กลไกการยับยั้งการเกิดสีน้ำตาลเมลลาร์ดของโปรตีนเวย์ไฮโดรไลเสทชนิดผงในผลิตภัณฑ์โปรตีนที่ผ่านการฆ่าเชื้อระดับสเตอริไลส์

Mechanisms of Maillard Browning Reduction of Whey Protein Hydrolysate Powder in Sterilized Protein Products

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บทคัดย่อ

งานวิจัยนี้ศึกษากลไกการลดการเกิดสีน้ำตาลจากปฏิกิริยาเมลลาร์ดของโปรตีนเวย์ไฮโดรไลเสทชนิดผงที่เตรียมจากการย่อยโปรตีนเวย์เข้มข้นเชิงการค้าด้วยเอนไซม์ทริพซิน (E.C.3.4.21.4) ที่ 37 องศาเซลเซียส เป็นเวลา 60 นาทีและทำแห้งแบบพ่นฝอยร่วมกับน้ำตาลทรีฮาโลส เมื่อนำไปเติมในสารละลายโปรตีนเวย์เข้มข้นเพื่อให้มีโปรตีนสุดท้าย ร้อยละ 1.3 โดยเป็นโปรตีนจากโปรตีนเวย์เข้มข้นร้อยละ 1.0 และจากโปรตีนเวย์ไฮโดรไลเสท ร้อยละ 0.3 ทำการเติมน้ำตาลแลกโทส 0 - 0.25 โมลต่อลิตร และสเตอริไลส์สารละลายโปรตีนผสมที่อุณหภูมิ 121 องศาเซลเซียส เป็นเวลา 15 นาที พบว่า ความสามารถในการลดการเกิดสีน้ำตาลของโปรตีนเวย์ไฮโดรไลเสทผงมาจากความสามารถในการต้านอนุมูลอิสระของโปรตีนเวย์ไฮโดรไลเสทที่เพิ่มขึ้นโดยประเมินจากความสามารถในการต้านอนุมูลอิสระด้วยวิธีดูดซับออกซิเจนแรดิคอล (oxygen radical absorbance capacity assay) และความสามารถในการกำจัดอนุมูลอิสระของ $ABTS^{\bullet+}$ ($ABTS^{\bullet+}$ radical scavenging assay) เนื่องจากโปรตีนเวย์ไฮโดรไลเสทมีเปปไทด์สายสั้นขนาดเล็กกว่า 6.5 กิโลดาลตันเพิ่มขึ้น นอกจากนั้นยังพบว่า หมู่ไทออลของกรดแอมิโนมีบทบาทสำคัญในการลดการเกิดสีน้ำตาลเมลานอยดินส์ด้วย ซึ่งอาจเกิดจากการที่หมู่ไทออลเข้าร่วมในปฏิกิริยาเมลลาร์ดเกิดเป็นสารวาวแสงโดยตรงระหว่างวิถีของปฏิกิริยาเมลลาร์ดหรือทำหน้าที่ให้อะตอมไฮโดรเจนในการต้านอนุมูลอิสระแล้วเปลี่ยนรูปเป็นสารประกอบซัลเฟอร์ที่คงตัว ทำให้ลดการเกิดสีน้ำตาลจากปฏิกิริยาเมลลาร์ดเต็มรูปแบบในสารละลายโปรตีน – น้ำตาลแลกโทสที่ผ่านการสเตอริไลส์ได้

คำสำคัญ: ปฏิกิริยาเมลลาร์ด สีน้ำตาล การยับยั้ง เวย์ โปรตีนไฮโดรไลเสท

ABSTRACT

This research investigated the mechanisms in the reduction of Maillard browning reactions of whey protein hydrolysate powder (WPH) prepared by hydrolysis of whey protein concentrate (WPC) with trypsin (E.C.3.4.21.4) at 37 °C for 60 min and spray-dried with trehalose when used in the mixed solutions containing protein 1.3 % (w/v) from WPC 1.0 % and WPH 0.3 %, added with lactose 0 – 0.25 mol L^{-1}, and sterilized at 121 °C for 15 min. The mechanisms of browning reduction of reconstituted WPH powder involved the increasing antioxidant activities of WPH, assessed by oxygen radical absorbance assay and $ABTS^{\bullet+}$ radical scavenging capacity assay of the short-chain peptides of less than 6.5 kDa. Besides, the thiol groups played an essential role in reducing the occurrence of brown melanoidin pigments, probably by directly involved in the Maillard reactions that generated fluorescent products or donating the hydrogen atom to the free radicals, and subsequently transforming to stable intermediates, thus suppressing the formation of brown pigments in full Maillard reactions of protein – lactose solutions subjected to sterilization.

