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Formation of protein-bound N^{ϵ}-carboxymethyllysine and N^{ϵ}-carboxyethyllysine in ground pork during commercial sterilization as affected by the type and concentration of sugars

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ABSTRACT

This research was aimed to investigate the formation of protein-bound N^{ϵ}-carboxymethyllysine (CML) and N^{ϵ}-carboxyethyllysine (CEL) in ground pork at 121 °C (5–30 min) as affected by sugars (1–9% w/w, glucose, fructose, lactose, and sucrose).The addition of reducing sugar significantly ($P < 0.05$) increased the levels of CML and CEL in heat treated pork samples. Even adding 1% of glucose in pork could lead to 3.8 and 4.0 times increase in the formation rate constant (zero-order) of CML and CEL, respectively. In a typical commercial sterilization process (121 °C, 30 min), adding glucose, fructose or lactose in pork resulted in an average increase of 224–581%, 26–276%, and 8–189% CML, and 217–720%, 213%–15.8 times, and 20–150% CEL, respectively, depending on the sugar concentration. Sucrose did not promote the formation of CML and CEL in pork during heating.

1. Introduction

Meat products are rich in proteins and fats, and in general subjected to more severe heat treatments compared to plant-source foods, which favor the formation of advanced glycation end-products (AGEs), a group of relatively stable compounds with potentially negative effects on human health (Hellwig et al., 2019; Sun et al., 2015; Zhu, Huang, Cheng, Khan, & Huang, 2020). Dietary AGEs are mainly formed from different stages of the Maillard reaction and possibly other approaches such as lipid oxidation and ascorbate oxidation, depending on the structural characterization of the target compound(s) (Srey et al., 2010). Typically, N^{ϵ}-carboxymethyllysine (CML), as the most well-known AGE, could be formed through oxidative degradation of Amadori rearrangement products (ARPs) like fructosyllysine and Heyns rearrangement products (HRPs) like glucosyllysine, as well as through the reaction between lysine and glyoxal (Treibmann, Hellwig, Hellwig, & Henle, 2017). N^{ϵ}-carboxyethyllysine (CEL) is also among the most studied AGEs, but to a much lesser extent as compared to CML. The known pathways of CEL formation is mainly linked to the reaction between lysine and methylglyoxal (Srey et al., 2010; Treibmann et al., 2017). Both glyoxal and methylglyoxal could be formed through lipid oxidation and degradation of sugars, ARPs, HRPs and ascorbic acid (Jiao et al., 2019; Srey et al., 2010; Sun et al., 2017).

Sugar, particularly reducing sugar, as one of the two reactants for the Maillard reaction and the major source of glyoxal and methylglyoxal, plays a key role on the formation of AGEs. In general, at the same sugar level, monosaccharides in food or model systems result in higher amounts of AGEs than disaccharides, while reducing disaccharides (such as lactose and maltose) lead to the formation of more AGEs than non-reducing disaccharides (such as sucrose) (Srey et al., 2010; Treibmann et al., 2017; Villaverde & Estévez, 2013). However, due to the disparity in the samples used and the difference in the test or heating conditions among different studies on the levels of AGEs as affected by sugars, the reported results may be quite different. For example, Treibmann et al. (2017) showed that cookies (baked at 175 °C for 10 min) prepared using glucose, fructose (about 17% sugar w/w) had similar amount of CML, and much higher than that prepared with sucrose; but the study of Charissou, Ait-Ameur, & Birlouez-Aragon (2007) indicated that cookies (200–300 °C, 7–16 min; 30% w/w of sugar) with fructose had the least amounts of CML, and the cookies with sucrose contained the highest CML level when baked at 200 °C and a lower CML level than the cookies with glucose when baked at

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250–300 °C.

To date, the majority of reported studies on the formation of dietary AGEs as affected by sugar were based upon model systems, such as myofibrillar proteins with reducing sugar (Villaverde & Estévez, 2013), or model foods, such as cookies and sponge cakes (Charissou et al., 2007; Srey et al., 2010; Treibmann et al., 2017). Little is known about the effects of sugar on the levels of AGEs in meat, although sugar is often added directly or as the main ingredient in cooking sauces in meat products to improve their flavor characteristics (Chao, Hsu, & Yin, 2009). The composition, particularly the sugar level, and the processing condition (such as temperature) for meat products are quite different from that of the reported model foods. Thus, systematic studies for understanding the formation of AGEs in meat during heating as affected by sugars are much needed. Such information would help proper design meat processing or preparation operations to limit the formation of AGEs in meat products Therefore, the objective of this study was to investigate the formation of CML and CEL in ground pork during commercial sterilization (121 °C, 5–30 min) as affected by the type and concentration (1–9% w/w) of sugars including glucose, fructose, lactose and sucrose.

