Kasetsart J. (Nat. Sci.) 48 : 759 - 768 (2014)

Effect of pH and Whey Protein Isolate to Glucose Ratios on the Formation of Maillard Reaction Products as Antioxidants

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ABSTRACT

A Maillard reaction is one of the common non-enzymatic browning reactions in foods subjected to heat treatment and involves an interaction between protein and reducing sugars. Whey protein isolate (WPI) contains at least 90% soluble proteins. It was hypothesized that WPI could interact with glucose (Glu) leading to the formation of Maillard reaction products (MRPs) and the development of antioxidant activities. The results of this study showed that WPI-Glu developed browning MRPs after heating for 20 min at 90 °C and pH 7 or pH 8. Additionally, the generated browning MRPs developed some antioxidant activities. WPI-Glu mixtures heated at pH 8 revealed the highest free radicals scavenging activity among all examined samples. The highest 2,2-diphenyl-1-picrylhydrazyl scavenging activities of heated WPI-Glu mixtures were 18 and 26% at pH 7 and pH 8, respectively. The results also showed a positive relationship between antioxidant quantities and the amount of added glucose. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis results proved that bovine serum albumin and β-lactoglobulin were the main proteins that reacted with glucose. High performance liquid chromatography analysis detected the presence of hydroxy methyl furfural (HMF) at all tested pH values. However, the data showed that the HMF quantity reached the highest value (6.58 ± 1.79 μg.mL^{-1}) at pH 8 and a WPI:Glu ratio of 1:3. These findings demonstrated that WPI in heat processed foods could be considered as a good source of proteins as well as antioxidants.

Keywords: antioxidants, Maillard reaction, whey protein isolate

INTRODUCTION

A Maillard reaction is a non-enzymatic, complicated browning reaction, which involves a series of chemical reactions between reducing sugars and amines (Nursten, 2005)., A Maillard reaction normally requires heat, and can be promoted by a low moisture content, and an alkaline environment (Frazier, 2009). The Maillard reaction involves three main stages—the initial stage includes sugar-amine condensation, followed by rearrangement and the formation of *Amadori* compounds, while in the third and final stage, the *Amadori* compounds will be degraded in two ways, via either 3-deoxyhexosone or methyl α-dicarbonyl leading to the formation of melanoidin pigments and a large number of products with complex structures (Morales and Jimenez-Perez, 2001; Wagner *et al*., 2002; Wan *et al*., 2005;). Nursten (2005) reported that a

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Received date : 01/04/14 Accepted date : 11/09/14

Maillard reaction could generate more than 100 detectable components, with hydroxymethylfurforal being one of these intermediate compounds. Researchers have also characterized some Maillard reaction products (MRPs) as having antioxidants activities. For example, Jiang and Bordkorb (2012) examined the antioxidants activity of MRPs generated via an α-lactalbumin and β-lactoglobulin interaction with ribose, and Dong *et al*. (2012) investigated the antioxidant activity of MRPs from a hydrolyzed β-lactoglubulin and glucose system. Currently, some synthetic antioxidants such as butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT) and propyl gallate are used as free radical scavengers to delay or prevent food rancidity or both (Peng *et al*., 2009). However, there are increasing concerns about the safety of these synthetic antioxidants due to their potential health risks (Peng *et al*., 2009). Therefore, it is essential to continue searching for some natural antioxidant compounds, such as MRPs that can replace BHA and BHT.

This study examined the antioxidant properties of MRPs upon heating of whey protein isolate (WPI) along with different concentrations of glucose at different pH values. It is expected that the results will provide a better understanding of changes in the chemical composition of WPI when heated in a food system containing reducing sugars. The present study also determined the optimal pH value and protein and sugar concentrations needed for the highest formation of MRPs.