Keywords: maillard reaction, browning, inhibition, whey, hydrolysate

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INTRODUCTION

The Maillard reaction or glycation is essential in food processing and storage. It is also a reaction that occurs in living organisms. Although this reaction enhances the functional properties of foods such as water hydrophilicity, antioxidative activity, and functional properties of proteins and starches [1], the Maillard reaction end products (MRPs) may lead to the loss of nutrients and reducing the proportion of digested protein [2]. The formation of MRPs has both benefits and disadvantages in the food and beverage industry. On the positive side, MRPs are the source of color and taste. The advanced glycation end products (AGEs) and the melanoidins consist of eneaminol, enediol, and reductone, which had antioxidant capacity [3]. However, recent reports have shown toxicity of the MRPs to both mice and human cells [3, 4]. The accumulation of MRPs in biological systems has been linked to important pathogenesis in diabetes, vascular disease, Alzheimer's disease, and age-related diseases [3].

The initial Maillard reaction is due to the condensation of carbonyl groups of aldehydes and amino groups of proteins or amino acids, which leads to the formation of Schiff-base compounds and Amadori rearrangement reaction [5]. Although the ε-amino group of lysyl sidechain in proteins preferably reacts with carbonyl groups, other amino acids, such as methionine, cysteine, and tryptophan, can be involved in a Maillard reaction [2] due to the reactions of their side chains.

After the Amadori rearrangement, further Maillard reactions generated the highly reactive compounds or AGEs through condensation and fragmentation. The formation of AGEs is primarily related to redox reactions when Amadori products create intermediates such as dicarbonyls, oxoaldehydes, glyoxal, and methylglyoxal. Most AGEs are unstable and cause autooxidation between AGEs and cross-linking reactions [5].

The final stage of the Maillard reaction is due to the condensation of aldol and the condensation of aldehyde-amine, leading to the formation of a high MW N-containing brown pigment called melanoidins [5, 6]. The suppression of the occurrence of AGEs and MRPs can be achieved by inhibiting the oxidation mechanism using natural compounds, for example by using polyphenolic compounds [7, 8], amino acids [9], and peptides and proteins [4, 10, 11] to hinder the cross-linking pathway among reactive molecules in the Maillard pathway.

The production of peptides and bioactive protein hydrolysate and the sequence of amino acid residues in short-chain peptides has been extensively investigated [12, 13] for the inhibition of oxidation in cell models. However, little is known about the potential use of antioxidative food peptides and protein hydrolysates to reduce the Maillard reaction in sterilized foods. Moreover, the mechanism of inhibiting the brown pigment formation from the Maillard reaction is mostly not known. The objective of this study was to elucidate the mechanisms of Maillard browning inhibition of hydrolyzed whey protein (WPH) when used in sterilized commercial whey protein concentrate (WPC) solutions at different lactose levels. The

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insights from this research can be used in controlling the Maillard browning reaction in sterilized milk protein products. Trypsin (E.C.3.4.21.4) was chosen because it is a significant protease in mammals and specific in the hydrolyzing C-terminal to arginine and lysine [14], which will not interfere with cystine content.

MATERIALS AND METHODS

1. Materials

Commercial WPC was imported and repacked by a local distributor in Thailand. It contained 75.83% protein (w.b.), 6.5% moisture content (w.b.), 2.71% ash (w.b.), 1.05% fat (w.b.) and 13.91% carbohydrate (w.b.) [15]. Trypsin (E.C. 3.4.21.4; 10,000 BAEE U mg^{-1}), *o*-phthaldialdehyde (OPA), fluorescein (Na salt), and lactose monohydrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate, 1-anilino-8-naphthalenesulfonate (ANS), quinine, and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (Steinheim, Germany); 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide (NEM) were purchased from Fluka (Buchs, Switzerland). Dithiothreitol 99% (DTT) was purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate dihydrate, and sodium chloride were purchased from Ajax Finechem (Taren Point, NSW, Australia). Trehalose (kindly supplied by the East Asiatic Company, Bangkok, Thailand) was used as a carbohydrate excipient during spray-drying.

