Inhibitory effect of sugarcane molasses extract on the formation of

N^ε-(carboxymethyl)lysine and N^ε-(carboxyethyl)lysine

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Abstract

Molasses, the main byproduct of sugar production, is a well-known source of phenolic compounds. In this study, the effect of sugarcane molasses extract on the formation of N^ε-(carboxymethyl)lysine (CML) and N^ε-(carboxyethyl)lysine (CEL) was investigated in glucose-lysine model reaction systems. Results showed that sugarcane molasses extract significantly and dose dependently inhibited the formation of CML and CEL in model reaction systems. The antiglycation activities of sugarcane molasses extract were not only brought about by its antioxidant activities but also associated with its trapping abilities of reactive carbonyl species, such as glyoxal (GO) and methylglyoxal (MGO), the key intermediate compound for the formation of CML and CEL. Besides, the presence of sugarcane molasses extract effectively reduced the extent of browning. The present study emphasized the potential health effects of sugarcane molasses and the possibility of using byproducts as effective ingredients to reduce dietary Maillard reaction end products.

Keyword: sugarcane molasses extract, N^ε-(carboxymethyl)lysine, N^ε-(carboxyethyl)-lysine, reactive carbonyl species

1. Introduction

Advanced glycation end-products (AGEs) are a group of complex and heterogeneous compounds formed mainly by nonenzymatic glycation (Semba, Ferruci, & Lakatta, 2011; Zhang, Huang, Xiao, & Mitchell, 2011). N^ε-(carboxymethyl)lysine (CML) is a well-characterized and extensively studied AGEs formed *in vivo* and during food processing. CML can be formed through different pathways including the oxidation of fructoselysine (Ahmed, Thorpe, & Baynes, 1986) and the direct reaction of glyoxal (GO) with the ε-amino group in lysine (Glomb & Monnier, 1995; Thornalley, Langborg, & Minhas, 1999). N^ε-(carboxyethyl) lysine (CEL), the homolog of CML, is formed mainly by the reaction between lysine residues and methylglyoxal (MGO) (He, Zeng, Zheng, He, & Chen, 2014). *In vivo*, the accumulation of CML and CEL may contribute to the complications of diabetes and other health disorders, such as Alzheimer's disease, atherosclerosis and normal aging (Assar, Moloney, Lima, Magee, & Ames, 2009; Peng, Zheng, Cheng, Shan, Ren, Chen, et al., 2008). Many studies have also reported that dietary AGEs, such as CML and CEL, formed from food processing or storage could increase the total level of AGEs *in vivo* and have potential risk to cause health disorders *in vivo* (Heizmann, 2007; Stirban, Negrean, Götting, Uribarri, Gawlowski, Stratmann, et al., 2008). Therefore, during food processing, the discovery and investigation of AGEs inhibitors would reduce the level of dietary AGEs level and offer a potential approach to prevent the diabetic complications or other health disorders caused by the accumulation of dietary AGEs *in vivo*.

Sugarcane molasses, the dark viscous syrup obtained as a main byproduct of the manufacture of sugarcane into sugar, is mainly used as an additive in animal feed and as a carbon source in fermentation processes. Molasses is generally regarded as safe (GRAS) for use by the U.S. Food and Drug Administration (FDA) and it has long been sold as a health supplement. In addition to containing significant amounts of minerals and trace amounts of vitamins, some studies have confirmed that molasses is a rich source of phenolic compounds (Grabek-Lejko & Tomczyk-Ulanowska, 2013; Guimaraes, Glao, Martinez, Pintado, Bento, et al., 2007; Valli, Gómez-Caravaca, Di Nunzio, Danesi, Caboni, & Bordoni, 2012). In recent years, some plant extracts have been evaluated for their effects on the formation of AGEs (Sun, Peng, Liu, Fan, Wang, & Chen, 2010; Tsuji-Naito, Saeki, & Hamano, 2009; Wang, Yagiz, Buran, Nunes, & Gu, 2011; Wu, Hsieh, Wang, & Chen, 2009). It is found that the inhibitory effects of most of these plant extracts on AGEs formation are mainly ascribable to the large amount of phenolic compounds they contain which possess strong antioxidative activity. As free radicals are involved in the formation of AGEs, it is reasonable to expect that phenolic compounds in sugarcane molasses extract can also inhibit the formation of AGEs.