2. Materials and methods

2.1. Preparation of ground pork containing different types and levels of sugars

Fresh lean pork from the hind leg of the pig was bought from a supermarket in Shanghai. The pork was cut into small pieces, mixed well, and stored in plastic bags (*ca.* 1 kg/bag) at − 80 °C. Before an experiment, a bag of pork sample was thawed overnight in a refrigerator, and then ground in a blender at low speed for three times with 10 s each time (8010 s, Waring, Inc., Torrington, Connecticut, USA). The pork samples with different levels of sugars (1%, 3%, and 9% w/w) were prepared by adding 1–9 g of D-glucose, D-fructose, D-lactose and D-sucrose to 99–91 g of ground pork. All four sugars were 99% of purity and purchased from J&K Scientific Ltd. (Shanghai, China).

2.2. Thermal treatments

About 13 g of ground pork with or without sugar was sealed in an aluminum cylindrical cell (diameter, 50 mm; height 5 mm) (Kong, Tang, Rasco, Crapo, & Smiley, 2007; Sun et al., 2015; Niu et al., 2018), heated at 121 °C for 5, 10, 15, or 30 min in a dimethyl silicone oil bath (HAAKE PC 300-S7; Thermo Fisher Scientific Inc., Waltham MA), and immediately cooled down in ice-water. The come-up time for the ground pork in the aluminum cell was around 4–5 min, and 10 min of heating was the minimum time required to obtain commercial sterile pork with an adequate margin for safety purpose (Heinz & Hautzinger, 2007; Sun et al., 2016). For retort canning, the required processing time at 121 °C could be 30 min or longer, and thus 30 min of heating time was also used in the study (Tang, 2015).

All heat treatments for pork samples containing different types and levels of sugars were repeated two times on different days. The concentrations of protein-bound CML and CEL in each sample were analyzed twice with the following HPLC-MS/MS method (Sun et al., 2015).

2.3. Analysis for protein-bound CML and CEL in pork

Since heating had minor effects on the free forms of CML and CEL in meat, poultry and fish based upon our previous investigation (Niu et al., 2017; Sun et al., 2016), only protein-bound CML and CEL in pork were determined with an HPLC-MS/MS method following the same procedure as that in Sun et al. (2015). First of all, to prepare a pork sample for the HPLC-MS/MS analysis, raw or heat treated pork (*ca.* 0.2000 g) was reduced (4 °C, 8 h) in a mixture of borate buffer (0.2 M, 2 mL) and sodium borohydride (2 M in 0.1 N NaOH, 0.4 mL) to prevent the production of AGEs in the later acid hydrolysis step (Niquet-Léridon & Tessier, 2011). Then, methanol-chloroform (1:2, v/v; 4 mL) was added to remove fat and precipitate protein. Following this, the protein was separated through centrifugation and hydrolyzed (110 °C, 24 h) in hydrochloric acid (6 M, 4 mL). The protein hydrolysate (4 mL) was spiked with an internal standard (d_4-CML, 8 mg/L), dried at 50 °C in a vacuum oven, reconstituted in water, and further purified using an MCX cartridge, dried in nitrogen, reconstituted in methanol–water, and finally cleaned up with a membrane filter.

A Waters 2695 HPLC system (Waters Corp., Milford, MA, USA) and a Waters Quattro Micro triple-quadrupole tandem mass spectrometer (MS/MS) was used for the determination of CML and CEL. All test conditions or the instrument settings for both HPLC and MS/MS were the same as that described in Sun et al. (2015) except for the concentrations of the internal standard (d_4-CML, 8 mg/L) and AGEs standard mixture (CML, CEL: 300 μg/L; d_4-CML: 400 μg/L). All three AGEs standards were of 98% purity and bought from Toronto Research Chemicals Inc. (Ontario, Canada). The other chemicals were bought from Sinopharm Group Co., Ltd (Shanghai, China).

The limit of detection (4–5 μg/kg) and recovery of CML and CEL in pork samples were conducted in our previous study (Sun et al., 2016). The recovery (n = 6) was 79.0–124.3% for CML and 97.4–114.7% for CEL, depending upon the concentration (100, 500, or 1000 μg/kg) of the spiked CML or CEL (Sun et al., 2016).

2.4. Data analysis

For each sugar, two-way analysis of variance (ANOVA) was applied to analyze whether the single and interactive effects of sugar concentration and heating time on the average amount of CML or CEL in heat treated pork were significant ($\alpha = 0.05$) or not, and a post-hoc least significant difference (LSD; $\alpha = 0.05$) test was further used for the pair comparison of the mean values of CML or CEL in pork with different sugar levels or heated for different length of time (SPSS Version 21, IBM Corp. Armonk, NY).

