Changes in the initial stages of a glucose-proline Maillard reaction model system influences dairy product quality during thermal processing

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ABSTRACT

The Maillard reaction always occurs during the thermal processing of dairy products, which significantly influences their quality. In the present study, the initial stages of a glucose-proline model system were investigated in water and different types of buffer solutions. Results showed that phosphate buffer accelerated the reversible degradation of the initial stages of the reaction. The proposed catalysis mechanism was that hydrogenous and dihydric phosphate radical anions simultaneously accepted and donated protons for the conversion of the intermediates into *N*-glycosylamine. The catalysis mechanism was confirmed via testing and no reducing of hydrogenous and dihydric phosphate radical anions was observed during the reaction. Moreover, both *N*-(1-deoxy-D-fructos-1-yl)proline and its degradation compounds were analyzed. Results showed that degradation of *N*-(1-deoxy-D-fructos-1-yl)proline to form 5-hydroxymethyl-2-furaldehyde and formic acid was also accelerated by phosphate buffer. An interesting phenomenon was that citrate decreased 5-hydroxymethyl-2-furaldehyde formation, which might be because Strecker-type degradation occurred more easily than 1,2-enolization reaction in citrate buffer solution. However, this hypothesis has not been confirmed, and element label experiments should be carried out in the future.

Key words: dairy thermal processing, *N*-(1-deoxy-D-fructos-1-yl)proline, Maillard reaction

INTRODUCTION

Dairy proteins are widely used in the food industry as functional ingredients because of their simple production, excellent nutritional value, and versatile techno-functional properties (Corzo-Martínez et al., 2011). Dairy protein is commercially used in many food systems, such as ice cream, cream liqueurs, whipped toppings and products for infant nutrition and enteral

formulas (Rufian-Henares et al., 2002). Dairy proteins can be hydrolyzed to release different amino acids such as proline, which could react with reducing sugars during dairy thermal processing (Habibi-Najafi and Lee, 1994; Basch et al., 1997; Mizuno et al., 2004). Because dairy processing always produces new nutritive or harmful compounds, it is necessary to detail the products and the processes applied.

The Maillard reaction is one of the most important and complex processes in food chemistry (Hodge, 1955; Moreno et al., 2003). Many components are able to participate through different pathways that form a complex mixture of products called Maillard reaction products (**MRP**; Rufián-Henares et al., 2004, 2007; Brenna et al., 2009). Early researchers on the Maillard reaction found that the type of amine and carbonyl compounds influence the rate of reaction as well as the products formed (Willits et al., 1958; Wolfrom et al., 1974; Bunn and Higgins, 1981; Ashoor and Zent, 1984; Feather and Nelson, 1984; Labuza and Massaro, 1990). The concentration and ratio of reactants also significantly changed the Maillard reaction trend (Wolfrom et al., 1974; Warmbier et al., 1976; Baisier and Labuza, 1992). In addition, the pH value significantly influences the Maillard reaction rate and the types of products formed (Wolfrom et al., 1953; Willits et al., 1958; Underwood et al., 1959; Lento et al., 1960; Ashoor and Zent, 1984; Lee et al., 1984; Apriyantono and Ames, 1993). However, the effect of buffer ions on the Maillard reaction has been always ignored.

Amadori compounds (i.e., *N*-substituted 1-amino-1-deoxyketoses) are formed in the initial phase of the Maillard reaction. Generally, the Amadori rearrangement product (**ARP**) is always produced via a degradation of the Schiff base, and the latter is obtained by the Amadori rearrangement of the corresponding *N*-glycosylamines (Hodge, 1953). Degradation of ARP to form intermediate products has been proven by several pathways. Enolization has been suggested as one of the most important mechanisms among the proposed pathways for ARP degradation (Hodge, 1953). For example, 5-hydroxymethyl-2-furaldehyde (**HMF**) is formed by 1,2-enolization, which leads to the formation of 3-deoxy-2-hexosulose, and the latter undergoes dehydration

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from HMF. Other pathways of ARP degradation that form a variety of cyclic substances have been proposed via various mechanisms such as the Strecker-type reaction or ARP acting as nucleophiles and reacting with other sugar molecules to form diketosyl derivatives (Hodge, 1955; Moreno et al., 2003). Moreover, acetic and formic acid always have been tested at the early stages of the degradation of the Amadori compounds, which has been reported in the literature (Brands and van Boekel, 2001; Rufián-Henares et al., 2006).