The present study aimed to evaluate the effect of sugarcane molasses extract on the formation of dietary CML and CEL during food processing at mild condition. The extract obtained was further tested for their activities in direct trapping of GO and MGO, the key intermediate compound for the formation of CML and CEL. Our research will provide additional evidence to support the possibility of using sugarcane

molasses extract as effective ingredients to reduce dietary AGEs formation in food processing.

2. Materials and methods

2.1. Chemicals

Sugarcane molasses was obtained from DONTA GROUP (Dongguan, China). CML and CEL were purchased from NeoMPS (Strasbourg, France). D101 macroporous resin for column chromatography was purchased from Zhengguang Resin CO., Ltd (Hangzhou, China). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Preparation of sugarcane molasses extract

The sugarcane molasses (1kg) was diluted to 15°Bx with deionized water and adjusted to pH 5 with 18 M sulfuric acid. The solution was then heated for 1.5 h at 90°C and cooled to room temperature immediately after the defined time. After adding 600 ppm phosphoric acid and adjusting to pH 8 with Ca(OH)₂, 15 ppm polyacrylamide was added at 90°C for the flocculation and precipitation of colloidal matter. The insoluble components were removed by centrifugation, the supernatant solution was partly dried under vacuum at 50 °C using a rotary evaporator, and the residue was then extracted with 70% ethanol solution through sonication for 1 h. After vacuum filtration, the 70% ethanol extract was concentrated under vacuum at 50°C using a rotary evaporator. The concentrated extract was loaded onto a glass column (800 mm×35 mm) (D101 macroporous resin) (Zhengguang Ltd., Zhejiang, China) at a flow rate of 800 ml/h. Five bed volumes of deionized water was used to remove

water-soluble components (primarily simple sugars), then followed by elution with 5 bed volumes of 95% ethanol. The ethanol eluent was evaporated to dryness with a rotary evaporator under vacuum. The dried extract obtained was stored in a desiccator before being used for antiglycation tests.

2.3. Determination of total phenolic content in sugarcane molasses extract

Total phenolic content of the molasses extract was determined according to the Folin-Ciocalteu spectrophotometric method of Vinson et al. (2001) with some modifications (Vinson, Su, Zubik, & Bose, 2001). Briefly, 200 µl of extract solution containing 2mg extract was diluted with 4.4 ml of methanol. 400 µl of Folin–Ciocalteu reagent was added and mixed thoroughly. After five minutes, 1 ml of 20% sodium carbonate was added and the mixture was incubated at room temperature for 1 h. The absorbance of the blue colour that developed was read at 765 nm. Quantification was based on the standard curve of gallic acid (GA) solutions prepared at the same time. Results were expressed as mg gallic acid equivalent (GAE) /g of extract. All determinations were carried out in triplicate.

2.4. DPPH radical scavenging activity

DPPH radical scavenging activity of sugarcane molasses extract was determined according to the method of Brand-Williams et al. (1995) with minor changes (Brand-Williams, Cuvelier, & Berset, 1995). 0.2 mM solution of DPPH in methanol was prepared and 2.5 ml of this solution was added to 2.5 ml of extract solution in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. Ascorbic acid was chosen as the reference compound. The

percentage inhibition activity was calculated from $(A_0 - A_t)/A_0 \times 100$, where A_0 was the absorbance of the control and A_t was the absorbance in the presence of the extract. The radical scavenging activity was expressed as IC₅₀ (the concentration of the tested sample required to scavenge 50% of DPPH), calculated by a linear regression analysis. The experiment was carried out in triplicate.