The following kinetic function was used to evaluate how fast CML or CEL was formed in ground pork with or without sugar at 121 °C,

$$\frac{dC}{dt} = kC^n \quad (1)$$

where C is the concentration of CML or CEL in pork after being heated for t min, and k is the rate constant. The order of reaction (n) was selected based upon the best linear fitting between t and C ($n = 0$), t and ln C ($n = 1$), and t and $1/C$ ($n = 2$) (Microsoft Excel 2019, Redmond, WA) (Sun et al., 2015). Since the come-up time for ground pork in the test cell was about 4–5 min, the 5 min of actual sample heating time was considered as time zero ($t = 0$), and the t values for other heat treated pork samples were obtained by subtracting 5 min from the actual heating time of the samples.

3. Results and discussion

3.1. Sample information

The lean hind leg pork used in this study contained 77.5 ± 0.1% of water, 20.0 ± 0.5% of protein, and 0.5 ± 0.1% of fat based on sample weight (w/w), which were analyzed by a certified laboratory. The protein levels in the ground pork added with three different levels of sugar were calculated as 19.8% (1% sugar), 19.4% (3% sugar) and 18.2% (9% sugar), respectively. Since the addition of sugar in pork would change the percentage level of protein, the CML and CEL concentrations in pork presented in this article were based upon the protein weight (mg/kg protein) instead of sample weight. The raw pork contained 11.5 ± 0.8 mg/kg protein of CML and 8.0 ± 2.2 mg/kg protein of CEL.

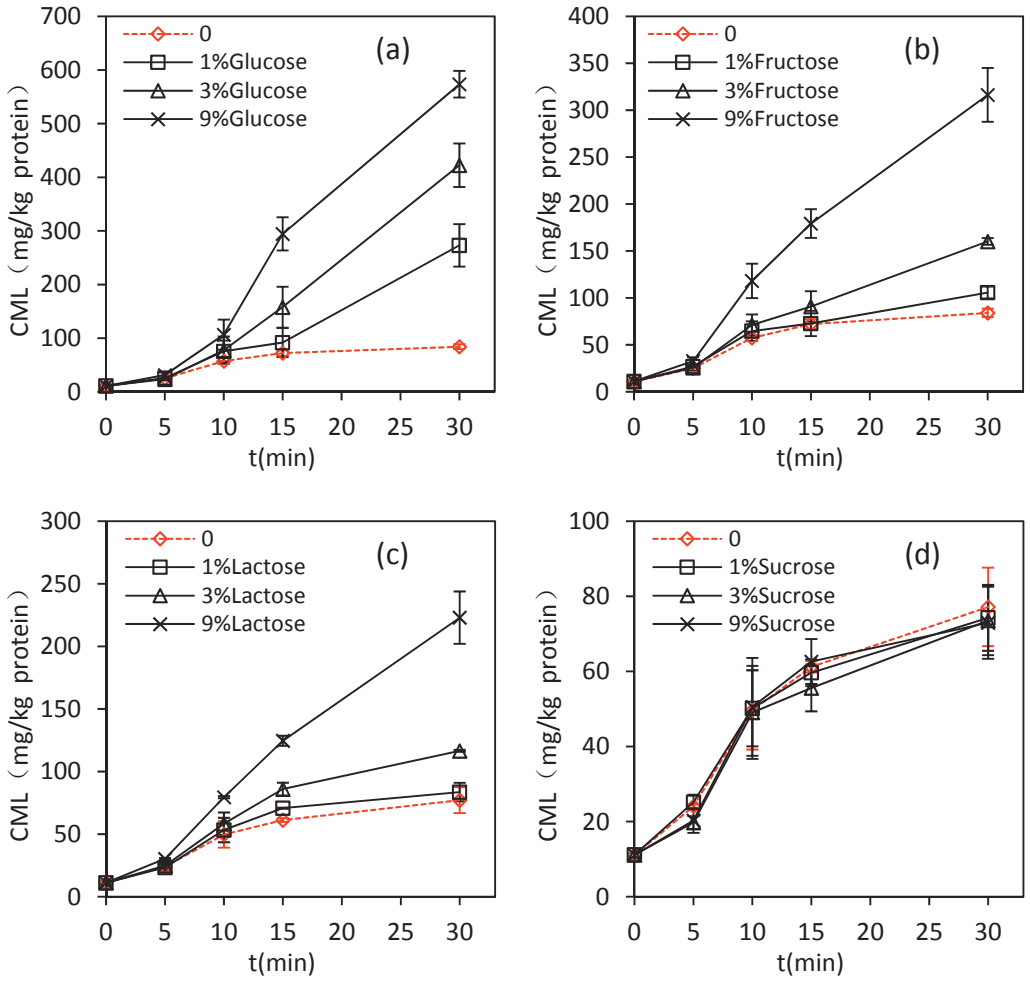

Fig. 1. Effects of (a) glucose, (b) fructose, (c) lactose, and (d) sucrose and heat treatments at 121 °C on the levels of N^{ϵ}-carboxymethyllysine (CML) in ground pork.

