

Study on Maillard Reaction Products Derived from Aqueous and Ethanolic Fructose-Glycine and Its Oligomer Solutions

Ji-Sang Kim

Department of Food and Nutritional Science, Kyungnam University, Gyeongnam 631-701, Korea

Abstract

The present study compared the Maillard reaction products (MRPs) derived from aqueous and ethanolic fructose-glycine and its oligomer (dimer and trimer) solutions. The pH was lower in glycine (G) than in diglycine (DG) and triglycine (TG) in both aqueous and ethanolic solutions, but the pH difference between the DG and TG was not significant. MRPs derived from the DG had a greater absorbance at 294 and 420 nm in ethanolic solution than in an aqueous solution. In particular, the loss of sugar was higher in ethanolic solution than in aqueous solution. Enolization of fructose was observed in both aqueous and ethanolic MRP solutions; however, enolization was not observed for the G in aqueous MRP solutions. The glycine oligomer content in ethanolic MRP solutions remained higher than that in aqueous MRP solutions. Furthermore, neither diglycine nor triglycine were detected in the G aqueous or ethanolic MRP solutions, while triglycine was detected in both the DG aqueous and ethanolic MRP solutions. Absorption in the ultraviolet-visible (UV-Vis) spectra was higher with MRPs derived from the ethanolic solution than with those derived from the aqueous solution. MRPs derived from the DG in an ethanolic solution showed the highest absorption intensity.

Key words: glycine oligomer, Maillard reaction products, peptide chain lengths, ethanol

INTRODUCTION

The Maillard reaction is a class of nonenzymatic browning that involves the interaction of reducing sugars and free amino acids or a free amino group of an amino acid that is a part of the protein chain (1). The factors that influence the rate of the Maillard reaction in the processing and storage of food include the composition of food, the time-temperature condition, pH, water activity (aw), oxygen tension, and the presence of promoters and inhibitors (2,3). In aqueous systems, the key chemical components that directly influence the progress of the Maillard reaction are sugars and amino acids. Del Pilar Buera et al. (4) compared the degradation of various monosaccharides and disaccharides below pH 6 and found that the level of sugar degradation, in descending order, is xylose, fructose, glucose, lactose, maltose, and sucrose. Brands et al. (5) investigated the Maillard reaction in a sugar-casein system and found that those sugars having a higher proportion of acyclic forms in the solution brown more rapidly. They also found that aldoses brown more slowly than their corresponding ketoses and that nonreducing disaccharides brown even slower than aldoses because the disaccharides need to be hydrolyzed to reducing sugars first. Ashoor and Zent (6) compared the rate of Maillard browning among common

amino acids and grouped them into high, intermediate, and low browning producing groups. Higher temperature and longer time result in more serious browning (4,7). However, only a limited number of reports have dealt with non-aqueous systems. The following papers reported the Maillard reaction in non-aqueous systems: the Maillard reaction between glucose and p-chloroaniline in water/ethanol (1:1) model solutions (8), acetic acid-ethanol solution (9,10), pure methanolic or aqueous methanolic solutions (11), and methanol/water (2:1) solutions (12).

Oligopeptides have been extensively isolated and identified in both natural and artificial protein hydrolysates of foods, such as seafood, coffee beans, soy, and wheat gluten (13,14). They have been recognized as precursors of the Maillard reaction, which lead to the color of processed foods (13). Furthermore, glycine, being the simplest amino acid, with no side chains to complicate the bonding of the proton, yet one of the most important amino acids, has long been a subject of intense theoretical and experimental investigation. Studies on the simple glycine oligomer may reveal important information regarding the peptide chain lengths effect in the Maillard reaction. Recently, sugar-amino acids have been extensively studied; however, peptides and/or proteins as a source of amino acids are still a problem due to their

complexity. Since the majority of studies were carried out on sugar-amino acid model systems, little is known about sugar-oligopeptides model systems. Lu et al. (15) reported that the effects of water content on volatile generation and peptide degradation in the Maillard reaction of glycine, diglycine, and triglycine. Oh et al. (16) studied the reactivity of glycine and its simple peptides toward glucose in the generation of volatile flavor compounds in the Maillard reaction. Therefore, the objective of this study was to evaluate the profiles of Maillard reaction products derived from fructose-glycine and its oligomers in aqueous and ethanolic solutions. In the present study, Maillard reaction products have been browned to a similar degree to elucidate the different Maillard browning mechanisms in the two solvent systems.