2. Characteristics of whey protein hydrolysates (WPH)

2.1 Effect of hydrolysis time on the molecular weight of proteins

WPC suspension (2% protein w/v) was prepared in distilled water and hydrolyzed by trypsin at 37°C, pH 8.0, for 30, 60, and 90 min using protein to enzyme ratio of 100:1. The hydrolysis was inactivated by adjusting the pH to 2.0 using 5 mol L^{-1} HCl. The WPC and hydrolysates were characterized for molecular weight (MW) by glycine SDS-PAGE [16] using 12% separating gel and 4% stacking gel. An aliquot of the sample solution containing 7 µg protein, or 4 µL low-range MW standards (6,500 – 66,000 Da, Sigma-Aldrich, M3913) was loaded into each well. Electrophoresis was run at a constant current of 150 V for 45 min. Gel slabs were fixed and stained simultaneously using stain solution (10% acetic acid, 40% methanol, and 0.01% Coomassie Brilliant Blue R-250 (Bio-Rad)) for 30 min, and then destained (10% methanol, 7.5% acetic acid, and 82.5% deionized water).

2.2 Preparation of whey protein hydrolysate powder

Commercial WPC suspension 7.5% w/v, which contained 5.69% protein w/v, was prepared in distilled water and hydrolyzed by trypsin using protein to enzyme ratio of 100:1 and hydrolysis time of 60 min at 37°C using method described earlier [4]. Trypsin was inactivated by adjusting the pH to 2.0 using 5 mol L^{-1} HCl. The hydrolysate was mixed with trehalose (TH)

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excipient using a ratio of protein to the carbohydrate of 0.3:0.7. Trehalose was used to embed proteins and peptides in its amorphous matrix upon drying and storage due to the high glass transition temperature of TH around 115-117°C [17]. The suspensions containing the total solid of 20 % w/v were fed at a rate of 4.0 ± 0.5 mL min^{-1}, and dried using an inlet air of 130 °C and outlet air of 100°C at a flow rate of around 600 Nm^3 min^{-1} using a Mini Spray Dryer B-190 (Büchi, Flawil, Switzerland). The WPH-TH powder was packed in a sealed aluminum foil bag and stored at -20 °C before analysis.

2.3 Determination of amino groups

Amino group content was determined by *the o*-phthaldialdehyde (OPA) method [18]. The amount of reactive NH_2 groups was obtained by measuring the absorbance at 340 nm using a Tecan multifunctional microplate reader (Infinite® M200 PRO; Männedorf, Switzerland). All samples were determined in triplicate, using distilled water as a blank; values were reported as absorbance at 340 nm.

2.4 Determination of thiol groups

Thiol group contents in reconstituted commercial WPC and WPH-TH were determined using Ellman's reagent [19]. The absorbance was measured at 412 nm using a Tecan multifunctional microplate reader (Infinite® M200 PRO; Männedorf, Switzerland).). The thiol contents were calculated according to Eq. (1):

$$SH\ content\ (\mu mole\ g^{-1}\ protein) = \frac{73.54 \times Abs_{412} \times 6.04}{Sample\ concentration\ (mg\ mL^{-1})} \quad (1)$$

2.5 Oxygen radical absorbance capacity ($ORAC_{FL}$)

Reconstituted commercial WPC and WPH-TH (1.3% protein w/v) were prepared in 0.1 mol L^{-1} phosphate buffer, pH 8.0, and evaluated for oxygen radical absorbance capacity-fluorescein ($ORAC_{FL}$) as determined by fluorescence assay [20, 21] in comparison with commercial WPC suspensions. The $ORAC_{FL}$ assays were carried out on a FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany) and reported as µmol Trolox equivalent per mg of protein (µmol TE mg^{-1} protein).

2.6 $ABTS^{\bullet+}$ radical scavenging capacity (Trolox equivalent antioxidant capacity, TEAC)

Suspensions of commercial WPC and WPH-TH (1.3% protein w/v) were prepared in 5 mmol L^{-1} phosphate-buffered saline (PBS), pH 7.4, and evaluated for Trolox equivalent antioxidant capacity (TEAC) [22]. The $ABTS^{\bullet+}$ solution was diluted with PBS to an absorbance of 0.700 at 734 nm as measured by a spectrophotometer (Spectronic 20+; Thermo Fisher Scientific, Waltham, MA, USA). Thirty µL of the sample was added to 3 mL of $ABTS^{\bullet+}$ solution, equilibrated at 30 °C for 4 min. Then, it was measured for absorbance at 734 nm using Trolox as an antioxidant standard. PBS was used as a blank. All determinations were carried out in triplicate. The absorbance at 734 nm was plotted as a function of antioxidant concentration. Each sample's antioxidant capacity was calculated as µmol Trolox equivalent per mg of protein (µmol TE mg^{-1} protein).