MATERIALS AND METHODS

Whey protein isolate (WPI) was obtained from the Murray Goulburn Co-operative Co. Ltd (Melbourne, VIC, Australia) and contained 90.7% protein, 0.58% lactose, 0.6% fat, 4.3% ash and 3% moisture. 2,2-Diphenyl-1-picrylhydrazyl (DPPH; 97%), 5-(hydroxymethyl)furfural, β-mercaptoethanol, (±)-6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid and methanol (high performance liquid chromatography (HPLC) grade) were purchased from Sigma-Aldrich Co. Ltd.; (Sydney, NSW, Australia). Acrylamide/Bis 37.5:1 premixed powder, sodium dodecyl sulfate (SDS), ammonium persulfate, 10×Tris/glycine/SDS buffer, Coomassie brilliant blue R-250 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein standard board range were purchased from Bio-Rad Laboratories Pty. Ltd. (Sydney, NSW, Australia). Tris-hydroxymethyl-methylamine was purchased from Merck KgaA (Melbourne, VIC, Australia). D-Glucose (Glu) anhydrous was purchased from Chem-Supply Pty. Ltd. (Gillman SA, Australia). Ethanol and acetic acid were purchased from VWR International, Pty. Ltd. (Murarrie, QLD, Australia).

Preparation of whey protein isolate and glucose mixtures

WPI (6 g each) was mixed with 2, 3, 6, 12 and 18 g of Glu to yield WPI:Glu ratios of 3:1, 2:1, 1:1, 1:2, and 1:3, respectively. Each mixture was dissolved in 100 mL Milli-Q water using a volumetric flask. The solutions were adjusted to pH6, pH7 and pH8. Samples were then transferred into 50 mL plastic centrifuge tubes and heated for 20 min at 90 °C in a water bath (RATEK Instruments; Melbourne, VIC, Australia). Each treatment within each trial was repeated three times.

Color measurement

The degree of browning was assessed by measuring the absorbance of the WPI:Glu mixtures using a spectrophotometer (Pharmacia Novaspec, LKB Blochrom, England) at an optical density (OD) of 420 nm according to Rao *et al*. (2011), and by the measuring color intensity using a tristimulus Minolta colorimeter with the CIE Lab system (CIE, 2013). The colorimeter was calibrated using the white plate provided by maker of the colorimeter. The tristimulus colorimeter measures three indices one of which—namely, b*—is an index for indicating blue and yellow with

a value from -60 to +60 and the color is closer to yellow when b* has a high positive value (CIE, 2013).

Determination of 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

DPPH was used to determine the free radical scavenging activity of the MRPs. The assay was carried out based on Yu *et al*. (2013) with some modification. The DPPH solution (0.1 mM) in 80% ethanol was prepared freshly and protected from light. An aliquot (2 mL) of DPPH solution was mixed with 1 mL of sample. The mixtures were kept at room temperature in the dark for 20 min. The absorbance of the mixtures was measured at λ 517 nm using a spectrophotometer (M501 CamSpec Ltd.; Cambridge, UK). The radical scavenging activity was estimated using Equation 1:

$$\text{Radical scavenging activity } \% = [(A - B) / A] \times 100\% \quad (1)$$

where A is the control absorbance and B is the sample absorbance.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Protein cross-linkage was analyzed using SDS-PAGE using a Mini-Protean Tetra Cell system (Bio-Rad Laboratories, Inc.; Sydney, NSW, Australia). Analysis was performed using 5% stacking gel and 15% resolving gel. The samples were diluted 10 fold using Milli-Q water, mixed with sample buffer and heated at 95 °C for 4 min (Laemmli,1970). Board range SDS-PAGE standard was used as the molecular weight marker. It was treated with standard buffer and heated at 94 °C for 5 min before use (Bio-Rad Laboratories Inc., 2013). Each aliquot (5 µL) of treated sample or standard was injected into a well using a micro syringe. The electrophoresis was carried out according to Gu *et al.* (2010) with slight modifications. The gels were stained with Coomassie Brilliant Blue R-250 and were shaken gently in a shaking bed (John Morris Scientific Pty. Ltd; Deepdene, VIC, Australia) for 1 hr. The gels were de-stained using 40% methanol and 10% acetic acid solutions.