Effects of buffer solutions on the Maillard reaction derived from reducing sugars have been investigated in early studies (Burton and McWeeny, 1963; Saunders and Jervis, 1966; Clever, 1968). A study from the literature detailed that a phosphate buffer solution significantly accelerated the Maillard reaction to form color at pH 7.0, but slight changes were found in citrate buffer solutions (Burton and McWeeny, 1963). Saunders and Jervis (1966) observed that the browning was higher in citrate compared with phosphate at pH 3. Bell (1997) investigated the effects of buffer type and concentration on the Maillard reaction, with the bifunctional catalytic ability of the phosphate anion being the explanation of the different effects of the buffers. However, limited information is available about the effects of various buffer solutions on the degradation of Amadori compounds, which are important intermediate products on the early steps of the Maillard reaction.

In this paper, the decrease in glucose as the reactant, degradation of *N*-(1-deoxy-D-fructos-1-yl)proline (DFP) to form reducing sugars, formic acid, and HMF via different pathways were investigated to obtain a better understanding of the pathway of the early stages of the glucose-proline Maillard reaction. Moreover, hydrogenous and dihydric phosphate radicals were also detected to confirm whether it participated in or catalyzed this important reaction.

MATERIALS AND METHODS

Chemicals

D-Glucose, D-mannose, sodium dihydrogen phosphate, disodium hydrogen phosphate, ethylic acid, sodium citrate, citric acid, hydrochloric acid, and ammonia water were purchased from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China). Methanol and sodium acetate were of chromatographic grade and purchased from Merck KGaA (Darmstadt, Germany). Sodium hydrogen carbonate, sodium hydroxide, HMF, acetic acid, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of MRP

Maillard reaction products were prepared using a high-pressure vessel (Parr 4522M; Parr Instrument Co., Moline, IL). Eighteen grams of glucose and 5.75 g of proline were mixed in 18 mL of deionized water and different buffer solutions (i.e., phosphate, acetate, and citrate buffer solution) with concentrations of 0.1, 0.2, and 0.3 M, respectively, at a pH of 5.8 (25°C). The sample mixture was heated at 110 and 130°C for different times (i.e., 0, 10, 20, 30, 40, 50, and 60 min), and then cooled to room temperature by iced water immediately to stop the reaction.

Preparation and Separation of DFP

A high-pressure vessel (Parr 4522M; Parr Instrument Co.) with a kettle volume of 2 L was applied to produce glucose-proline Maillard reaction solutions. In this experiment, 360 g of glucose and 115 g of proline were mixed in 360 mL of deionized water. The sample mixture was heated at 90°C for 60 min with a pressure of 14 MPa, and then cooled immediately to room temperature with iced water.

The ion resin ZG C107 (Hangzhou Zhengguang Resin Co. Ltd., Hangzhou, China) was used for DFP isolation, and the pretreatment method was as follows: 1 L of ZG C107 ion exchange resin (Na⁺ form) was washed with 5% NaOH solution (1 L) and water (2 L), respectively, and then eluted with 4% HCl (1 L) to transform the Na⁺ form to the H⁺ form. After the ion exchange resin was abundantly transformed to the H⁺ form, deionized water was used to elute the resin until the pH of the eluent was higher than 6.5.

The Amadori compound glucose-proline [i.e., *N*-(1-deoxy-D-glucose-1-yl)proline] was separated and isolated as described in the literature, with slight modification (Davidek et al., 2005). The reaction mixture was dissolved in an ethanol/water mixture (70 + 30, vol/vol; 1 L), and the solution was passed through a column (3.5 × 50 cm; Tianjin Instrument Co. Ltd., Guangzhou, China) filled with the pretreated resin. Then, the resin was washed with an ethanol/water mixture (70 + 30, vol/vol; 5 L) and water (1.5 L), respectively, and then eluted with ammonium hydroxide (0.2 mol/L), collecting 100-mL fractions. Each fraction was tested using the HPLC-MS/MS and high-performance anion exchange chromatography (HPAEC)-electrochemical detector (ECD) analyses. The DFP was obtained as an amorphous white powder (2.2 g, 0.46% yield) with a purity of higher than 98%, measured by HPLC-MS/MS. The product

was hygroscopic and was stored in a desiccator at 4°C before further tests within 72 h.