MATERIALS AND METHODS

Materials

Ethanol (99% v/v), D-fructose, glycine, diglycine and triglycine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile, methanol, and HPLC-grade water were purchased from J.T. Baker (Phillipsburg, NJ, USA). *o*-phthalaldehyde was purchased from Agilent Technologies (Wilmington, DE, USA). All chemicals used were of analytical grade and were obtained from Merck (Darmstadt, Germany), unless mentioned otherwise.

Preparation of Maillard reaction products (MRPs)

The Maillard reaction products were prepared according to the modified Shen and Wu method (17) with some modifications. Equimolar (0.2 M) amounts of glycine, diglycine, and triglycine were dissolved, individually, with 0.2 M of fructose in about 90 mL of deionized water and 50% (v/v) ethanol solution. Three model systems were prepared for each aqueous and ethanolic solution: fructose/glycine (G), fructose/diglycine (DG) and fructose/triglycine (TG). The solution was then brought to a final volume of 100 mL with deionized water and 50% (v/v) ethanol solution adjusted to 7.8 with 6 N NaOH. The pH value was used to minimize hydrolysis of peptide bonds. Diglycine, especially, requires an optimum pH of 7.5 to 8.9 (18). Aliquots of 10 mL of the solutions were then transferred to 25 mL screw-sealed tubes (Pyrex®, NY, USA), tightly capped and heated in a thermostatic oil bath at 100°C for 36 hrs as an accelerated storage test to obtain the aqueous MRPs solution. The ethanolic MRPs solution was obtained by following a similar procedure, except that the heating time was 18 hr. After the heating period, the tubes were immedi-

ately cooled in ice water. Part of the MRP samples was used directly for final pH measurements, while the remainder was kept in an incubator (model MIR 153, Sanyo Electric Co., Ora-Gun, Gunma, Japan) at 4°C until further use, since storage for up to 2 days did not alter the sugar and glycine oligopeptide content. All model systems were prepared in triplicate.

Measurement of pH

The pH was measured using Corning 440 pH meter (Corning Life Sciences, New York, USA) calibrated with buffer solution of pH 4.0 and 10.0.

Absorbance measurements of MRPs

The UV-absorbance and browning of MRPs were measured according to the method of Ajandouz et al. (19). The absorbance of MRPs was measured using a spectrophotometer (Shimadzu UV 160A, Shimadzu Co., Kyoto, Japan) at 294 nm, as an indication of the formation of intermediate products of nonenzymatic browning, and at 420 nm, as an index of the brown polymers formed in more advanced stages.

Determination of sugars in MRPs

The reducing sugars in MRP samples were determined using an HP 1100 liquid chromatograph (Hewlett Packard, Wilmington, DE, USA). An Agilent quaternary pump connected to a refractive index detector (Model: G1362A, Hewlett Packard) was used with a Zorbax carbohydrate column (4.6 × 250 mm i.d., 5 µm particle size, Agilent Technologies, CA, USA). The mobile phase consisting of acetonitrile/water (75:25, v/v) was delivered at a flow rate of 2.0 mL/min. The column temperature was 30°C and 1 µL of sample was injected into the HPLC system. The data analysis was performed using Chemstation software (Rev. A. 10.01, Hewlett Packard).

Determination of glycine oligomer in MRPs

The glycine, diglycine, and triglycine in MRPs samples were analyzed using HP 1100 liquid chromatograph (Hewlett Packard) with a diode array detector DAD HP 1100 operating at 338 nm (Excitation=340 nm). Separation was carried out with a Zorbax Eclipse AAA Rapid Resolution column (150 × 4.6 mm i.d., 5 µm particle size, Agilent Technologies). A linear gradient profile of mobile phase, comprising 40 mM Na₂HPO₄, pH 7.8 (solvent A) and ACN/MeOH/water 45:45:10 (v/v) (solvent B), 0% B (0~1.9 min), 0~57% (1.9~18.1 min), 57~100% (18.1~18.8 min), 100% (18.8~22.3 min), 100~0% (22.3~23.2 min) and 0% (23.2~26 min) was applied at a flow rate of 2.0 mL/min. The column was equilibrated for 5 min under initial conditions prior to injection of the next samples. The column temperature

was 40°C. To determine amino acids from MRPs, pre-column derivatization with *o*-phthalaldehyde (OPA) was used and 0.5 μ L of prepared sample was injected into the HPLC system. The data analysis was performed using Chemstation software (Hewlett Packard).