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3. Antibrowning characteristics of reconstituted whey protein hydrolysate in sterilized mixed WPC-lactose suspensions

3.1 Effect of thiol-blocking agent and sterilization on Maillard reactions in WPH.

Five mL of mixed WPH-TH (1.3% protein w/v) and 0.25 mol L^{-1} added lactose was prepared in a 20 mL test tube with a cap and sterilized at 121°C for 15 min in an autoclave in the absence or presence of thiol-blocking agent *N*-ethylmaleimide (NEM). Before sterilization, NEM was added to block SH groups in reconstituted WPH. The final concentrations of NEM were 0, 15, 20, and 25 mmol L^{-1}. After sterilizing and cooling to 80 °C in an autoclave, the sterilized WPH was cooled down to 27°C (room temperature) using running water within 30 min. The samples were then determined for AGEs and brown pigment formation using the methods described below.

3.2 Effect of WPH-TH addition on Maillard reactions in sterilized whey protein – lactose mixed suspension

Commercial WPC suspension (1–1.3% protein w/v) in 0.1 mol L^{-1} phosphate buffer, pH 8.0, was supplemented with appropriate concentration of lactose solution, with or without WPH-TH (0–0.3% protein w/v). The final concentration of proteins in suspension was 1.3% (w/v) and added lactose concentration of 0, 0.0625, 0.125, 0.188 and 0.25 mol L^{-1}. The mixed suspensions were sterilized at 121°C for 15 min, cooled at room temperature and characterized for reactive NH_2 group contents after sterilization by the OPA method described above. The antibrowning activity was evaluated as AGE formation and brown pigment formation.

3.3 Determination of advanced glycation end products (AGEs)

The amount of advanced glycation end products (AGEs) was measured as % fluorescence intensity using an excitation wavelength of 380 nm and an emission wavelength of 465 nm, as measured by a Tecan multifunctional microplate reader (Infinite® M200 PRO; Männedorf, Switzerland). All samples were determined in triplicate. Quinine sulfate (1 µg mL^{-1} in 0.1 N H_2SO_4) was used as a reference. The formation of AGEs was reported as % fluorescence intensity compared to that of quinine sulfate as 100% [23].

3.4 Evaluation of brown pigment formation

The end-stage of Maillard reactions was detected as brown pigment formation by measuring the absorbance at 420 nm and correcting for turbidity by subtracting the absorbance at 620 nm [24] using a Tecan multifunction microplate reader (Infinite® M200 PRO; Männedorf, Switzerland).

4. Statistical analysis

Experiments were carried out in two independent trials of spray-dried WPH-TH preparation; each trial was analyzed in triplicate. The data were analyzed by analysis of variance (ANOVA) with significance at $P<0.05$. Tukey's test determined significant differences among mean values. All statistical analyses were performed using Graphpad Prizm 8.4.2 (GraphPad Software Inc., San Diego, CA, USA).

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RESULTS AND DISCUSSION

1. Characteristics of whey protein hydrolysates

The molecular weight profiles of commercial whey protein concentrate (WPC) and hydrolyzed whey protein (WPH) obtained after hydrolysis by trypsin for 30, 60, and 90 min, compared to the suspension before hydrolysis (WPH 0) are shown in Figure 1. SDS-PAGE showed that the major proteins in commercial WPC (β-lactoglobulin (β-Lg, MW 18 kDa) and α-lactalbumin (α-La, MW 14 kDa)) retained after 90 min incubation with trypsin. Commercial WPC before hydrolysis, however, contained additional proteins having MWs between 29 and 36 kDa, between 45 and 66 kDa, and above 66 kDa. These proteins were likely polymerized products of the indigenous milk proteins that existed in commercial WPC before tryptic hydrolysis.