3.2. Formation of CML as affected by heating time and sugars

The amounts of CML in the control (pork without added sugar) were 58.5 ± 1.60 mg/kg protein after 10 min of heating and 85.4 ± 4.9 mg/kg protein after 30 min of heating at 121 °C, which were consistent with other reported studies (Chen & Smith, 2015; Sun et al., 2016).The effects of sugars and heat treatments at 121 °C on the levels of CML in pork are shown in Fig. 1. As expected, a continuous increase of CML during heating was observed, regardless of the type and the level of sugar added. Except for sucrose, the addition of a higher level of sugar resulted in more CML formed in pork heated for 10 min or longer time. For examples, after 10 min of heating, pork with 1%, 3% and 9% of glucose contained an average of 31%, 35% and 85% more CML than their control counterpart, while after 30 min of heating, the corresponding CML were 224%, 402% and 581% more than their control. At the same sugar level, the addition of glucose in pork in general led to the highest amount of CML formed during heating, followed by fructose, lactose, and sucrose. As compared to their control counterparts without sugar, pork with fructose had 12–105% and 26–276% more CML after 10 min and 30 min of heating at 121 °C, respectively; while pork with lactose contained 7–60% and 8–189% more CML after 10 min and 30 min of heating, respectively.

Two-way ANOVA indicated that for each sugar, except for sucrose, the sugar concentration ($P < 0.001$), heating time ($P < 0.001$) and the interaction of both factors ($P < 0.001$) significantly affected the mean values of CML in heated pork samples; the addition of sucrose caused no significant difference ($P = 0.928$) in the mean values of CML in heated pork, though different heating time still significantly influenced ($P < 0.001$) the CML level in pork with sucrose. A post-hoc LSD test for the effects of four heat treatments varying in heating time (5–30 min) on the formation of CML in pork with a specific type of sugar showed that any two different heating time led to a significant difference in the mean values of CML, regardless of the sugar concentration (data not shown). As for the influence of sugar concentrations on CML levels in heated pork, the results from post-hoc LSD test for each sugar are shown in Fig. 2. The addition of different amounts of glucose, even at a 1% level, significantly affected the mean values of CML in heated pork samples, regardless of the heating time (Fig. 2a). However, for heated pork samples with 1% fructose or lactose, the mean of their CML levels was not significantly different from that of the control, and only the addition of 3% or higher concentration of fructose or lactose caused a significant increase in the mean of CML levels (Fig. 2b and c).The addition of sucrose did not significantly influence the mean values of CML in heated pork samples (Fig. 2d).

Little is known about the effects of sugars on the levels of CML in meat, but the results from this study were consistent with some other studies using model foods. For examples, some studies indicated that cookies or sponge cakes prepared with glucose (*ca.* 17%–30% w/w) contained higher CML content compared to those with the same levels of fructose (Charissou et al., 2007; Srey et al., 2010; Treibmann et al., 2017). Also, the study of Villaverde and Estévez (2013) showed that glucose and fructose were more reactive than lactose for the formation of fluorescence AGEs in a myofibrillar proteins and reducing sugar system. Nevertheless, although sucrose in cookies baked at high temperature (200–300 °C, 10–16 min) led to the acceleration of CML formation likely due to glucose and fructose produced from the hydrolysis