High performance liquid chromatography analysis

Samples were prepared by mixing 10 mL aliquots of each sample treated with WPI-Glu with 5 mL of methanol (80%). The mixtures were mixed vigorously and centrifuged for 5 min at 5,000 rpm to separate any sediment. The supernatant was then subjected to roto-evaporation (John Morris Scientific Pty. Ltd; Deepdene, VIC, Australia) for 10 min at 100 rpm and 40 °C. The concentrated samples were brought to a final volume of 5 mL using Milli-Q water. All samples were filtered through 0.45 µm acrodisc syringe filters before injection for HPLC analysis. The analysis was carried out according to Porretta (1992) using Shimadzu HPLC equipment (Kyoto, Japan), equipped with an SCL-10A System Controller, an LC-10AT Liquid Chromatographer, a DGU-14A Degasser, an RID-10A Refractive Index Detector, and an SIL-10AD Auto Injector. Each aliquot (10 µL) sample extract was injected into the Phenomenex C18 column (25 cm × 4.6 mm packed with C^{18} reversed-phase material with a particle size of 5 µm)). The samples were eluted using the isocratic mode and degassed with CH_3OH:H_2O (10:90, volume per volume) for 20 min and the peaks recorded at 280 nm. The column temperature was controlled at 40 °C by a CH-30 column heater. Aliquots (5 µL each) of various concentrations (0, 0.025, 0.05, 0.1 and 0.2%) of pure hydroxy methyl furfural (HMF) were used as external standards.

Statistical analysis

All experiments were repeated twice and every measurement within each trial was replicated three times. All the data were subjected to one way analysis of variance and a least significance difference test to separate the means at the 95% confidence level using SAS software (version 9.1.3; SAS Institute; Cary, NC, USA).

The difference between variables was considered as significantly different when $P < 0.05$.

RESULTS AND DISCUSSION

The WPI and glucose mixtures heated for 20 min at 90 °C and pH 7 and 8 developed a brown color with different intensity levels. The degree of browning development was measured using both OD and b* values:

Changes in optical density of whey protein isolate and glucose mixtures

Changes in the degree of browning due to heat treatment of the WPI:Glu mixtures were recorded at 420 nm. The results in Table 1 show a positive correlation between the Glu to WPI ratios and increments in OD values. The WPI:Glu ratio of 1:3 produced the largest OD value (0.27 ± 0.0) at pH 7 suggesting that the Maillard reaction was more active and developed more browning MRPs when the glucose concentration was three times greater than the WPI concentration, as the presence of more reducing sugar at a fixed WPI concentration would yield more Maillard reaction products.

Changes in b* values;

The results in Figure 1 show significant ($P < 0.05$) changes in the b* values of the WPI:Glu ratios after heating at 90 °C for 20 min at different pH values. The WPI-Glu mixture heated at pH 8 produced the highest b* value after heating, followed by pH 7. Additionally, the yellow-brown MRPs formed under pH 8 were visually noticeable. Samples heated at pH 6 showed a significant ($P < 0.05$) decrease in b* values in comparison with the b* values at pH 7 and 8. Such results might indicate that the WPI:Glu ratio could react more actively upon heating in alkaline conditions (pH 8) which was in agreement with those reported by Nursten (2005) and Owusu-apenten (2005) who reported that the amine groups were in a highly reactive state and more browning MRPs were formed under alkaline conditions. The data in Figure 1 show no significant differences ($P > 0.05$) between the unheated WPI:Glu mixtures at different pH levels, which confirmed that the browning and changes in b* values were due to the heat treatments of the WPI:Glu mixtures. Similar to the results for the OD values (Table 1), there was also a positive correlation between the b* values and an increase in the Glu:WPI ratios. The largest b* value was recorded at pH 8 and a WPI:Glu ratio of 1:3 (Figure 1). The highest b* values at pH 8 were in agreement with the fact that the Maillard reaction is promoted in an alkaline environment (Frazier, 2009). Brown color development is always used as an indicator of the extent of a Maillard reaction, as generally, the higher the extent of the Maillard reaction in advanced stages the more likely it is to generate more browning MRPs leading to a larger b* value (Owusu-apenten, 2005).