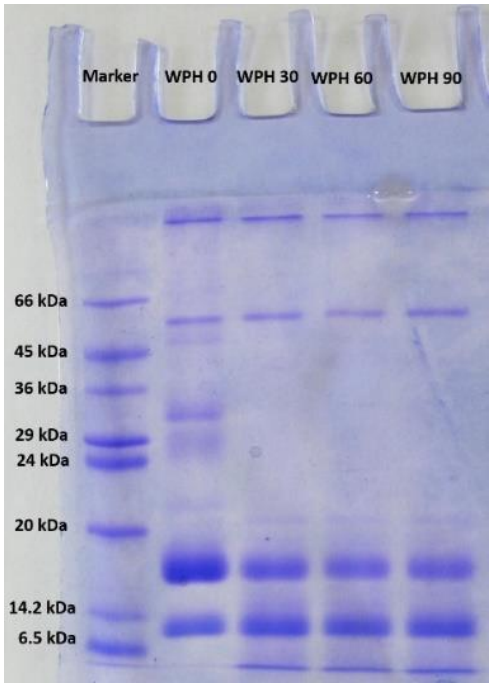

Figure 1 Effect of hydrolysis time on the MW profiles of commercial WPC before hydrolysis (WPH 0) and after hydrolysis at 37 °C for 30, 60, and 90 min designated as WPH 30, WPH 60 and WPH 90, respectively.

Trypsin partially hydrolyzed some proteins in the commercial WPC (Figure 1). However, β-Lg and α-La were hydrolyzed to some extent, observed as their less intense bands. Trypsin specifically hydrolyzes C-terminal to arginine and lysine [14]. The native whey proteins are quite resistant to tryptic hydrolysis and could be hydrolyzed by trypsin for less than 20% [25]. The complete degradation of α-La in WPCs by trypsin at 37°C and pH 8 could take 120 min while β-Lg would take longer time for trypsin to hydrolyze [26]. Nonetheless, the presence of low-MW peptides of less than 6.5 kDa after hydrolysis for 30 – 90 min was observed in this study. The proteins having MWs within the ranges of 29 to 36 kDa and 45 to 66 kDa were entirely hydrolyzed after 30 min hydrolysis.

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The characteristics of reconstituted commercial WPC and WPH-TH are shown in Table 1. The preparation of spray-dried WPH-TH increased NH_2 group content significantly but did not affect the thiol (SH) group. Approximately 1.5 fold increase in the NH_2 group content of WPH-TH determined by the OPA method suggested that the degree of hydrolysis was quite low, probably due to the compact globular structure of whey proteins not easily digested unless they were unfolded and exposed specific peptide bonds of arginine and lysine before hydrolysis as suggested by [25].

The increase in antioxidant capacities measured as $ORAC_{FL}$ and TEAC in WPH-TH, compared to that of commercial WPC, was probably due to greater exposure of reactive groups responsible for antioxidant activities in the hydrolysates, such as thiol (SH) group in cysteine residue. The small MWs peptides in WPH obtained after tryptic hydrolysis could also play significant roles in hydrogen atom transfer and electron transfer, especially if they contained aromatic ring side chain such as histidine, tryptophan, phenylalanine, tyrosine and proline. However, the tryptic hydrolysis for 60 min investigated in this study was quite limited to minimize the exposure of aromatic amino acid that could generate bitter taste. The $ORAC_{FL}$ of commercial WPC was 0.20 μmol TE mg^{-1} protein before tryptic hydrolysis, which increased to 0.34 μmol TE mg^{-1} protein in WPH-TH ($P<0.05$). Likewise, the TEAC of WPH-TH was higher than that of the unhydrolyzed commercial WPC ($P<0.05$).

Table 1 Chemical characteristics of reconstituted whey protein products.

Types of whey protein powder	OPA NH_2 content reported as absorbance at 340 nm	Accessible SH content (μ mole g^{-1} protein)	Oxygen radical absorbance capacity ($ORAC_{FL}$) (μmol TE mg^{-1} protein)	Trolox equivalent antioxidant capacity (TEAC) (μmol TE mg^{-1} protein)
Commercial WPC	0.239^{b}±0.034	21.3^{a}±0.2	0.20^{b}±0.05	0.08^{b}±0.01
WPH-TH	0.361^{a}±0.007	20.8^{a}±0.5	0.34^{a}±0.00	0.12^{a}±0.00

Means ± SD in the same column from 2 independent trials, followed by different superscripts, are significantly different ($P < 0.05$).

2. Antibrowning characteristics of whey protein hydrolysates

The indigenous lactose of 0.007 mol L^{-1} in the 1.3% whey protein suspension was high enough to induce brown pigment formation when sterilization. The sterilized commercial WPC suspensions were light brown (Figure 2a). However, the addition of WPH to WPC before sterilization lowered the brown color in sterilized WPC+WPH having similar protein and lactose contents (Figure 2b).

