 min decreased the solubility significantly to $1.86 \pm 0.08\%$ ($p < 0.05$).

Changes in emulsion capacity and stability of heat-induced polymerized whey proteins

Effects of thermal treatment on the emulsifying properties of whey protein were studied. Emulsions were formed by homogenization which dispersed the oil phase into the aqueous dispersing phase. Whey protein being amphiphilic with both hydrophilic and hydrophobic groups is able to form cohesive viscoelastic films at oil/water interfaces, thus form finely dispersed emulsion droplets. The absorbance values of the emulsion droplets can be used to evaluate the emulsifying properties of whey protein.

Emulsion capacity of heat-induced polymerized whey proteins

Emulsion capacity of polymerized whey proteins as functions of pH, protein concentration, heating temperature and time is displayed in Fig. 1. Obviously, conversion of whey protein to polymerized whey protein by heating was accompanied by loss of emulsion capacity. The formation of thicker layer on the oil–water interface by PWP may be responsible for this decrease. Similarly, Britten et al. (1994) found that high level of heat treated whey protein decreased the emulsifying activity index of a mixture of heat denatured and native whey proteins. All factors involved in this study influenced the emulsion capacity of whey proteins. Variation of pH influenced the EC values significantly ($p < 0.05$) and may due to the great impact of pH on protein denaturation and aggregation (Fig. 1A). Polymerized whey protein behaved gradually lower EC with protein concentration increased may due to the increased aggregates size caused by higher protein concentration (Fig. 1B) (Havea et al., 2001). EC values increased with temperature elevated from 70 to 85 °C ($p < 0.05$), indicating more protein molecules could migrate to the oil–water interface and increase the formation of stable emulsions (Fig. 1C). No significant difference was observed between samples heated at 85–95 °C ($p > 0.05$). Heating time affected the EC value negatively with values decreased with heating time increased from 5 to 30 min (Fig. 1D).

Emulsion stability of heat-induced polymerized whey proteins

Emulsion stability (ES) reflects the ability of the proteins to impart strength to an emulsion and resistance to stress (Palatnik et al., 2015). Figure 2 displays the ES values of all samples prepared under various conditions. All polymerized whey proteins showed significantly lower ES values than respective controls ($p < 0.05$).

Fig. 1 Emulsion capacity of polymerized whey proteins at different pH (A), protein concentration (B), heating temperature (C) and time (D). Note: Different lower case letters denote significant difference between samples at $p < 0.05$

ES values showed a positive correlation with pH, which increased from 26.72 ± 2.61 to 163.31 ± 16.17 min with pH changing from 6 to 8 ($p < 0.05$) (Fig. 2A). The increased negative charges may cause a barrier for the close approach of droplets and thus decreased the rate to coalescence (Zayas, 1997). As for protein concentration, emulsion stability decreased with protein concentration increased (Fig. 2B) ($p < 0.05$). Emulsion stability showed significant variation between treatment at temperature from 70 to 95 °C (Fig. 2C) ($p < 0.05$). A remarkable low emulsion stability was observed for samples heated at 70 °C, which was the critical temperature for whey protein denaturation. An increased level of disulfide bonds in polymerized whey protein isolate resulted in noticeable improvement in emulsion stability (Qi et al., 2017). ES values significantly decreased with heating time increased from 5 to 30 min (Fig. 2D) ($p < 0.05$). During heat treatment, small aggregates are formed at the beginning, and then larger aggregates are formed at increasing temperature or heating time (Raikos, 2010). Following heat treatment, large protein aggregates may be unable to cover the fat droplets efficiently, leading to emulsion instability.

Changes in antioxidant capacity of heat-induced polymerized whey proteins

DPPH scavenging ability of heat-induced polymerized whey proteins

DPPH, a stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule, is widely used to investigate radical scavenging activity (Gad et al., 2011). All samples showed dose-dependent DPPH radical scavenging behaviors at concentrations of 1–10 mg/mL (Table S1 in Supplementary File). Compared with control samples, PWP samples showed significantly lower DPPH eliminating ability, $p < 0.05$, suggesting the lower electron-donating ability. Similar results were reported for white bean proteins (Arcan and Yemenicioglu, 2007). Some of the antioxidant groups in whey proteins may be reduced or masked by heating which caused the aggregation of whey protein (De la Fuente et al., 2002).

The IC_{50} values were calculated and these values represent the concentrations which had the ability to scavenge 50% of radicals. A lower IC_{50} value corresponds to a larger scavenging activity (Kerasioti et al., 2014). Table 3 shows the calculated IC_{50} values in DPPH radical scavenging assay for all samples. The control samples have the best

Fig. 2 Emulsion stability of polymerized whey proteins at different pH (A), protein concentration (B), heating temperature (C) and time (D). Note: Different lower case letters denote significant difference between samples at $p < 0.05$