HPLC-MS/MS Analysis of DFP

The HPLC-MS/MS analysis of DFP was performed using an integrated Surveyor system including LC Pump Plus, autosampler, a PDA Plus Detector (Thermo Finnigan LLC, San Jose, CA) and a LCQ DECA XP MAX trap tandem mass spectrometer (Thermo Finnigan LLC). Analytical separation was achieved on a 5- μ m Waters Atlantis T3 150 $\times$ 4.6 mm column (Waters Corp., Milford, MA). The mobile phase was 100% water, 0 to 10 min; then, a linear gradient of 100% water to 100% methanol, from 10 to 30 min; and finally, 100% methanol, from 30 to 40 min at a flow rate of 0.5 mL/min. The column temperature was maintained at 25°C with a column heater. The injection volume was 20 μ L. The Amadori compounds were detected in positive and negative electrospray ionization (**ESI**⁺ and **ESI**⁻, respectively). The capillary temperature was 250°C. The source voltage was 4,500 V for the positive polarity and 5,300 V for the negative polarity and the source current was 80 μ A for both positive and negative polarities. The capillary voltage was 20 V for positive polarity and -37 V for negative polarity. Scans were acquired over the range 50 to 800 amu.

Thermal Treatment of DFP

The DFP was dissolved in deionized water and 0.1 *M* different buffer solutions with an initial pH of 5.8, and heated at 110 and 130°C in a closed container for 10, 20, 30, 40, 50, and 60 min, respectively. After thermal treatment, a 5-mL solution was taken out and cooled immediately to room temperature. After that, HPAEC-ECD analysis for DFP, reducing sugar, and formic acid content were performed within 72 h. While part of the thermal-treated solution was freeze-dried, HPLC analysis for HMF was performed.

Analysis of Glucose Derived from Glucose-Proline Reaction

Samples were diluted 40 times and then filtered through a Millex-HN nylon clarification kit of 0.45- μ m pore size (Millipore Corp., Bedford, MA) for the HPLC test. The HPLC system was a Waters 600 chromatogram controller (Waters Corp.), Waters 600E pump (Waters Corp.), Rheodyne 7725i manual injector (Waters Corp.), and Waters 2414 refractive index detector (Waters Corp.). The HPLC peaks were identified to be glucose by comparing the reaction time with the standard compound. The regression equation for glucose

standard tested by HPLC was $a^* = 277,777 \times C_1 + 225$ ($R^2 = 0.9997$), where C_1 = glucose concentration (g/L) and a^* = peak area of glucose. The retention time of glucose was 10.5 min.

Analysis of Hydrogenous and Dihydric Phosphate Radicals

The samples were filtered through a Millex-HN nylon clarification kit of 0.45- μ m pore size (Millipore Corp.), and then analyzed on a DX 5000 ion chromatography system (Dionex Corp., Sunnyvale, CA) composed by a gradient pump (model EG40) and a conductivity detector. The anion self-regenerating suppressor (ASRS 300, 4 mm) was working in the autosuppression recycle mode. The gradient separation was accomplished on an IonPac AS-23 anion-exchange column (250 $\times$ 4 mm; Dionex Corp.) and an IonPac AG-23 guard column (50 $\times$ 4 mm; Dionex Corp.) as follows: the mobile phases were A: water, and D: NaHCO₃ (0.8 mM) and Na₂CO₃ (4.5 mM) solution. The settings were as follows: 0 to approximately 40 min, from 50% A and 50% D changed to 0% A and 100% D, linearly; 40 to approximately 50 min, isocratic elution of 0% A and 100% D; and 50 to approximately 60 min, isocratic elution of 50% A and 50% D for column equilibration. All tests used a constant flow rate of 0.8 mL/min in the program. The injection volume was 10 μ L.