Table 1 Changes in optical density at 420nm of whey protein isolate (WPI):glucose (Glu) mixtures after heating for 20 min at 90 °C.

pH	WPI:Glu ratio					
	3:0	3:1	2:1	1:1	1:2	1:3
7	$0.09\pm0.00^{e,A}$	$0.19\pm0.00^{d,A}$	$0.20\pm0.01^{d,A}$	$0.23\pm0.00^{c,A}$	$0.26\pm0.00^{b,A}$	$0.27\pm0.00^{a,A}$
8	$0.03\pm0.00^{f,B}$	$0.08\pm0.00^{e,B}$	$0.10\pm0.00^{d,B}$	$0.14\pm0.00^{c,B}$	$0.21\pm0.00^{b,B}$	$0.22\pm0.01^{a,B}$

Values represent the average ± SD of six measurements.

Means within a row followed with different lowercase superscripts ($^{a\text{-}e}$) indicate significant differences ($P < 0.05$) in the samples at different WPI:GLU ratios.

Means within a column followed with different uppercase superscripts ($^{A\text{-}B}$) indicate significant differences in the samples at different pH values.

2,2-Diphenyl-2-picrylhydrazyl radical scavenging activity

There were significant ($P < 0.05$) differences in the DPPH scavenging activities when the WPI:Glu mixtures were heated at different pH values (Figure 2). The WPI:Glu mixtures which were heated at pH 6 did not show any detectable antioxidant activity. These findings contradicted a previous study (Wang *et al*., 2013) which reported that whey protein and sugar could generate MRPs with some scavenging capacity at pH 6. However, the treatment conditions in Wang *et al*. (2013) were different as they treated the WPI and glucose mixture at 50 °C for 7 d and recorded a DPPH free radical scavenging activity of 20% at pH 6. Consequently, that reported scavenging activity at pH 6 by Wang *et al.* (2013) might be attributed to the long incubation time (7 d) at a

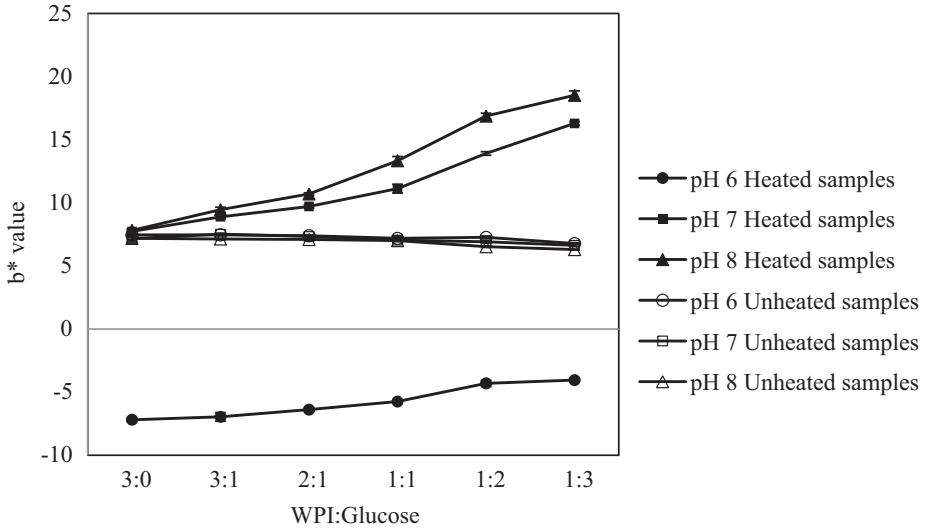

Figure 1 Changes in b* values of whey protein isolate (WPI)-glucose mixtures before and after heating at different pH values.