Table 3 Changes in DPPH radical scavenging activity (IC_{50} values) of heat-induced polymerized whey proteins

pH	6 (Non-heated)	7 (Non-heated)	8 (Non-heated)	6	7	8	
IC ₅₀ (mg/mL)	15.47 ± 1.32 ^a	6.01 ± 0.52 ^b	6.32 ± 0.47 ^b	27.20 ± 1.83 ^c	25.81 ± 1.72 ^d	37.37 ± 2.21 ^e	
Pro. Conc. (%)	Non-heated	6	7	8	9	10	11
IC ₅₀ (mg/mL)	6.01 ± 0.52 ^a	15.14 ± 1.21 ^b	17.71 ± 1.31 ^c	24.28 ± 1.96 ^d	24.64 ± 1.86 ^d	28.32 ± 1.45 ^e	35.21 ± 2.44 ^f
Temperature (°C)	Non-heated	70	75	80	85	90	95
IC ₅₀ (mg/mL)	6.01 ± 0.52 ^a	17.63 ± 1.47 ^b	20.90 ± 1.26 ^c	21.39 ± 2.09 ^c	26.95 ± 1.46 ^d	27.01 ± 1.53 ^d	28.28 ± 0.85 ^e
Time	Non-heated	5	10	15	20	25	30
IC ₅₀ (mg/mL)	6.01 ± 0.52 ^a	15.05 ± 1.29 ^b	15.95 ± 1.34 ^b	16.40 ± 1.21 ^b	17.25 ± 1.37 ^c	17.91 ± 0.97 ^c	26.21 ± 1.32 ^d

Different lower case letters denote significant difference between samples within a row at $p < 0.05$

scavenging ability with an IC_{50} value of 6.01 ± 0.52 mg/mL for control sample at pH 7. Compared with controls, all PWP samples showed significantly higher IC_{50} values ($p < 0.05$). Similar result was reported by Liu et al. (2007). As for pH, samples heated at pH 7 showed the lowest IC_{50} value among all the pH values. Generally, IC_{50} value of samples increased with increasing protein concentration, heating temperature, and heating time ($p < 0.05$).

ABTS scavenging ability of heat-induced polymerized whey proteins

ABTS radical cation decolourisation test is also a spectrophotometric method widely used for the assessment of antioxidant activity of various substances (Miliauskas et al., 2004). Results (Supplementary File Table S2) show that scavenging activity to ABTS radical is proportional to

Table 4 Changes in ABTS radical scavenging activity (IC₅₀ values) of heat-induced polymerized whey proteins

pH	6 (Non-heated)	7 (Non-heated)	8 (Non-heated)	6	7	8	
IC ₅₀ (mg/mL)	3.16 ± 0.21 ^a	1.98 ± 0.09 ^b	1.43 ± 0.08 ^c	1.63 ± 0.08 ^d	0.71 ± 0.04 ^e	0.40 ± 0.01 ^f	
Pro. Conc. (%)	Non-heated	6	7	8	9	10	11
IC ₅₀ (mg/mL)	1.98 ± 0.09 ^a	0.63 ± 0.04 ^b	0.68 ± 0.05 ^{bc}	0.70 ± 0.04 ^c	0.74 ± 0.05 ^d	0.79 ± 0.06 ^e	0.88 ± 0.05 ^f
Temperature (°C)	Non-heated	70	75	80	85	90	95
IC ₅₀ (mg/mL)	1.98 ± 0.09 ^a	0.34 ± 0.04 ^b	0.45 ± 0.05 ^c	0.56 ± 0.06 ^c	0.75 ± 0.05 ^d	0.83 ± 0.04 ^d	0.85 ± 0.03 ^e
Time	Non-heated	5	10	15	20	25	30
IC ₅₀ (mg/mL)	1.98 ± 0.09 ^a	1.75 ± 0.06 ^b	1.54 ± 0.07 ^c	1.31 ± 0.05 ^d	1.13 ± 0.06 ^e	1.01 ± 0.07 ^f	0.76 ± 0.06 ^g

Different lower case letters denote significant difference between samples within a row at $p < 0.05$

concentration (1–10 mg/mL) for all samples under various conditions. All PWP exhibited more potent ABTS scavenging ability than respective control samples. Similar results were reported for heated pea protein isolate (Zilic et al., 2012). The authors reported that hydrogen and –S–S bonds may promote reactions of proteins with free radicals.

Table 4 presents the IC₅₀ values in ABTS radical scavenging assay. It was found that PWP was significantly more active than native whey protein. As for pH, the order of IC₅₀ value of PWP was as follows: pH 6 (IC₅₀ 1.63 ± 0.08) > pH 7 (IC₅₀ 0.71 ± 0.04) > pH 8 (IC₅₀ 0.40 ± 0.01). ABTS scavenging ability significantly increased with pH elevated from 6 to 8, $p < 0.05$. At pH 8, the thiol group of β-lactoglobulin is more readily available for reactions, increasing disulfide bonds contents (Havea et al., 2001). The IC₅₀ value increased with protein concentration increased. Samples under different temperatures from 70 to 95 °C exhibited activity with IC₅₀ values of 0.34 ± 0.04, 0.45 ± 0.05, 0.56 ± 0.06, 0.75 ± 0.05, 0.83 ± 0.04, and 0.85 ± 0.03 mg/mL, respectively. Polymerized whey protein showed decreased IC₅₀ values with increasing heating time (5–30 min).

Whey protein under heat treatment undergone denaturation and aggregation. All factors including pH, protein concentration, heating temperature, and heating time had significant influence on the solubility, emulsifying and antioxidative properties. Decreased solubility is accompanied with decrease in emulsifying properties. Protein denaturation and aggregation caused a decreased DPPH but increased ABTS radicals scavenging ability.

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