Analysis of DFP and Reducing Sugars Derived from DFP Degradation

Samples were filtered through a Millex-HN nylon, 0.45- μ m pore size (Millipore Corp.), then analyzed on a DX 5000 Dionex system (Dionex Corp.), composed of a gradient pump (model EG40) with on-line degassing, and an ECD (model ED40). Separation was accomplished on a CarboPac PA1 anion-exchange column (250 $\times$ 4 mm; Dionex Corp.) and a CarboPac PA1 guard column (50 $\times$ 4 mm; Dionex Corp.) using the gradient as follows: the mobile phases were A: water, B: NaOH solution (300 mM), and C: sodium acetate solution (300 mM). The settings were as follows: 0 to approximately 30 min, from 97% A and 3% B changed to 95% A, 3% B, and 2% C, linearly; 30 to approximately 35 min, 95% A, 3% B, and 2% C changed to 49% A, 3% B, and 48% C, linearly; and 35 to approximately 40 min, isocratic elution of 49% A, 3% B, and 48% C; and finally, 40 to approximately 50 min, isocratic elution of 97% A and 3% B for column equilibration. All tests used a constant flow rate of 1 mL/min. The injection volume was 10 μ L. The regression equation for DFP was $b^* = 14,104 \times C_2 + 17.999$ ($R^2 = 0.9923$), where C_2 = DFP concentration (mmol/L) and b^* = peak area

of DFP [nano (1 over a billion) coulomb interactions $\times$ minute ($\text{nC} \times \text{min}$)]. The experimental range was 0.0004 to 0.0018 mmol/L in this test. The regression equation for the glucose standard tested by HPAEC was $c^* = 62,816 \times C_3 + 7.878$ ($R^2 = 0.9791$), where C_3 = glucose concentration (mmol/L) and c^* = peak area of glucose ($\text{nC} \times \text{min}$). The experimental range was 0.05 to 1 mmol/L in this test. The regression equation for mannose standard was $d^* = 69,933 \times C_4 + 3.7348$ ($R^2 = 0.9935$), where C_4 = mannose concentration (mmol/L) and d^* = peak area of mannose ($\text{nC} \times \text{min}$). The experimental range was 0.05 to 1 mmol/L in this test.

Analysis of Formic Acid Derived from DFP Degradation

The formic acid analyses were performed using a DX 5000 Dionex system (Dionex Corp.), a gradient pump (model EG40) with on-line degassing, and a conductivity detector. The anion self-regenerating suppressor (ASRS 300, 4 mm) was working in the autosuppression recycle mode. The isocratic separation was accomplished on an IonPac AS-23 anion-exchange column (250×4 mm; Dionex Corp.) and an IonPac AG-23 guard column (50×4 mm; Dionex Corp.) using sodium hydrogen carbonate (2.5 mmol/L) as the mobile phase delivered at a flow rate of 1 mL/min. Formic acid (retention time, 6.4 min) was quantified on the basis of a calibration curves by comparing the peak area with those of the standard solution containing known amounts of pure compound. The regression equation for the formic acid standard was $e^* = 542.92 \times C_5 - 0.1592$ ($R^2 = 0.9980$), where C_5 = formic acid concentration (mmol/L) and e^* = peak area of acetic acid ($\text{nC} \times \text{min}$). The experimental range was 0.3 to 10 mmol/L in this test.

Analysis of HMF Derived from DFP Degradation

Ten milligrams of freeze-dried powder was dissolved in 10 mL of deionized water and then filtered through a Millex-HN nylon clarification kit of 0.45- μm pore size (Millipore Corp.). The analysis was performed with an HPLC-Diode Array Detector (DAD) system, which consisted of a 5- μm Waters Atlantis T3 150×4.6 mm column (Waters Corp.), a Waters 600 pump, and a Waters 2998 diode array detector (Waters Corp.). The injection volume was 20 μL . The mobile phase was 100% water (0 to 10 min), then a linear gradient of 100% water to 100% methanol (from 10 to 55 min), and finally, 100% methanol (from 55 to 70 min) delivered at a flow rate of 0.5 mL/min. The column temperature was set at 25°C. Spectral data from all peaks were accumulated in the range 200 to 800 nm; chromatograms

were recorded at 284 nm. The UV-visible spectrum and retention time of all peaks and HMF standard were compared to confirm which peak was HMF. The regression equation for the HMF standard was $f^* = 2 \times 10^7 \times C_6 + 23,778$ ($R^2 = 0.9999$), where C_6 = HMF concentration (mmol/L) and f^* = peak area of HMF. The experimental range was 0.1 to 1.6 mmol/L in this test.