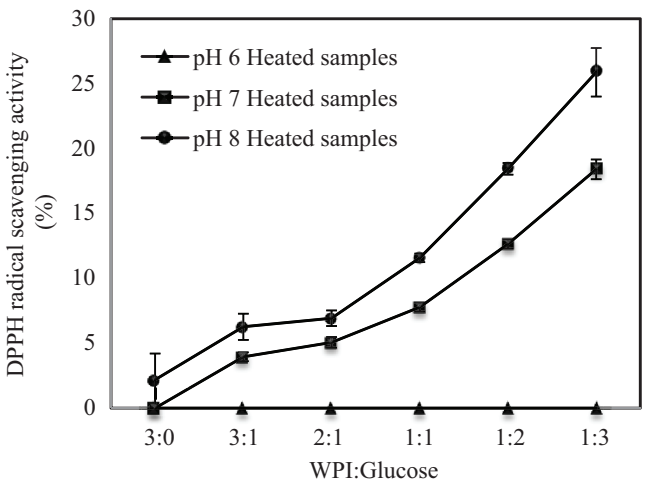

Figure 2 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of whey protein isolate (WPI):Glucose mixtures at different ratios after heating at 90 °C for 20 min at different pH values.

mild temperature (50 °C) which were substantially different conditions from those used in the current study. Another study by Stanic-Vucinic *et al*. (2013) subjected β-lactoglobulin to a high intensity ultrasonication treatment at pH 6.5. They reported the formation of MRPs with 5–18% DPPH free radical scavenging activity. Once more, the ultrasonication treatment by Stanic-Vucinic *et al*. (2013), involving heating at 90 °C for 20 min, was different from the current investigation. Those observations clearly indicated that the formation of free radical scavenging activity MRPs products at pH 6–6.5 will depend on the processing conditions. However, the results from the current study proved that heating a mixture of WPI and Glu at 90 °C for 20 min at pH 6 did not generate any detectable DPPH scavenging activity which concurred with the OD and color measurements results (Table 1 and Figure 1)

Changes in the color of the DPPH ethanol solution after interaction with the generated MRPs indicated the level of radical scavenging activity of the examined WPI:Glu solutions. The original color of the DPPH is purple and it turns light yellow when it reacts with radical scavenging reagents (Kim *et al*., 2009). DPPH free radical scavenging activities were detected when the WPI:Glu mixtures were heated at both pH 7 and pH 8 (Figure 2). Those treatments revealed also significantly (P<0.05) larger DPPH scavenging activities at pH 8 in comparison with pH 7. The highest DPPH scavenging activities at a WPI:Glu ratio of 1:3 were 18.0 and 26% at pH 7 and 8, respectively. These results were in agreement with those reported by Wang *et al*. (2013), who found that WPI and reducing sugar developed MRPs with higher DPPH scavenging activities under alkaline conditions. Tareke *et al*. (2002) explained the higher Maillard reaction rate under alkaline conditions based on the fact that the reactive carbonyl group of the sugar reacts with an amino group of the amino acid much faster in an alkaline environment, since the amino groups (RNH_3^+) are deprotenated and, hence, have an increased nucleophilicity under alkaline conditions. The same data (Figure 2) revealed a positive relationship between scavenging activity and the amount of glucose in the WPI:Glu mixtures. The DPPH scavenging activity increased from 0% to 18% when the glucose to WPI ratios increased from 0:3 to 3:1 at pH 7 (Figure 2).

Impact of Maillard reaction on protein composition

The SDS-PAGE results showed three main proteins in the unheated WPI:Glu mixtures—namely, bovine serum albumin (BSA), β-lactoglobulin and α-lactalbumin, with molecular weights of 66.2 kDa, 18.2 kDa, and 14.2 kDa, respectively (Figure 3). β-Lactoglobulin and α-lactalbumin, which are the main proteins in WPI (Vanaman *et al*., 1970; Smith *et al*., 1990; Sigma-Aldrich, 2013) appeared as more dense bands than BSA in both the heated and unheated samples (Figure 3).