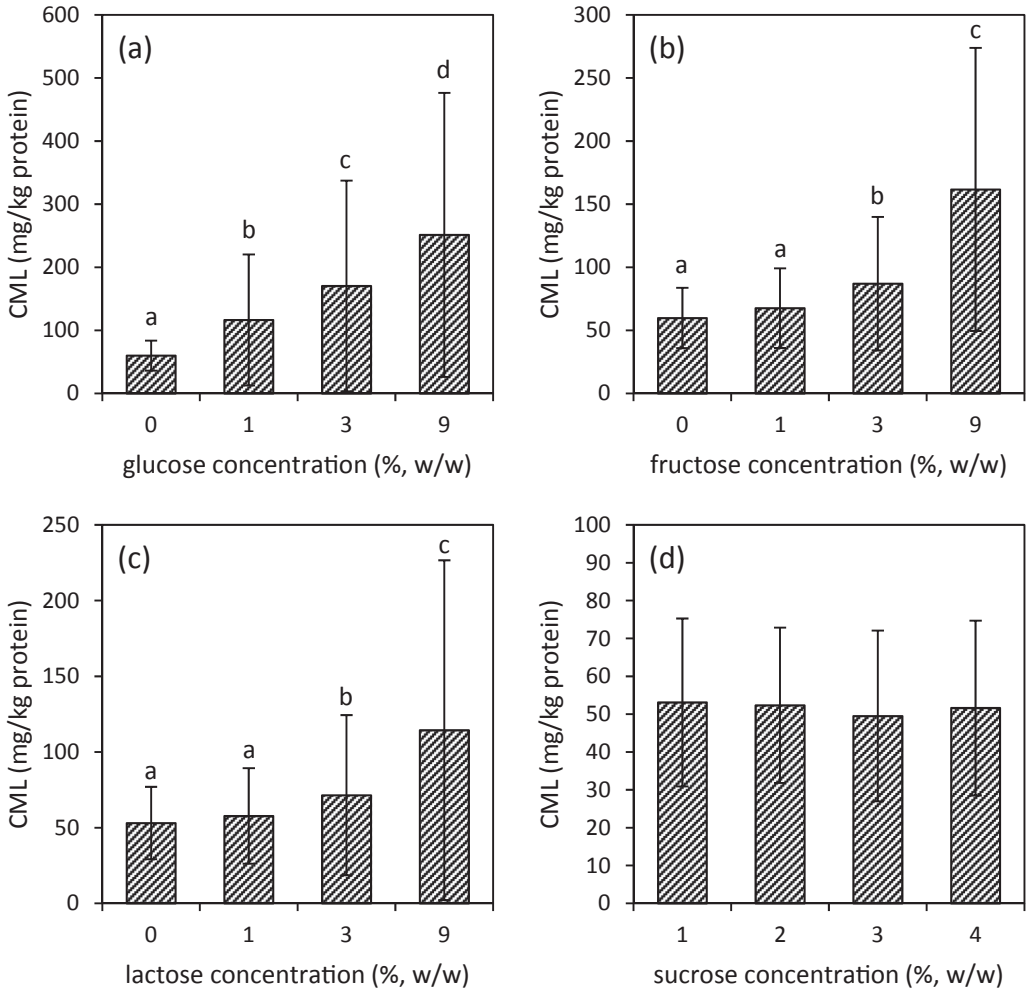

Fig. 2. Effects of different concentrations of (a) glucose, (b) fructose, (c) lactose, and (d) sucrose on the average amounts of N^{ε}-carboxymethyllysine (CML) in heat treated pork (121 °C, 5–30 min). Different letters indicate significant difference (n = 8) based upon a post-hoc least significant difference (α = 0.05) test.

of sucrose (Charissou et al., 2007), no similar effect was found for sucrose in pork heated at 121 °C for up to 30 min, possibly due to the less severe heat treatment used in this study.

3.3. Formation of CEL as affected by heating time and sugars

The amounts of CEL in pork without added sugar were 29.8 ± 8.2 mg/kg protein after 10 min of heating and 43.5 ± 7.2 mg/kg protein after 30 min of heating at 121 °C, and both CEL levels were only about 51% of the CML levels in pork with the same heat treatments. Similar to that for CML, the concentration of CEL in heat treated pork increased as the heating time increased, and adding a higher level of reducing sugar led to more CEL formed in pork heated for 10 min or longer time (Fig. 3). Except for pork samples with fructose, the amounts of CEL formed in all other sugar treated pork during the same heat treatment were less than that of CML. Nevertheless, as compared to their control pork without sugar added, adding 1–9% of glucose, fructose, and lactose into pork resulted in 46–154%, 59–435%, and 5–47% more CEL after 10 min of heating, respectively, and 217–720%, 213%–15.8 times, 20–150% more CEL after 30 min of heating, respectively. Actually, the addition of fructose or glucose in general resulted in greater increase of CEL in percentage than that of CML in pork with the same heat treatments. At the same sugar level, the addition of fructose in pork in general led to the highest amount of CEL formed during heating, followed by glucose, and lactose, and sucrose had no obvious effect on the formation of CEL in pork during heating.

Two factor ANOVA for the levels of CEL in pork as influenced by sugar concentration and heating time also resulted in similar results as that for CML in pork. The sugar concentration ($P < 0.001$), heating time ($P < 0.001$) and the interaction of both factors ($P \leq 0.016$) significantly affected the CEL level in pork with glucose, fructose or lactose, but the addition of sucrose had no significant effect ($P = 0.623$) on the CEL formation during heating. The results from post-hoc LSD test for the effect of concentrations of each sugar on CEL levels in heated pork are shown in Fig. 4. The addition of different levels of either glucose or fructose, as little as 1%, significantly affected the mean of CEL levels in heated pork samples (Fig. 4a and b). The effects of two disaccharides on the CEL levels were similar to that for CML levels in heated pork. The addition of 3% or more lactose (Fig. 4c) led to significant increase of CEL in heated pork in comparison with the control without sugar added, and the addition of sucrose did not significantly affect the CEL level (Fig. 4d). However, the effect of fructose on the CEL formation in pork was somewhat different from that on the CML formation. Firstly, even with 1% fructose, the average amount of heat induced CEL in pork was 163% more than that of the control, a significant increase in the mean of CEL levels, but there was no such significant increase in the mean of CML levels (Fig. 4b vs. Fig. 2b). Secondly, the average heat induced CEL levels was higher than that of CML in pork with the same level of fructose, which indicated that probably a higher amount of methylglyoxal (precursor for CEL) was formed than glyoxal (precursor for CML) in fructose treated pork during heating (Srey et al., 2010). This result was in line with that of Srey et al.