Statistical Analysis

All experiments were carried out in triplicate. Means and standard deviations of the data were calculated for each treatment. Analysis of variance was carried out to determine any significant differences ($P < 0.05$) among the applied treatments using the SPSS package (SPSS 10.0 for Windows; SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Changes in Glucose and Hydrogenous and Dihydric Phosphate Radical Anions

As shown in Figure 1A and B, glucose was significantly reduced along with the increase in the concentration of phosphate buffer, which indicated that phosphates enhanced the Maillard reaction. An explanation might be that phosphate was a base catalyst for the formation of *N*-glycosylamine at the first step of the Maillard reaction. However, no clear data for the proposed mechanism was demonstrated. In the present study, hydrogenous and dihydric phosphate radical anions were tested to demonstrate this hypothesis. An interesting finding was that hydrogenous and dihydric phosphate radical anions had the same retention time as did phosphate radical anions, which showed 1 peak in the HPAEC. This should be explained as follows: OH^- reacting with hydrogenous and dihydric phosphate radical anions to form phosphate radical anion was carried out in the anion self-regenerating suppressor; the tested peak (retention time was about 36 min) should be the sum of hydrogenous and dihydric phosphate radical anions, which were designated as phosphate radicals. On the other hand, little change of the phosphate radical anion was found as the reaction was extended. Therefore, it could be confirmed that hydrogenous and dihydric phosphate radical anions should be the base catalysts to accelerate the decrease of glucose in the Maillard reaction. Moreover, only phosphate buffer accelerated glucose decrease, almost no change in glucose was found as the citrate and acetate buffer concentration increased.

Figure 2 explains the phenomenon stated above as follows: charged intermediates were formed after the nucleophilic attack of the amine to the aldehyde. Hy-

Figure 1. Changes in glucose derived from the glucose-proline reaction at 110°C (A) and 130°C (B).

drogenous and dihydric phosphate radical anions, having both hydrogen-accepting and -donating groups in close proximity, simultaneously accepted and donated the protons for the conversion of the intermediates into *N*-glycosylamine. The faster formation of *N*-glycosylamine and the continued catalytic potential of the hydrogenous and dihydric phosphate radical anions to

accelerate the decrease in glucose in the initial stage of the Maillard reaction have been suggested by Bell (1997). However, acetate and citric acid radical anions could not catalyze the *N*-glycosylamine formation. A possible explanation is that acetate radical anions had no hydrogen-accepting and -donating groups simultaneously, and citric acid radical anions were significantly

Figure 2. Proposed mechanism of the effect of phosphate anions on the glucose-proline reaction (Bell, 1997).

bigger than phosphate radical anions, which led to steric hindrance, making the simultaneous acceptance and donation of the protons for the conversion of the intermediates into *N*-glycosylamine more difficult. The same results were also found in the literature (Bell and Wetzel, 1995), suggesting that aspartame could not be degraded in citrate buffer, but significantly degraded in phosphate buffer, which was due to the ability of the small phosphate anion to simultaneously donate and accept the proton necessary for the ultimate conversion into diketopiperazine.

DFP Detection

As shown in Figure 3A and B, DFP was observed at a retention time of 35.5 min, and no glucose (retention time, about 6 min) was observed. However, neither qualitative analysis nor the purity of the compound could be carried out by HPAEC-ECD. Therefore, HPLC-MS/MS should be used for further analysis of this compound. The total ion chromatogram (TIC) was required for the detection by ESI⁺-MS/MS and ESI⁻-MS/MS, shown in Figure 3C and D, respectively. Mass spectrum data with positive and negative ion models are shown in Figure 4, which agreed with those described in the literature (Davidek et al., 2005).