Absorption spectra of MRPs

Absorption spectra of MRPs were recorded by a UV-Vis spectrophotometer (Shimadzu UV 160A, Shimadzu Co.), with the wavelength ranging from 200 nm to 700 nm.

Statistical analysis

All experimental data were subjected to analysis of variance (ANOVA) and significant differences among means from triplicate analysis at ($p < 0.05$) were determined by Duncan's multiple range tests using the statistical analysis system (SPSS 12.0 for windows, SPSS Inc, Chicago, IL).

RESULTS AND DISCUSSION

Changes in pH

The changes in pH of MRPs derived from aqueous and ethanolic fructose-glycine and its oligomer solutions are shown in Fig. 1. Although all MRP samples started with the same initial pH, the values decreased in both aqueous and ethanolic solutions. Among the glycine oligomers tested, pH was lower in the G compared to the DG and TG in both aqueous and ethanolic solutions; the difference between the DG and TG was not significant ($p > 0.05$). The results were in agreement with the results obtained in studies conducted by Morales and Jimenez-Perez (20) and Kim and Lee (21,22), who re-

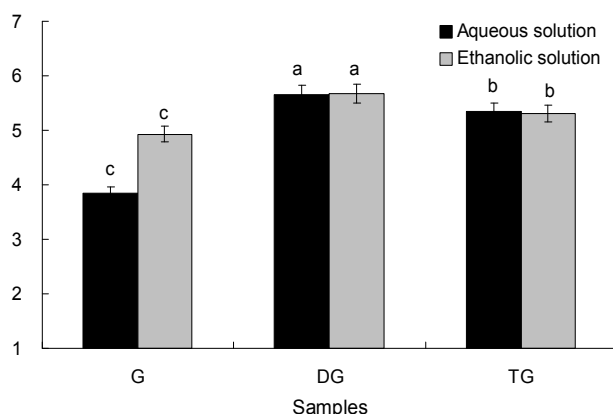


Fig. 1. The changes in pH of MRPs derived from aqueous and ethanolic fructose-glycine and its oligomer solutions. Bars indicate the standard deviation from triplicate determinations. G, glycine with fructose; DG, diglycine with fructose; TG, triglycine with fructose. Different letters (a-c) indicate significant differences according to the samples in the same solvent system at $p < 0.05$ level.

ported frequent pH decreases with increasing heating time during the Maillard reaction. Ogura et al. (23) reported that the formation of the acid form of glycine, or glycyl-glycine, was a result of a decrease in pH, which, in turn, was attributed to oxygen evolution occurring with increasing positive polarization. Huber and Wächtershäuser (24) reported that a decrease in pH can be explained by the formation of acids from CO during the heating process of peptides. The molecule size could also play a role in the smaller reactivity of peptides compared to glycine (25). In addition, Shen and Wu (17) reported that pH in an ethanolic glucose-glycine solution decreased with an increase in ethanol concentration.

Changes in absorbance at 294 nm and browning intensity

An absorbance at 294 nm was used to determine the intermediate compounds of the Maillard reaction (26). Development of a brown color (A_{420} nm) is the easiest measurable consequence of the Maillard reaction because it offers a visual estimate. The changes in absorbance at 294 nm and browning intensity at 420 nm of MRPs derived from aqueous and ethanolic fructose-gly-