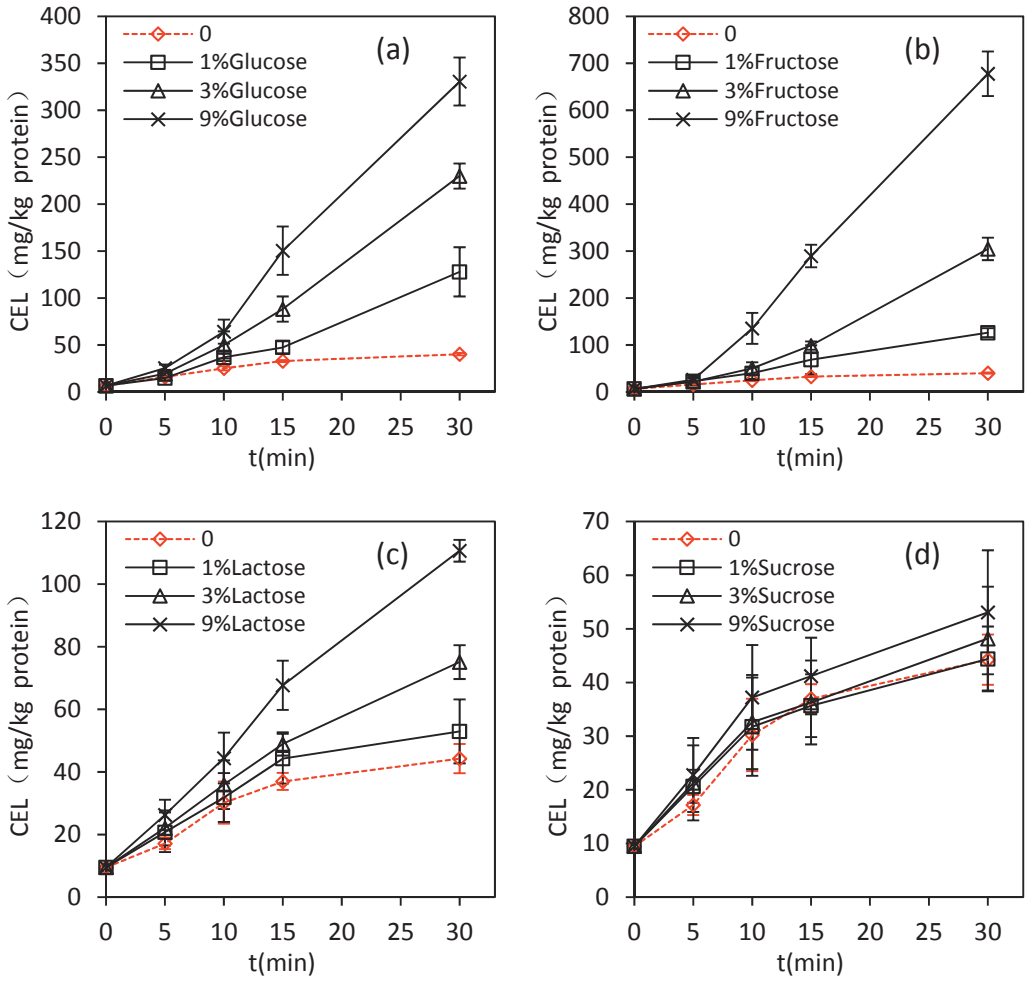

Fig. 3. Effects of (a) glucose, (b) fructose, (c) lactose, and (d) sucrose and heat treatments at 121 °C on the levels of N^{ε}-carboxyethyllysine (CEL) in ground pork.

(2010) and Treibmann et al. (2017). Srey et al. (2010) showed that the CEL level in sponge cake (*ca.* 27% w/w sugar, 190 °C, 30 min) with fructose was 6.8 and 14.5 times higher than that with glucose and refined sucrose. Similarly, the study of Treibmann et al. (2017) indicated that 2.1 times more CEL was found in cookies (175 °C, 10 min) added with fructose (*ca.* 17% w/w) than that with the same level of glucose. The formation of CEL is generally attributed to the reaction of lysine and methylglyoxal, and higher amount of methylglyoxal was produced in a fructose-lysine system in comparison with its corresponding glucose-lysine system (Chen & Kitts, 2011; Novotný, Cejpek, &Velíšek, 2007; Treibmann et al., 2017).