2.5. *Glucose-lysine assay*

200 millimole of glucose and lysine were mixed with 5 ml of phosphate buffer solutions (PBS, pH 7.4) (with or without sugarcane molasses extracts at concentrations of 10, 6, 2, and 0 mg/ml, respectively). The mixtures were incubated in a water bath at 37 °C for 3 days. After the defined time, the mixtures were taken out and stored at 4°C for further analyses.

2.6. *Evaluation of direct MGO and GO trapping capacity*

Direct MGO and GO trapping capacity was tested using the method described by Peng et al. (2008) with a slight modification (Peng, Zheng, et al., 2008). Briefly, MGO (5 mM), GO (5 mM) and o-Phenylenediamine (OPD, derivatization agent, 20 mM) were freshly prepared in PBS (pH 7.4); 2.5 ml of the prepared MGO or GO solution was mixed with 2.5 ml of PBS (pH 7.4) (with or without sugarcane molasses extracts at concentrations of 10, 6, 2, and 0 mg/ml, respectively). The mixtures were incubated in a water bath at 37 °C for 1 h. Then 1.0 ml of OPD was added to 1.0 ml of reaction solution and kept at 37°C for 0.5 h (when the derivatization reaction was completed). Quantification of MGO and GO as quinoxaline derivatives was performed according to the method of Gobert et al. (2009) with slight modifications

(Gobert & Glomb, 2009). The analysis was carried out in an HPLC-DAD system, which consisted of a Waters 600 pump, a Waters 2998 diode array detector (Waters Co., USA) and a 5 μ m Waters Atlantis T3 150 $\times$ 4.6 mm column (Waters Co., USA). The sample injection volume was 20 μ l (sterilized through 0.45 μ m filters). The mobile phase was composed of water and methanol, and set as 5 % methanol, 0-5min, and then a linear gradient from 5 % methanol to 30 % methanol, 5-10 min, subsequently, 30 % methanol to 40 % methanol, 10-45min, finally, 100 % methanol was used from 45 to 65 min at a flow rate of 1 ml/min. Column temperature was set at 25 $^{\circ}$ C. Spectral data from all peaks were accumulated in the range of 200-600 nm, and the chromatograms were obtained at 315 nm.

2.7. *Measurement of the browning*

The UV-absorbance and browning of the Maillard reaction products were measured according to the method of Davidek et al. (2002) with a slight modification (Davidek, Clety, Aubin, & Blank, 2002). The treated samples were diluted 10 times with distilled water, and the data was obtained at 420 nm in a UV-1901 spectrophotometer (Puxi, Beijing, China), which consisted of halogen lamp and 1 cm sample cell.

2.8. *Determination of CML and CEL*

The determination of CML and CEL was according to the method of Xu et al. (2013) with a slight modification (Xu, Ma, Yu, & Guan, 2013). The analysis was carried out using a high-performance anion-exchange chromatography with pulsed integrated amperometric electrochemical detection. The reaction mixtures were filtered through a Millex-HN nylon clarification kit of 0.45- μ m pore size (Millipore

Corp., Bedford, MA), and analyzed on a DX 5000 Dionex system (Dionex Corp., Sunnyvale, CA), which consisted of a pulsed integrated amperometric electrochemical detector (ED40; Dionex Corp.) and a gradient pump (model EG40) with online degassing. Separation was achieved on a CarboPac PA1 anion-exchange column (250 × 4 mm; Dionex Corp.) and a CarboPac PA1 guard column (50 × 4 mm; Dionex Corp.) using the gradient as follows: The mobile phases were water (solvent A) and NaOH (500 mM, solvent B). The elutions started with 5% solvent B, linear gradient to 0.5% solvent B in 10 min. Then it was 0.5 to 20% solvent B from 10 to 30 min and finally kept at 70% solvent B for 20 min. The flow rate was 1 ml/min and the injection volume was 20 µl. The column temperature was set at 30°C.