The WPI:Glu mixtures heated at pH 7 and pH 8 showed some extra bands at the top of the gels, which were greater than 200 kDa. Those bands were not detected in the WPI:Glu mixtures after heating at pH 6. Such observations were in agreement with the previously reported DPPH results, which revealed the highest DPPH scavenging activity at pH 7 and 8. The results were also consistent with the colorimeter results, which illustrated stronger browning formation at pH 7 and pH 8. Consequently, it was concluded that high molecular weight MRPs could be formed when the WPI:Glu mixtures are heated at pH 7 and pH 8.

SDS-PAGE techniques were used to test if a conjugation between reducing sugars and proteins would lead to the formation of proteins with a larger molecular weight. The formation of covalent, cross-linked protein aggregates is one of the consequences of an advanced Maillard reaction (Nagaraj*et al*., 1996). The protein cross-link could occur via a Maillard reaction with intermediate or final stage products, such as di-

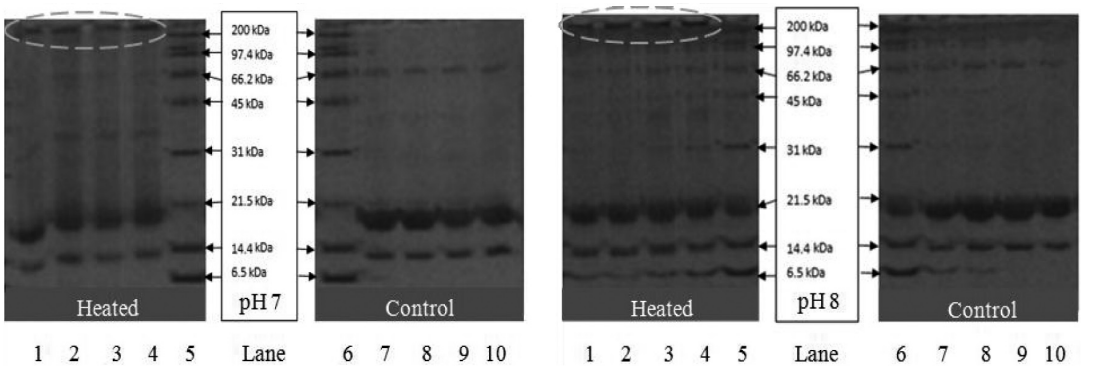

Figure 3 SDS-PAGE of whey protein isolate(WPI):glucose (Glu) mixtures after heating at pH 7 and 8 with different WPI:Glu ratios. Lanes 5 and 6 are protein standards. Lanes 1, 2, 3 and 4 represent samples after heating and lanes 7, 8, 9 and 10 before heating at WPI:Glu ratios of 3:1, 2:1, 1:1 and 1:2, respectively. Arrows indicate the value (in kilo daltons) of different isolated protein standards.

carbonyl compounds glyoxal, methylglyoxal and 3-deoxyglucosone. These products could react with amino acid residues and could form intra- or inter- molecular cross-links (Le *et al*., 2013).

Changes in the protein components as a result of a Maillard reaction were studied by Wang *et al*. (2013). However, they incubated different types of sugars with WPI in an oven at 50 °C for 7 d. When compared with the observations of Wang *et al*. (2013), heating the WPI and glucose at 90 °C for 20 min produced proteins with a higher molecular weight as the protein cross-linking phenomenon was stronger when heated at 90°C for 20 min. Although Wang et al. (2013) incubated the WPI and glucose for 5 d, the Maillard reaction was not as efficient as the one resulting from heating at 90 °C for 20 min. That was in agreement with the theory that temperature is more important than time for a heat-induced Maillard reaction (Nursten, 2005). High molecular weight proteins were also detected during irradiation at 20–100 kGy Chawla *et al*. (2009) and although irradiation is not the same as thermal processing, it could induce a higher intensity of Maillard reaction products than heating.

