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Comparison of the antioxidative and cytotoxic properties of glucose-lysine and fructose-lysine Maillard reaction products

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Abstract

The antioxidative activity and cytotoxic effect of different fractions generated from whole mixtures of model Maillard reaction products (MRPs) made from glucose-lysine (Glc-Lys) and fructose-lysine (Fru-Lys) reactants were tested. MRPs were derived from Glc-Lys and Fru-Lys by autoclave heating, and ethanol fractionation. All MRP fractions showed various degrees of antioxidative activity when evaluated by a deoxyribose oxidative degradation assay. A significant difference ($P < 0.05$) was seen in the affinity to inhibit Fenton-reaction-induced free radical production, for both Glc-Lys and Fru-Lys ethanol precipitates, and also the Fru-Lys ethanol extract. Similar protective antioxidative activity of the different MRP fractions was not exhibited in a DNA nicking assay. Cytotoxicity potential of the MRPs was investigated using Caco-2 cells. The greater cytotoxicity observed for both Glc-Lys and Fru-Lys ethanol precipitates was specific to the higher molecular weight fractions. Cytotoxicity also depended on incubation time, MRP concentration, and sugar source.

1. Introduction

The Maillard reaction refers to the interaction initiated between the terminal α- or ϵ-amino group of lysine residues in peptides or proteins and the carbonyl moiety of reducing sugars. These products are found in roasted foods (e.g. meats and fish), browned confectionery (e.g. toast, crackers, and potato chips), and luxury food and seasonings (e.g. cane sugar, coffee, black tea, oolong tea, dried fruits, soybean paste, soy sauce, beer, and tomato paste) (Hayase & Kato, 1994). A cascade of complex reactions follows the formation of the initial intermediates, which include Amadori and Heyn's products (Hodge, 1953; Yaylayan, 1997). The complex Maillard reaction consists of both early and advanced Maillard reactions, which can be characterized by absorption spectra and molecular weights (Wijewickreme, Kitts & Durance, 1997). Early reactions of MRP correspond to well-defined chemical steps without browning and progress by fragmentation and polymerization to volatile or soluble substances, and finally to insoluble brown polymers (Mauron, 1981).

The antioxidant activity of Maillard reaction products has been studied by a number of investigators (Alaiz, Zamora & Hidalgo, 1996; Chuyen, 1998; Park & Kim, 1983; Wijewickreme & Kitts, 1997). Incorporation of preformed MRPs, or the application of food processing practices to derive MRPs within the food can improve oxidative stability of food products (Wijewickreme & Kitts, 1998). Assessing specific antioxidative activity of fractions of MRP is important for evaluating use in potential food processing strategies.

MRPs have been reported to have cytotoxic effect on both rat and human cells (Vagnarelli, Sario, Cuzzoni, Mazza & Carli, 1991; Wang, Tseng, Yen, Shiow & Lin, 1987;). The whole mixtures of MRPs, made from model systems of glucose-, and fructose-lysine, have been shown to produce a cytotoxic effect on rat glioma cells (Wang et al., 1987). Since Caco-2 cell represents a useful model for human intestine-food nutrient interactions, numerous workers have used the Caco-2 cell line to study absorption of nutrients, and nutrient-induced effects on Caco-2 cell growth and differentiation (Finley & Monroe, 1998; Glahn, Lai, Hsu, Thompson, Guo & Van Campen, 1998). Assessment of toxicity potential of

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MRPs using Caco-2 cells could be an important in vitro test in evaluating MRP toxicity in humans.

This study was designed to investigate the antioxidative and cytotoxic activities of different fractions of model MRPs generated from whole mixtures made from aldose- and ketose-lysine reactants. Antioxidative properties of MRP were evaluated using in vitro procedures including deoxyribose and DNA degradation, whereas the cytotoxic effect of the MRPs on the human intestine epithelial cell model was examined in cultured Caco-2 cells as components of a complete nutrient medium.