The regression equation for CML was $y_1 = 2.144x_1 + 1.193$; $R^2 = 0.998$. Where x_1 = CML concentration, mg/l; y_1 = peak area of CML. The retention time of CML was about 34.2 min. The linear range was 0.8-25.0 mg/l.

The regression equation for CEL was $y_2 = 1.185x_2 + 0.444$; $R^2 = 0.997$. Where x_2 = CEL concentration, mg/l; y_2 = peak area of CML. The retention time of CML was about 32.1 min. The linear range was 0.8-25.0 mg/l.

2.9. Phenolic profile using HPLC-DAD analysis

The sugarcane molasses extracts were analyzed in an HPLC-DAD system (Valli, Gómez-Caravaca, Di Nunzio, Danesi, Caboni, & Bordoni, 2012), which consisted of a 5 µm SunFire TM 150 × 4.6 mm column (Waters Co., USA), a LC-20AD pump and a SPD-M20A diode array detector (DAD, Shimadzu Co., Japan). The gradient elution was designed as mobile phase A (1% acetic acid) and mobile phase B (40% of

acetonitrile with 1% acetic acid). The gradient was applied as follows: from 5 to 50% phase B, 0–25 min; from 50 to 100% phase B, 25–30 min. The flow rate was constant at 1 ml/min, and the column temperature was maintained at 25 °C. The injection volume was 10 µl, and UV spectra was recorded from 200 to 600 nm, whereas the chromatogram was registered at 330 nm.

2.10. Statistical analysis

Means and standard deviations of the data were calculated for each treatment. Analysis of variance (ANOVA) was carried out to establish any significant differences ($p < 0.05$) among the applied treatments by the SPSS software package (SPSS 10.0 for windows).

3. Result and discussion

3.1. Total phenolic content

The content of phenolic compounds (mg/g) in sugarcane molasses extract (SME) was found 25.5 mg GAE /g of extract. Peng et al. (2008) found that mung bean, black bean, soybean and cowpea contained 9.94, 8.04, 7.15, and 5.70 mg GAE/g of extract, respectively (Peng, Zheng, et al., 2008). Sun et al. (2010) reported that the content of phenolic compounds in microalgal ranged from 0.98 to 9.76 mg GAE/g of extract (Sun, Peng, Liu, Fan, Wang, & Chen, 2010). Thus the total phenolic content in sugarcane molasses extract is relatively high compared with other natural products. The relatively high value was not surprising as phenolic compounds were strongly involved in molasses products. In this study, the phenolic profile of sugarcane molasses extract was analyzed by HPLC. As shown in Fig. 1, five phenolic

compounds were identified using standards (peak 1, 2, 3, 4 and 5 was catechin, vanillic acid, syringic acid, tricetin 7-O-glucoside and *p*-coumaric acid, respectively). Meanwhile, the content of phenolic compounds was listed in Table 1, the level of vanillic acid (10.82 ± 2.31 mg/kg SME) and catechin (11.58 ± 3.12 mg/kg SME) was higher than that of other phenolic compounds. The identified phenolic compounds in sugarcane molasses extracts had also been separated previously in some literature, which showed the presence of significant amounts of phenolic acids (mainly syringic, vanillic and *p*-coumaric acid) and flavonoids (such as catechin, quercetin and tricetin derivatives) (Guimaraes, et al., 2007; Valli, Gómez-Caravaca, Di Nunzio, Danesi, Caboni, & Bordoni, 2012). The interests of phenolic compounds were increasing in the food industry since they could inhibit the oxidative pathways during food processing and thereby improve food quality and nutritional value (Wojdyło, Oszmiański, & Czemerys, 2007).