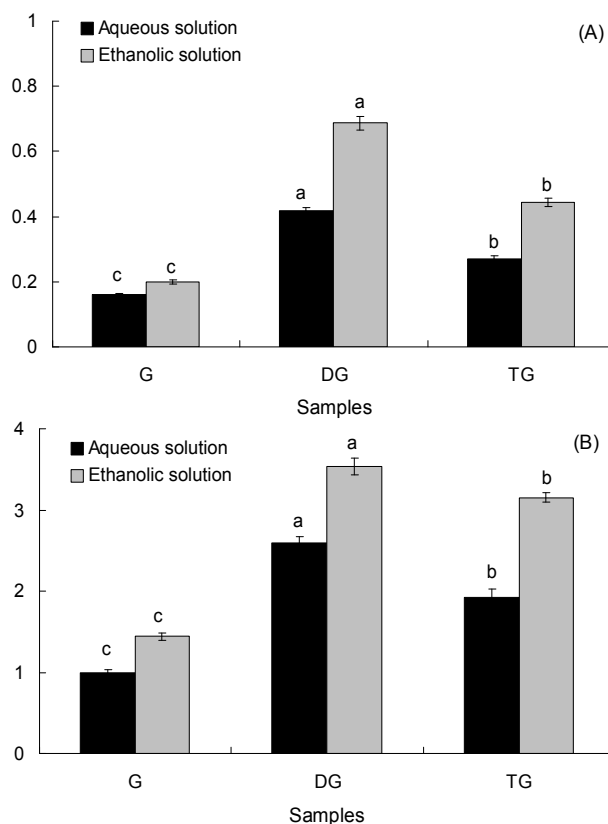


Fig. 2. The changes in browning intensity (A) and absorbance at 294 nm (B) of MRPs derived from aqueous and ethanolic fructose-glycine and its oligomer solutions. Bars indicate the standard deviation from triplicate determinations. Different letters (a-c) indicate significant differences according to the samples in the same solvent system at $p < 0.05$ level.

cine and its oligomer solutions are shown in Fig. 2. After the heating period, the absorbance at 294 nm for all MRP samples increased sharply in both aqueous and ethanolic solutions. The difference in absorbance at 294 nm was statistically significant based on the reaction media and the glycine oligomer ($p < 0.05$). The samples in ethanolic MRP solutions had a greater absorbance at 294 nm than those in aqueous MRP solutions. The increase in absorbance at 294 nm suggested the formation of an uncolored compound, which could be the precursor of the Maillard reaction. In addition, the browning intensities of all MRP samples increased with a concomitant increase in A_{294} . The MRPs derived from the DG solution showed the highest increase in absorbance at 294 and 420 nm, followed by those derived from the TG and G solutions. These results are in agreement with the results from Lu et al. (15), who reported that diglycine-glucose reaction mixtures had higher degrees of browning, followed by glycine-glucose, and triglycine-glucose reaction mixtures. Maillard himself first reported that the browning reaction with fructose occurred at a faster rate than that with glucose (27), an observation that was repeated numerous times (28-30). In many cases, however, the extent of the browning reactions with D-fructose and proteins was not as extensive as that with glucose (31). In this regard Suárez et al. (32) observed that protein previously glycosylated by glucose reacted more extensively with additional hexose than that previously glycosylated with fructose. Therefore, the result suggested that intermediate products and brown pigmentation might be dependent on hydrolysis and stability of the peptide bond. Particularly, this indicated that the peptide bond for triglycine was more stable than that of diglycine.

Loss and enolization of sugar

As depicted in Fig. 3, after the heating period, the

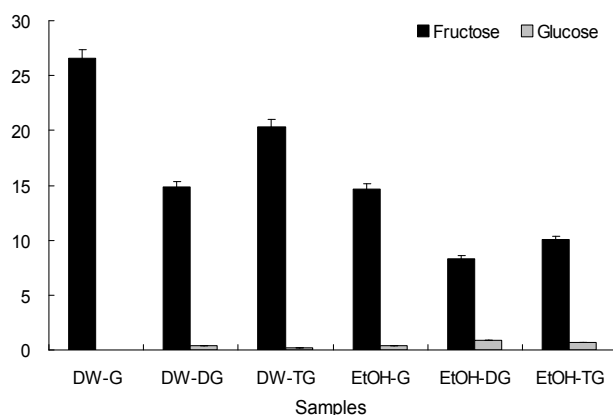


Fig. 3. The loss and enolization of sugar in MRPs derived from aqueous and ethanolic fructose-glycine and its oligomer solutions. Bars indicate the standard deviation from triplicate determinations.