3.4. Effects of sugars on formation rates of CML and CEL in pork

Formation rates of either CML or CEL in pork at 121 °C (up to 25 min) could be considered as zero-order reaction (R^2 for CML: control 0.754–0.832, glucose 0.953–0.999, fructose 0.805–0.991, lactose 0.751–0.998, sucrose 0.697–0.894; R^2 for CEL: control 0.784–0.913, glucose 0.972–0.999, fructose 0.831–1.000, lactose 0.848–0.995, sucrose 0.756–0.957). This indicated that the CML or CEL concentration in pork was minor compared to their precursors and thus did not influence their formation rates (Van Boekel, 2008), which was in line with the results from our previous study on AGEs in heat treated (65–100 °C) beef (Sun et al., 2015). Fig. 5 shows how the type and concentration of sugars affected the rate constant (k) of the zero-order reaction for the formation of CML or CEL in pork during heating. Except for sucrose, the formation rates of CML and CEL in pork greatly increased as the sugar level increased. For CML, its average of formation rate constants in pork with glucose, fructose and lactose were 479%–10.7-fold, 137–529%, and 113–396% of that of the control without sugar (k = 2.07 mg kg^{-1} min^{-1}), respectively, depending on the sugar level. The addition of glucose affected the most on the CML formation during heating at 121 °C. Even adding 1% of glucose led to 3.8 times faster in CML formation as compared to the control. At the same sugar level, the formation rates of CML in pork containing glucose were 2.0–3.5 times and 2.9–4.7 times of that in pork containing fructose and lactose, respectively (Fig. 5a). However, for the formation rate of CEL in pork, the addition of fructose at 3% and 9% levels led to 36% and 110% faster than the glucose treated counterparts, although at 1% level the formation rate was slower than the glucose treated pork (Fig. 5b). Nevertheless, both fructose and glucose had great effects on the formation rate of CEL. The addition of 1% of fructose or glucose led to 3.6–4.0 times faster in CEL formation as compared to the control (k = 0.90 mg kg^{-1} min^{-1}), while the addition of 9% of either sugar led to 12.9–28.2 times faster than the control. The impact of lactose on the formation rate of CEL was relatively small but still quite obviously, resulting in an average of 25–245% increase in the reaction rate constant as the lactose concentration increased from 1% to 9%.

4. Conclusions

The addition of glucose, fructose or lactose significantly increased the levels of CML and CEL in pork heated at 121 °C. Particularly for glucose and fructose, even at 1% level, it could lead to 379% and 37% increase in the formation rate of CML, respectively, as well as 402% and 363% increase in the formation rate of CEL, respectively, in comparison