Hydroxy methyl furforal formation in heated whey protein isloates:glucose mixtures

The HPLC results showed small amounts of HMF (1.33–3.17 $\mu g.mL^{-1}$) present in the controls (unheated samples). The presence of HMF in the unheated samples could be attributed to the reaction between protein and lactose in the WPI during processing and storage. Nursten (2005) indicated that a Maillard reaction could take place at room temperature over a long period of storage time. However, the data in Table 2 reveal a significant increase in the amounts of HMF after heating. The WPI:Glu mixtures at a 1:3 ratio, and pH 8 had significantly ($P < 0.05$) larger quantities of HMF than the controls. In addition, the same data (Table 2) indicated a positive relationship between the treatment pH and the amounts of HMF produced. The detected amounts of HMF reached (6.58 ± 1.79 µg.mL) when WPI:Glu (1:3) samples were heated at pH 8 in comparison with 3.7 ± 1.26 in the controls. Once more, those results illustrated the highest level of HMF formation under alkaline conditions (pH 8). Such observations were in agreement with other measurements reported in this study, such as the DPPH assay and colorimeter results. Additionally, those results were consistent with a previous study by Zhang and Zhou (2013),

who found a positive relationship between the DPPH scavenging activity and the amount of HMF generated during the Maillard reaction.

HMF is one of the intermediate by-products from a Maillard reaction that has been claimed to have antioxidant properties (Owusu-apenten, 2005). HPLC techniques were used to confirm whether HMF had been produced via a WPI and glucose interaction at high temperature. The HPLC results(Figure 4) showed that in addition to the HMF peak which emerged at a retention time of 10.488 min, additional peaks were also detected at shorter retention times (Figure 4). Nursten (2005) reported that a Maillard reaction could generate more than 100 detectable components. However, the results of the current study identified

Table 2 Hydroxy methyl furfular content (μg.mL^{-1}) in whey protein isolate (WPI) mixed with glucose (Glu) after heating at different ratios and pH values.

Treatment	pH	WPI:Glu ratio				
		3:1	2:1	1:1	1:2	1:3
Heated	6	1.96±0.66^{a,B}	2.16±1.17^{a,B}	2.08±1.33^{a,A}	1.88±0.21^{a,B}	2.06±0.37^{a,B}
	7	5.06±2.35^{ab,AB}	2.14±0.43^{b,B}	5.47±4.26^{a,A}	5.84±1.83^{ab,A}	5.64±0.70^{ab,A}
	8	5.49±2.06^{a,A}	4.85±2.65^{a,A}	4.53±1.12^{ab,A}	2.62±1.16^{b,B}	6.85±3.79^{ab,A}
Control	6	1.33±2.56^{a,A}	1.86±0.41^{a,B}	2.65±0.70^{a,A}	2.22±0.28^{a,A}	3.05±0.17^{a,A}
	7	3.17±0.14^{a,A}	3.72±0.45^{a,A}	2.90±0.44^{a,A}	2.59±0.94^{a,A}	3.40±0.90^{a,A}
	8	2.45±0.83^{a,A}	2.63±0.35^{a,B}	2.47±0.30^{a,A}	2.70±0.39^{a,A}	3.70±1.26^{a,A}

Results expressed as mean ± SD for each analysis.

Means within a row followed with different lowercase superscripts ($^{a-e}$) indicate significant differences ($P < 0.05$) in the samples at different WPI:GLU ratios.

Means within a column followed with different uppercase superscripts ($^{A-B}$) indicate significant differences in the samples at different pH values.