sugar content of all MRP samples was noticeably decreased in both aqueous and ethanolic solutions. The loss of sugar in ethanolic MRP solutions was higher than that in aqueous MRP solutions ($p < 0.05$); the difference in the loss of sugar was statistically significant based on the reaction media and the glycine oligomer ($p < 0.05$). The loss of sugar was highest in the DG solution, although enolization of fructose was observed in both aqueous and ethanolic MRP solutions, except for the G in aqueous MRP solutions. Enolization is generally accepted to be the rate-limiting step, whereas the subsequent β -elimination reactions are rapid in the presence of acid. In the case of 2-ketohexoses, such as fructose, the reaction rate is usually higher than that for aldoses because 1,2-enolization occurs more easily (33). However, the degree of sugar enolization is favored by an alkaline pH condition, leading to the transformation of these sugars (34). Of special interest, the rate of enolization was decreased with increasing ethanol concentration and decreasing hydroxyl-ion concentration (35). Strong evidence for sugar isomerization was provided by reports that the formation of sugar isomerization occurred mainly during the course of the Maillard reaction with an amino acid as well as an oligopeptide (22,36). Therefore, the results suggested that the loss and isomerization of sugar can be influenced by the reaction media and peptide chain length, and sugar isomerization can also occur during the course of the Maillard reaction in ethanolic solutions.

Changes in glycine oligomer

At an early stage of the MRPs, terminal α -amino groups of peptides react with the carbonyl function of reducing sugars present in the reaction medium. Thus, the loss of available primary amino groups is another indicator used to compare sugar reactivity in MRPs (37). Changes in the glycine oligomer of the MRPs derived from aqueous and ethanolic fructose-glycine and its oligomer solutions are shown in Table 1. The changes in the glycine oligomer were only statistically different in the TG solution. In aqueous MRP solutions, the glycine oligomer remained at 61.54, 87.69, and 22.06% (mol/mol) in the G, DG, and TG solutions, respectively. Diglycine degraded to glycine with an 11.44% (mol/mol) yield. Triglycine mainly degraded to glycine with a 40.99% (mol/mol) yield, and diglycine with a 6.38% (mol/mol) yield. In contrast, in ethanolic MRP solutions, the glycine oligomer remained at 62.15, 88.75, and 47.52% (mol/mol) in the G, DG, and TG solutions, respectively. Diglycine degraded to glycine with a 9.57% (mol/mol) yield. Triglycine mainly degraded to glycine with a 32.61% (mol/mol) yield, and diglycine with a

Table 1. The changes in glycine, diglycine, and triglycine (mol/mol %) in the MRPs derived from aqueous and ethanolic fructose-glycine and its oligomer solutions¹⁾

| Samples ²⁾ | | Glycine oligomer | | |
|-----------------------|----|---------------------------|---------------------------|---------------------------|
| | | Glycine | Diglycine | Triglycine |
| Aqueous solution | G | 61.54 ± 2.57 ^a | nd ³⁾ | nd |
| | DG | 11.44 ± 0.24 ^d | 87.69 ± 0.42 ^a | nd |
| | TG | 40.99 ± 0.23 ^b | 6.38 ± 0.03 ^b | 22.06 ± 0.26 ^b |
| Ethanolic solution | G | 62.15 ± 0.37 ^a | nd | nd |
| | DG | 9.57 ± 0.16 ^d | 88.75 ± 1.83 ^a | nd |
| | TG | 32.61 ± 0.02 ^c | 3.14 ± 0.06 ^c | 47.52 ± 0.04 ^a |

Different superscripts (a-d) indicate significant differences among samples in the same glycine oligomer at $p < 0.05$ level.

¹⁾Values represent mean ± SD (n=3).

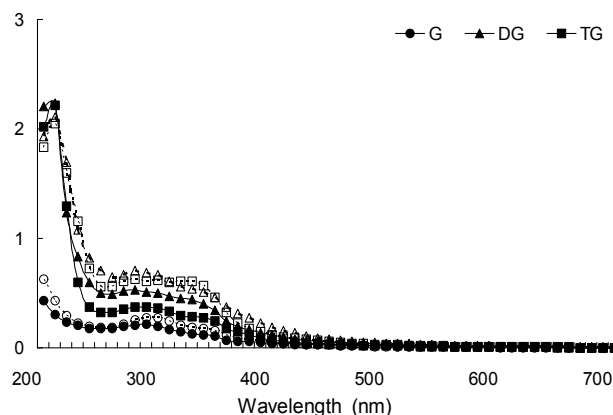
²⁾Glycine with fructose; Diglycine with fructose; Triglycine with fructose.

³⁾Not detected.