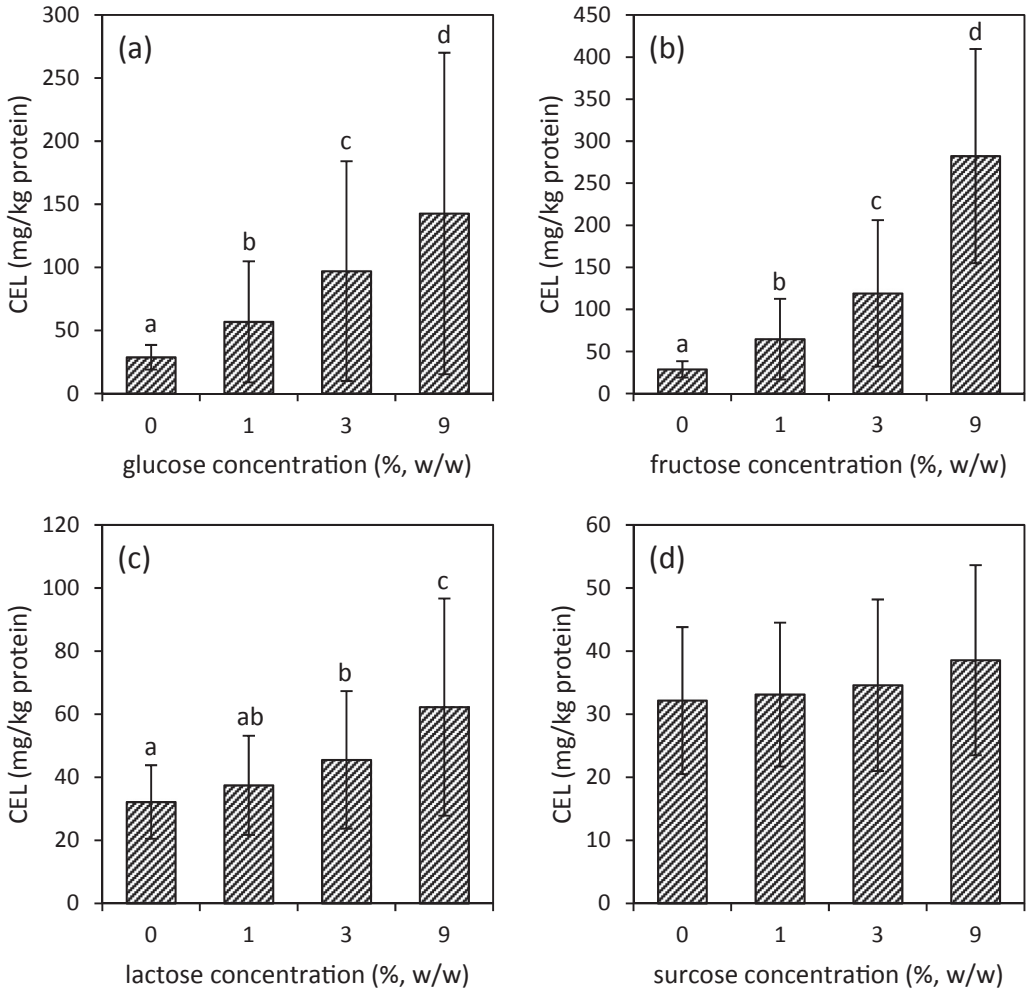

Fig. 4. Effects of different concentrations of (a) glucose, (b) fructose, (c) lactose, and (d) sucrose on the average amounts of N^{ε}-carboxyethyllysine (CEL) in heat treated pork (121 °C, 5–30 min). Different letters indicate significant difference (n = 8) based upon a post-hoc least significant difference (α = 0.05) test.

with their control counterpart without sugar. In a typical commercial sterilization process for retort canning (such as 121 °C, 30 min), the addition of just 1% of glucose, fructose or lactose resulted in an average of 224%, 26%, and 8% increase of CML, respectively, as well as 217%, 213% and 20% increase of CEL in sterile pork, respectively. If the heating time could be shortened to 10 min with an advanced thermal processing technology, such as microwave heating, the correspondingly increased CML level could be reduced to 31%, 12%, and 7%, and the increased CEL level could be reduced to 46%, 59% and 5%, respectively, indicating the importance of shorten thermal processing time to

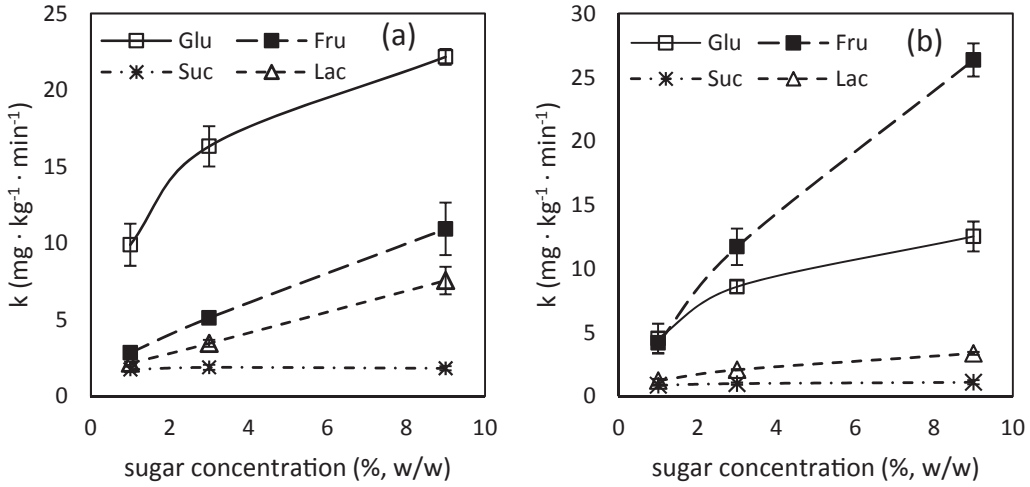

Fig. 5. The rate constant from the zero-order kinetic model for the formation of (a) N^{ε}-carboxymethyllysine and (b) N^{ε}-carboxyethyllysine as affected by the type and concentration of sugars.

limit the AGEs formation. As the level of glucose, fructose or lactose in pork was increased to 9%, the amounts of CML in pork heated for 30 min were increased 581%, 276%, and 189%, respectively, while the CEL were increased 720%, 15.8 times, and 150%, respectively, in comparison with the control pork without added sugar. Since the addition of reducing sugars in pork could lead to significant increases of CML and CEL formed during thermal treatment, ingredients such as honey, corn syrup that contain high levels of glucose and/or fructose should be avoided for thermal processed meat products. Instead, sucrose could be used as a sweetener in meat products since it did not significantly promote the formation of CML and CEL during commercial sterilization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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