Formation of the Amadori compound DFP in this experiment could happen from the dehydration reaction loss of 1 molecule of water between C1 and C2 of the

imine, forming the precursor of the Amadori compound DFP. The proposed mechanism above is different from that of early research on the formation of the Amadori compound *N*-(1-deoxy-D-fructos-1-yl)glycine, based on the degradation of the Schiff base (Davidek et al., 2002). However, it is not inconsistent, because in this experiment, proline, a secondary amine was used as the reactant. The Schiff base of glucose-proline could not be formed due to the existence of the C-N-C bond in the proline molecule. However, early research focused on the Maillard reaction derived from the primary amines such as glycine, which contains a C-N bond but not a C-N-C bond. An element label experiment will be carried out in further research to confirm the proposed mechanism in this experiment..

Degradation of DFP in Different Buffer Solutions

To better understand the change in the initial stage of the Maillard reaction, changes in Amadori compounds should be clearly studied, although the changes in glucose and hydrogenous and dihydric phosphate radical anions have been detailed above. *N*-(1-Deoxy-D-fructos-1-yl)proline was one of the most important Amadori compounds in the initial stage of the Maillard reaction. It was not stable during thermal treatment at 110 and 130°C in different buffer solutions (Figure 5A); DFP was easily degraded in phosphate buffer solution. For example, after heating for 60 min at 110 and 130°C,

Figure 3. High-performance anion exchange chromatography-electrochemical detector (HPAEC-ECD; in pico-Coulombs, pC) analysis of blank (water; A) and *N*-(1-deoxy-D-fructos-1-yl)proline (DFP) sample (B), and total ion chromatogram of DFP tested by HPLC-ESI⁺-MS/MS (C) and HPLC-ESI⁻-MS/MS (D). ESI⁺ and ESI⁻ are positive and negative electrospray ionization, respectively.

Figure 4. Mass spectrum of *N*-(1-deoxy-D-fructos-1-yl)proline (DFP) on the positive (A) and negative (B) ion model.

Figure 5. Changes in *N*-(1-deoxy-D-fructos-1-yl)proline (DFP; A), glucose (B), mannose (C), 5-hydroxymethyl-2-furaldehyde (HMF; D), and formic acid (E) during the degradation of DFP at the initial stages of the glucose-proline Maillard reaction.

about 3.2 and 2.3% of the DFP remained in the phosphate buffer, respectively. However, about 46.7 and 23.9% of the DFP remained in acetate buffer, whereas 40.7 and 19.6% of the DFP remained in citrate buffer. Moreover, increasing the reaction temperature favored the degradation of DFP. For example, 30 min was required to degrade about 77% of the DFP at 110°C in phosphate buffer at a pH of 5.8; only 10 min was needed to reach the same degree of degradation at 130°C in the same buffer solution. Figure 1 could explain the degradation of DFP in this experiment. First, DFP might perform a reversible degradation to form glucose and mannose. Second, HMF and formic acid might be formed via 1,2-enolization in a neutral or acidic condition. Third, DFP undergoes a 2,3-enolization, and forms 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one and acetic acid in alkaline conditions. Last but not least, a Strecker-type reaction should occur. However,

according to the recent literature, it is difficult to clearly understand all of the mechanisms of Strecker-type reactions, although DFP undergoing the Strecker-type degradation has been universally acknowledged. Moreover, to better understand which pathway is the main one (i.e., forming reducing sugars, HMF, and formic acid) of DFP degradation, reducing sugars, HMF, and formic acid derived from DFP were tested in this experiment. This result was in accordance with the literature, which has clearly demonstrated that degradation of DFP was found in phosphate buffer solution at the early stages of the Maillard reaction (Davidek et al., 2002).