3.2. Inhibition of DPPH radical

DPPH has been widely used to evaluate antioxidative activity of antioxidants (Hatano, Takagi, Ito, & Yoshida, 1997). The stable radical DPPH can be reduced to the non-radical form DPPH-H in the presence of a hydrogen donating antioxidant. Fig. 2 illustrated the scavenging rate was significantly increased with increasing sugarcane molasses extract concentration due to the scavenging ability of sugarcane molasses extract. On DPPH radical, sugarcane molasses extract had obvious scavenging effects in the range of 0.5–100 µg/ml. As phenolic acids and flavonoids were strongly involved in molasses products and typical phenolic compounds possessing antioxidant

activity had been characterized as flavonoids and phenolic acids (Guimaraes, et al., 2007; Javanmardi, Stushnoff, Locke, & Vivanco, 2003; Kähkönen, Hopia, Vuorela, Rauha, Pihlaja, Kujala, et al., 1999; Valli, Gómez-Caravaca, Di Nunzio, Danesi, Caboni, & Bordoni, 2012), thus it was observed that sugarcane molasses extract had significant DPPH radical scavenging ability. However, the scavenging activity of ascorbic acid, a known antioxidant, used as a positive control, was relatively higher than that of sugarcane molasses extract. The IC₅₀ values were found to be 54.2 and 1.9 µg/ml for sugarcane molasses extracts and ascorbic acid, respectively.

3.3. Direct MGO and GO trapping capacity

MGO and GO have been confirmed as reactive carbonyl intermediates of CEL and CML formation, respectively. As shown in Fig.3, sugarcane molasses extract could effectively scavenge GO. The levels of GO in all model systems with sugarcane molasses extract were lower than that in the model system without molasses extract. The GO trapping percentages were up to 69.1%, 73.6% and 76.4% in the model systems with 2, 6 or 10 mg/ml of sugarcane molasses extract addition, respectively. Meanwhile, sugarcane molasses extract showed slight MGO trapping abilities. The MGO trapping percentages were up to 11.4%, 18.4% and 21.6% in the presence of 2, 6 or 10 mg/ml of sugarcane molasses extract, respectively.

It was not surprising that sugarcane molasses extract could trap MGO and GO as it was rich in phenolic compounds, and some of which it contained (such as catechin in Fig.1) had been confirmed as the potential MGO and GO scavengers (Jiang, Chiaro, Maddali, Prabhu, & Peterson, 2009; Peng, Cheng, Ma, Chen, Ho, Lo, et al., 2008;

Valli, Gómez-Caravaca, Di Nunzio, Danesi, Caboni, & Bordoni, 2012; Wang, Yagiz, Buran, Nunes, & Gu, 2011).

3.4. Inhibitory effects on CML and CEL formation

As shown in Table 2, sugarcane molasses extracts displayed obvious inhibitory effects on the formation of CML and CEL in the system composed of glucose and lysine. Notably, the highest inhibition was achieved when 10 mg/ml sugarcane molasses extract was added with respect to 0 mg/ml sugarcane molasses extracts in model system (calculated from Table 2, 5.03 mg/l for CML reduction and 1.96 mg/l for CEL reduction, respectively). Obviously, the reduced level of CML was higher than that of CEL after 10 mg/ml sugarcane molasses extracts addition. The result was consistent with the decrease of their precursors (GO and MGO) after 10 mg/ml sugarcane molasses extracts addition. It was not a surprise to find sugarcane molasses extract showed antiglycation activities in glucose-lysine model as high contents of phenolic compounds was involved in it. On the basis of a literature search, many phenolic-rich plant extracts and purified phenolic compounds with strong antioxidant activity had been found to have significant AGEs inhibitory activity (Matsuda, Wang, Managi, & Yoshikawa, 2003; Yokozawa & Nakagawa, 2004). As the oxidative process was mainly involved in the formation of CML and CEL, antioxidant activity of sugarcane molasses extract may play an important role in the inhibition of CML and CEL formation. Moreover, CML and CEL could be also formed from the reactions between dicarbonyls (GO and MGO respectively) and lysine, and compounds or extracts possessing dicarbonyls scavenging capacities were supposed