3.14% (mol/mol) yield. The glycine oligomer content remaining in ethanolic MRP solutions was more than that in aqueous MRP solutions. Furthermore, neither diglycine nor triglycine was detected in the G aqueous or ethanolic MRP solutions, while triglycine was detected in the DG aqueous or ethanolic MRP solutions. However, Nagayama et al. (38) reported that the possible mechanisms of formation of triglycine are chain elongation or opening of the diketopiperazine (DKP) ring with addition of one glycine molecule. Imai et al. (39) reported that when repeated circulation of glycine through the hot (200~250°C) and cold (0°C) regions in a flow reactor at 24.0 MPa, glycine oligomers were made. In addition, typical monohydric alcohols such as methanol, ethanol, and *n*-propanol generally destabilize the native structure and stabilize the α -helical conformation in unfolded proteins and peptides; however, at lower concentrations, some alcohols have been found to slightly stabilize the native state of the protein (40). Lu et al. (15) found that diglycine generated less glycine than triglycine in both aqueous and ethanolic MRP solutions because of the high electron density of the peptide bond, which suppresses hydrolysis. The fact that glycine and diglycine were synthesized with no detectable amounts of diglycine or triglycine in either aqueous or ethanolic model systems suggests that diglycine and triglycine molecules can be rapidly hydrolyzed into molecules of glycine and diglycine, respectively. Therefore, this result suggested that the hydrolysis of the glycine oligomer during the Maillard reaction can be influenced by an ethanol reaction media and the stability of the peptide bond.

Absorption spectra of MRPs

The changes in the absorption spectra of the MRPs derived from aqueous and ethanolic fructose-glycine and

**Fig. 4.** The changes in the absorption spectra of MRPs derived from aqueous and ethanolic fructose-glycine and its oligomer solutions. Solid line: aqueous model systems, dotted line: ethanolic model systems.

its oligomer solutions are shown in Fig. 4. All MRP samples showed different absorptions in the ultra-violet-visible (UV-Vis) spectra, although these had similar shapes in both aqueous and ethanolic solutions. The absorption intensities of MRPs derived from an ethanolic solution were higher than from an aqueous solution. The higher intensity of all MRP samples was indicative of increased absorbance in the ultraviolet region, and the absorbance was gradually reduced in the visible region. Every peak had a maximum absorbance that appeared in the range 260~320 nm, which is characteristic of melanoidins. This trend was also described by other authors for melanoidin-type colorants (41). Moreover, the band intensities of MRPs derived from the DG and TG solutions were significantly higher than those of MRPs derived from the G solution. This wavelength intensity is likely due to a possible increase in the extent of chromophore conjugation in melanoidins, resulting in a co-pigmentation effect. The UV-Vis spectra, exhibiting both featureless end absorption and increased intensity with a decrease in the wavelength, were typical of melanoidins (42). UV-Vis radiation comprises only a small part of the electromagnetic spectrum (about 100~750 nm), and UV-Vis absorption spectroscopy can be described as spectroscopy involving the electronic energy levels of the molecule. Hence, the absorption of radiation leads to transitions among the electronic energy levels of the molecule (43). Therefore, this result indicated that the electronic energy levels of the MRP molecules varied according to the reaction media and the peptide chain length.

In conclusion, the pH value was lower in the G than in the DG and TG in both aqueous and ethanolic solutions, while the difference between the DG and TG was not significant. MRPs derived from the DG had a greater absorbance at 294 and 420 nm in ethanolic solution than

in an aqueous solution. In particular, the loss of sugar was higher in ethanolic solution than in aqueous solution. Enolization of fructose was observed in both aqueous and ethanolic MRP solutions; however, enolization was not observed for the G in aqueous MRP solutions. The glycine oligomer content in ethanolic MRP solutions remained higher than that in aqueous MRP solutions. Furthermore, neither diglycine nor triglycine was detected in the G aqueous or ethanolic MRP solutions, and triglycine was detected in the DG aqueous or ethanolic MRP solutions. Absorption in the UV-Vis spectra was higher in MRPs derived from the ethanolic solution than in those derived from the aqueous solution. MRPs derived from the DG in an ethanolic solution showed the highest absorption intensity. The results indicate that the reaction media and peptide chain length affect the Maillard reaction, which is more markedly accelerated in an ethanolic solution than in an aqueous solution. In addition, the diglycine molecule is more rapidly hydrolyzed than glycine and triglycine, regardless of the reaction media. Thus, the Maillard reaction rate might be related to the degree of hydrolysis of the peptide bond and the reaction media.

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