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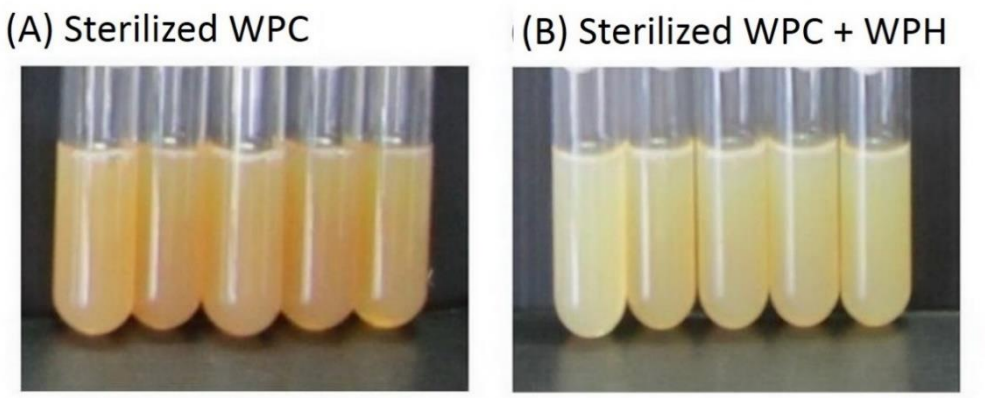

Figure 2 Appearance of (A) sterilized commercial WPC containing 1.3% protein (w/v) and (B) sterilized commercial WPC (1.0 % protein (w/v) added with WPH (0.3% protein (w/v)). Final concentration of protein in suspension was 1.3 % (w/v) and indigenous lactose was 0.007 mol L^{-1}.

Figure 3 shows that when lactose was added at the level of 0.25 mol L^{-1} (i.e., 8.55% w/v), the addition of antioxidative WPH-TH in WPC suspensions before sterilization suppressed the formation of AGEs (P <0.05). At a low concentration of indigenous lactose in WPC (0.007 mol L^{-1}), the fluorescence intensity of sterilized WPC and WPC+WPH-TH was around 22% of quinine sulfate, and the influence of WPH was not apparent. However, lactose 0.25 mol L^{-1} drastically increased the formation of AGEs in sterilized WPC (P<0.05). The presence of antioxidative WPH-TH helped lower the formation of fluorescent AGEs in sterilized WPC+WPH-TH) when lactose concentration was increased, suggesting that WPH-TH could suppress further redox reactions of Amadori products that formed fluorescence compounds.

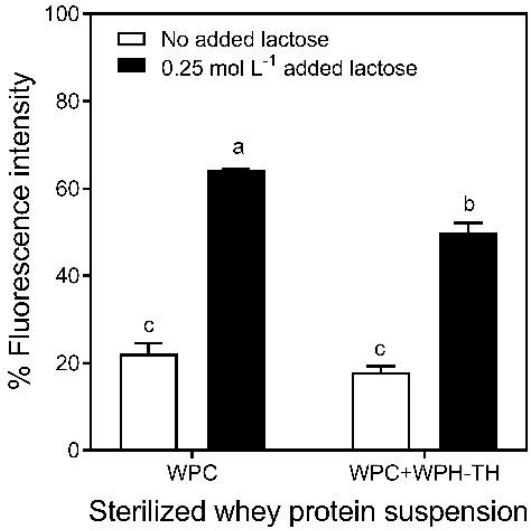

Figure 3 Effect of WPH-TH addition in WPC suspensions on the formation of advanced glycation end products (measured as % fluorescence intensity) in sterilized protein suspensions. Total protein concentration was 1.3 % (w/v). Bars represent the standard deviation of 2 independent trials.

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The SH-group roles in WPH-TH in antibrowning ability in the presence of 0.25 mol L^{-1} added lactose were further investigated. NEM blocked the SH groups in WPH-TH before sterilization. Figure 4a shows that in the absence of NEM, fluorescence AGEs found in sterilized WPH-TH were around 57% of quinine sulfate (Figure 4a). When NEM blocked SH group, sterilized WPH-TH had a lower formation of fluorescence AGEs ($P<0.05$) to 20%, suggesting that the SH group in reconstituted WPH-TH also participated in the formation of AGEs in addition to the well-known ε-NH_2 group of lysyl residue.