to affect the formation of CML and CEL. From direct MGO and GO trapping test, it was observed that sugarcane molasses extract as the potential MGO and GO scavenger could effectively reduce MGO and GO formation (Fig.3). Therefore, sugarcane molasses extract was supposed to inhibit CEL and CML formation through both their strong antioxidant activities and dicarbonyl scavenging abilities.

3.5. Changes in browning intensity

Browning intensity (A_{420}) as a marker of the advanced stage of the Maillard reaction is the easiest measurable consequence because it offers a visual estimate. A_{420} intensity is usually used as an indication of the extent to which the Maillard reaction takes place in foods (Kim & Lee, 2009). In this study, sugarcane molasses extract in model Maillard systems showed the great influence on the change of A_{420} (Fig. 4). The value of A_{420} was lower in all three model systems containing sugarcane molasses extracts (2-10 mg/ml) than that of control system. In addition, the value of A_{420} was decreased with increasing concentration of sugarcane molasses extract. The addition of sugarcane molasses extract to glycation model systems significantly inhibited the formation of brown compounds. According to the results published previously, as well as those presented here, it can be concluded that sugarcane molasses extract might prevent brown compounds formation by antioxidant activity and inhibition of melanoidin precursors (such as MGO and GO).

4. Conclusion

Sugarcane molasses extract possessed obvious inhibitory effects on the formation of dietary CML and CEL, which was largely attributed to both its antioxidant

activities and carbonyl scavenging capacities. Data herein reported emphasized that it was feasible to use sugarcane molasses extract for their antioxidant activity. Moreover, the present study emphasized the potential health benefits of sugarcane molasses extract and its possible use as effective ingredients to reduce dietary AGEs.

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Figure Legends:

Figure 1 Phenolic profile of sugarcane molasses extract. Peak 1, 2, 3, 4 and 5 was catechin, vanillic acid, syringic acid, triclin 7-O-glucoside and p-coumaric acid, respectively.

Figure 2 DPPH radical scavenging capacity of sugarcane molasses extract.

Figure 3A: Chromatograms of GO only (a), GO with 2 mg/ml sugarcane molasses extract (b), GO with 6 mg/ml sugarcane molasses extract (c) and GO with 10 mg/ml sugarcane molasses extract (d) after incubation and derivatization at 315 nm; B: Chromatograms of MGO only (a), MGO with 2 mg/ml sugarcane molasses extract (b), MGO with 6 mg/ml sugarcane molasses extract (c), and MGO with 10 mg/ml sugarcane molasses extract (d) after incubation and derivatization at 315 nm.

Figure 4 Changes in browning intensity as the function of sugarcane molasses extract concentration.

Table 1 Content of main phenolic compound from sugarcane molasses extract

(SME)

phenolic compound	retention time	concentration(mg/kg SME)
catechin	5.7	11.58 ± 3.12
vanillic acid	8.1	10.82 ± 2.31
syringic acid	8.3	2.95 ± 1.09
tricin 7-O-glucoside	11.5	0.87 ± 0.44
p-coumaric acid	12.1	8.12 ± 2.43

Table 2 Content of N^ε-(carboxymethyl)lysine (CML) and N^ε-(carboxyethyl)lysine (CEL) formed from the glucose-lysine model system with or without sugarcane molasses extract (SME)

SME (mg/l)	CML (mg/l)	CEL (mg/l)
0	5.86±0.09	1.96±0.06
2	3.82±0.12	0.91±0.11
6	1.98±0.07	<LOQ
10	0.83±0.04	<LOQ

LOQ: limit of quantification.

Figure 1

Figure 2

Figure 3

Figure 4