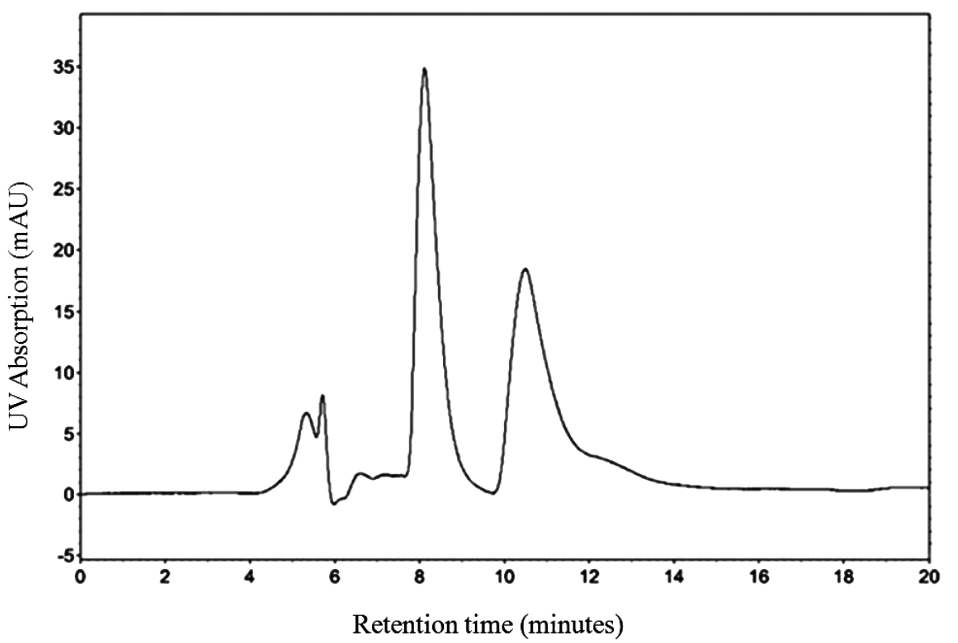

Figure 4 Chromatogram of extracts from WPI:glucose mixtures heated at 90 °C for 20 min at pH 8. Ultraviolet (UV) absorption measured in milli absorbance units (mAU).

and quantified only HMF as one of the major intermediate Maillard reaction compounds. It has been well documented that HMF is formed as a result of sugar dehydration during the formation of a Schiff base of hydroxymethylfurfural (HMF) or furfural which will further form HMF or furfural accordingly (Nursten, 2005). Furfural can react with the amino compound directly and form melanoidins (Frazier, 2009).

Jiang and Bordkorb (2012) examined the antioxidants activity of MRPs generated via an α-lactalbumin and β-lactoglobulin interaction with ribose, and Dong *et al*. (2012) investigated the antioxidant activity of MRPs from a hydrolysed β-lactoglubulin and glucose system. The results of these other studies were in agreement with the current results and supported the fact that the final MRPs (melanoidins) would be highly colored (as shown by the OD and b* values in Table 1 and Figure 1), and the source of the browning with some antioxidant activity, as illustrated by the DPPH results in the current study (Figure 2).

CONCLUSION

The properties were examined of a WPI:Glu mixture after heating at 90 °C for 20 min at different ratios (3:0, 3:1, 2:1, 1:1, 1:2 and 1:3) and different pH values (pH 6, 7 and 8). The WPI:Glu mixtures developed browning MRPs after heating at pH 7 and pH 8, whereas no browning MRPs formed at pH 6. The results showed a positive relationship between increased amounts of added glucose to WPI and browning intensity. Additionally, the WPI:Glu mixtures developed antioxidant activity after heating at pH 7 and pH 8, but not at pH 6. The SDS-PAGE results indicated that BSA and β-lactoglobulin were the main proteins in the WPI that reacted with glucose. High molecular weight protein bands (greater than 200kDa) were detected in the heated WPI:Glu mixtures confirming the interaction between proteins and reducing sugars and the formation of MRPs. HMF was detected in the extracts of the WPI:Glu mixtures heated under alkaline conditions. Such findings would support the idea that heating WPI in the presence of a reducing sugar could be considered as a new approach to produce food rich in natural antioxidants.

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