Formation of Reducing Sugars, HMF, and Formic Acid

Mannose is the isomere of glucose; it was formed by heating *N*-(1-deoxy-D-fructos-1-yl)glycine in an aque-

Figure 6. Proposed mechanism of the initial stages of the Maillard reaction at pH < 7 (pathway A: reversible degradation to form reducing sugars; pathway B: formation of HMF undergoing 1,2-enolization; and pathway C: Strecker-type reaction).

ous model system, which proved that the early stages of the glucose-glycine Maillard reaction were reversible (Davidek et al., 2002). Therefore, glucose and mannose should be detected to better understand the effects of buffer solutions on the reversible early stages of the glucose-proline Maillard reaction. On the other hand, formic acid is always formed during the Maillard reaction, which might be one of the main reasons to change the rate of the reaction system. Figure 5B and C showed that higher levels of glucose and mannose were formed during the degradation of DFP in phosphate buffer solution (about 1.3 and 1.5 times higher than in citrate and acetate buffer solutions after a 60-min reaction at 130°C). The formation of glucose and mannose might be due to a reverse Amadori rearrangement (Figure 6, pathway A). Early research has reported that Amadori rearrangement products were degraded by undergoing an enolization and then forming *N*-glycosylamines, which were not stable and formed glucose and mannose (Davidek et al., 2002). Those authors found that phosphate buffer favored the formation of glucose and mannose from *N*-(1-deoxy-D-fructos-1-yl)glycine, particularly at pH 5. However, the basic mechanism to explain

why phosphate buffer solution accelerates glucose and mannose formation is still unclear. 5-Hydroxymethyl-2-furaldehyde is a main intermediate product in the Maillard reaction. As shown in Figure 5D, high yields of HMF were detected in the phosphate buffer solution. For example, the yields of HMF in phosphate buffer after a 60-min reaction at 130°C were about 2.7 and 14.1 times higher than in acetate and citrate buffer solutions. Some reasons might explain these phenomena as follows: in acidic conditions, DFP could have easily undergone 1,2-enolization, and led to the formation of 3-deoxy-2-hexosulose, which formed HMF via cyclization and dehydration reactions (Figure 6, pathway B; Hall and Smith, 1998; Salvà et al., 2003; Shipar, 2004; Reddy and Beyaz, 2006; Jalbout et al., 2008; Jalbout and Shipar, 2008; Flores-Morales et al., 2010). The series reactions stated above might be catalyzed by hydrogenous and dihydric phosphate radical anions. On the other hand, HMF was a relative stable compound at pH 5.8. A long reaction may not significantly degrade HMF. An interesting phenomenon was that little HMF was formed in citrate buffer solution at pH 5.8. A possible explanation might be that citrate favored the deg-

radation of DFP via Strecker-type reaction more easily (Figure 6, pathway C) than 1,2-enolization. However, this hypothesis should be detailed by further research. This result was in accordance with the report in the literature, which found that the concentration of HMF was increased along with the extension of the reaction time (Davidek et al., 2005). On the other hand, formic acid was always formed along with HMF. Figure 5E shows that the concentration of formic acid increased with the extension of the reaction time and degree of DFP degradation. Obviously, phosphate buffer solution accelerated the formation of formic acid compared with citrate and acetate buffer solutions. The formation of formic acid could be explained by a C1-C2 cleavage of 3-deoxy-2-hexosulose. However, seemingly contradictory results were that the yield of formic acid was not the same as the formation of HMF. If yielding 1 mol of formic acid via degradation of 3-deoxy-2-hexosulose, it should form 1 mol of HMF, based on the proposed mechanism shown in pathway B (Figure 6). A possible explanation could be that Strecker-type degradation of DFP to form formic acid in buffer solutions might also have occurred in this experiment. Moreover, HMF might have been polymerized themselves, or polymerized with proline or other MRP to form advanced products, which led to an inequality of HMF and formic acid content tested in this experiment. Therefore, it can be confirmed that hydrogenous and dihydric phosphate radical anions should also catalyze the formation of formic acid at other steps of the Maillard reaction.

CONCLUSIONS

Phosphate buffer solution accelerated the reversible degradation of glucose and DFP at the initial stage of the Maillard reaction at pH 5.8. The proposed catalysis mechanism that hydrogenous and dihydric phosphate radical anions simultaneously accepted and donated protons for the conversion of the intermediates into the *N*-glycosylamine was confirmed via testing that no change in hydrogenous and dihydric phosphate radical anion concentrations occurred during the Maillard reaction. Citrate decreased HMF formation at pH 5.8 by inducing DFP to undergo mainly Strecker-type degradation, but not 1,2-enolization reaction. This is only a hypothesis, which needs to be confirmed by labeling experiments in further research.

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