The formation of brown pigment in sterilized WPH-TH in the presence of 0.25 mol L^{-1} added lactose was not affected by SH-blocking agent NEM ($P\geq0.05$), suggesting that the AGEs induced by SH group did not proceed to brown pigment formation. The SH group's involvement from cysteine residue was likely to form fluorescence intermediates from Amadori products rather than the condensation to polymerized brown-colored N-containing compound melanoidins in the final stage.

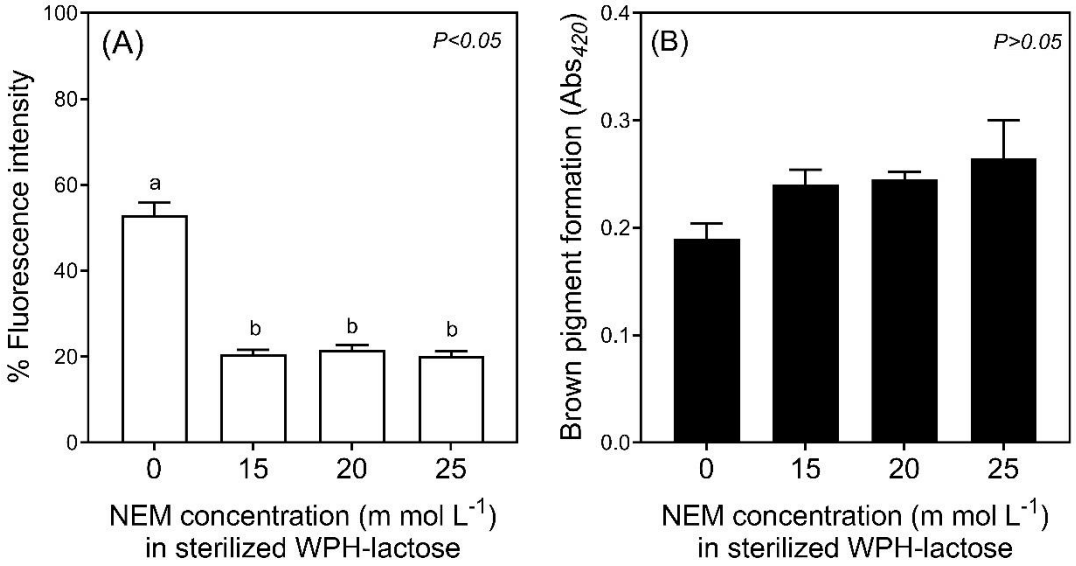

Figure 4 Effect of thiol-blocking agent *N*-ethylmaleimide (NEM) on the formation of (A) advanced glycation end products (measured as % fluorescence intensity) and (B) brown pigment formation (measured as absorbance at 420 nm) of sterilized WPH-lactose mixed suspensions containing 1.3% w/v protein and 0.25 mol L^{-1} added lactose. Bars represent the standard deviation of 2 independent trials.

Cysteine residue could donate H atom and form stable complexes [9] is, in part, responsible for the antibrowning ability of WPH-TH and reduced brown color shown in Figure 2. The SH group of cysteine not only inhibited the formation of AGEs by donating H atom to radicals in both early and advanced stages of Maillard reactions but could also react directly

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with dicarbonyl compounds to give stable *S*-carboxymethylcysteine [27], thus reducing the full length of Maillard brown color shown in Figure 2b.

The influences of lactose concentration on chemical characteristics of sterilized WPC, WPC+WPH-TH, and WPH-TH suspensions (1.3% protein) are shown in Figure 5. In the absence of added lactose, sterilized WPC+WPH-TH and WPH-TH suspensions (containing 0.007 mol L^{-1} indigenous lactose) had slightly higher NH_2 group content than did the sterilized WPC suspensions (Figure 5a; (P<0.05)) due to the presence of peptides and free NH_2 groups in the hydrolysates. Despite the higher NH_2 group, the presence of WPH-TH before sterilization helped lower the formation of AGEs (Figure 5b) and a much lower brown pigment formation (Figure 5c) in sterilized WPC+WPH and WPH. It should be noted that although WPH investigated in this study contained intact β-Lg and α-La with a slight amount of less than 6.5 kDa peptides (Figure 1) and similar contents of SH group (Table 1), the antioxidative WPH could help to reduce the formation of brown pigment.

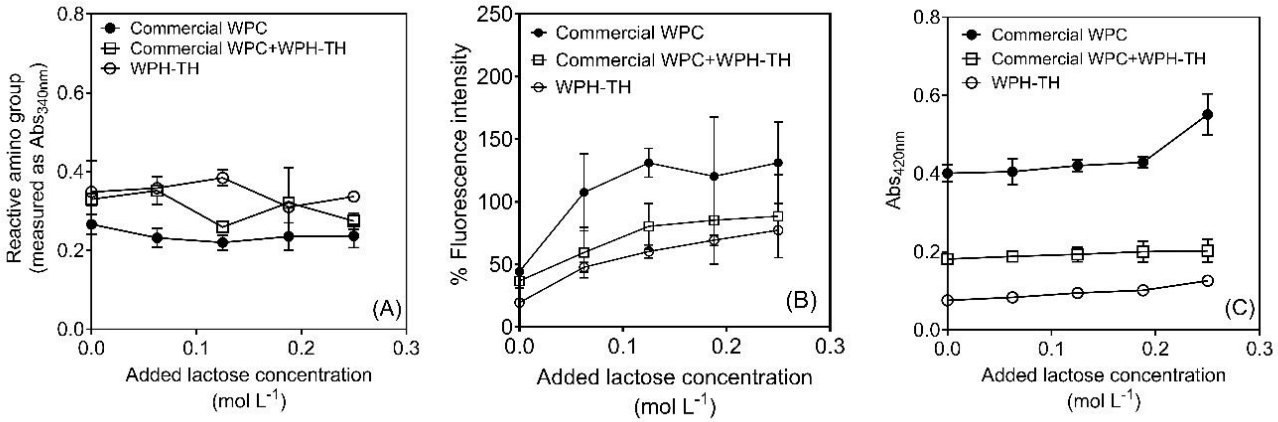

Figure 5 Effect of added lactose concentration on (A) reactive amino group content, (B) advanced glycation end products measured as % fluorescence intensity, and (C) brown pigment formation measured as absorbance at 420 nm in sterilized protein-lactose suspensions containing 1.3% protein (w/v). Bars represent the standard deviation of 2 independent trials.

Raising the lactose concentration to 0.25 mol L^{-1} enhanced the formation of AGEs (Figure 5b) and brown pigment (Figure 5c) in sterilized WPC. However, the presence of WPH suppressed brown pigment formation in sterilized suspensions, observed as low value of absorbance at 420 nm of sterilized WPC+WPH and WPH suspensions. In the absence of WPH, adding lactose at the level of 0.0625 mol L^{-1} increased the formation of AGEs (Figure 5b) in sterilized WPC. Further increase in lactose concentration did not significantly change % fluorescence intensity of sterilized WPC ($P \geq 0.05$). The brown pigment formation in sterilized WPC-lactose suspensions remained similar up to 0.20 mol L^{-1} added lactose, then dramatically increased when lactose concentration was added at 0.25 mol L^{-1} while

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%fluorescence intensity remained plateau (Figure 5c).

The effect of lactose concentration on the formation of AGEs in sterilized WPC+WPH and WPH was not as drastic as found in sterilized WPC, probably due to the effectiveness antioxidative WPH in suppressing the formation of fluorescence AGEs at high lactose concentration. The present study suggested that the antibrowning ability of antioxidative WPH was *via* hydrogen atom transfer reaction (measured by $ORAC_{FL}$ assay) and electron transfer reaction (measured by TEAC assay).

This study provides information on the possibility of tryptic protein hydrolysate powder prepared in the presence of trehalose (TH) excipient to suppress the formation of AGEs and further brown pigment formation of N-containing melanoidins in sterilized whey protein-lactose mixed suspension. TH itself is not a reducing sugar. It has no antibrowning or antioxidant activities (result not shown) and was added to immobilize antioxidative whey peptides in the powder's glassy matrix. Storage stability of WPC containing antibrowning WPH in different carbohydrate excipients is underway.

CONCLUSION

This study demonstrated that reconstituted antioxidative WPH obtained from tryptic hydrolysis for 60 min, which partially hydrolyzed proteins, could reduce Maillard browning reactions induced during sterilization of whey proteins and lactose at 121 °C for 15 min. The antibrowning mechanisms involved the inhibition of brown pigment formation by lowering the formation of AGEs via antioxidative activities of reconstituted WPH by short-chain peptides of less than 6.5 kDa not present in the commercial WPC. The insights from this research could help control the formation of brown color pigments in thermally processed protein drinks.

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