2. Materials and methods

2.1. Preparation of MRPs from sugar-lysine model system

An aqueous solution of 0.8 M D-glucose (or -fructose) and 0.8 M L-lysine was adjusted to pH 9 and heated at 121°C for 1 h in an autoclave (Barnstead Co., Boston, MA; # 0767). The solution was prepared by dissolving 0.1 M of sugar and 0.1 M of lysine in 100 ml of distilled water and then adjusting the solution to pH 9 using 10 M NaOH. The reaction solution was brought to 125 ml so that the final concentration of the reactants was 0.8 M. Reaction flasks were capped and autoclaved at 121°C for 1.0 h and after heating cooled in an ice bath. The heated solution was freeze-dried at a temperature below −40°C, which represented the entire mixture (e.g. "w") in this experiment. The heated solution was also used for further fractionating. Absolute ethanol was added to a level of 90% by volume and the ethanol suspension was centrifuged at 5000×g for 10 min. The resulting supernatant, containing both intermediate compounds and low molecular weight melanoidins of Maillard reaction represented an ethanol extract (e.g. "e"). A precipitate containing high molecular weight materials was processed further and identified herein as "precipitate" (e.g. "p"). The heated solutions were also dialyzed (MWCO = 15 000) against double distilled deionized water at 4°C until the dialysis water became colorless. All the fractions were freeze-dried prior to use (Kitts, Wu, Stich & Powrie, 1993).

2.2. Assay of 2-Deoxy-ribose degradation

Reaction mixtures of 1.2 ml contained the following reagents and final concentrations: deoxyribose (2.8 mM), $FeCl_3$ (25 mM), EDTA (100 μM), H_2O_2 (2.8 mM), KH_2PO_4/KOH buffer at pH 7.4 (10 mM), MRPs to be tested (1 mg/ml), and ascorbate (100 μM). The mixtures were incubated at 37°C for 1 h. MRP color was controlled by adding MRPs in 1.2 ml of a KH2PO4/KOH buffer (pH 7.4). The degradation of

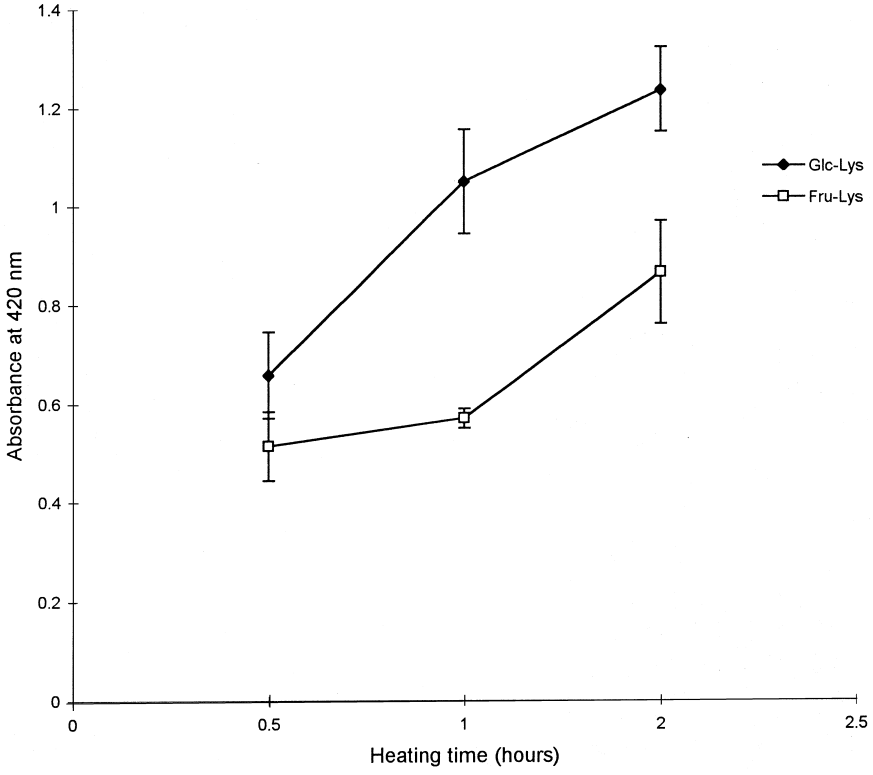


Fig. 1. Absorbance spectra of Glc-Lys and Fru-Lys MRP model systems derived at the different heating time. Value represents mean±S.E.M. —■—■— = Glc-Lys, —◇—◇— = Fru-Lys.

deoxyribose was measured by adding 1 ml of 1% (w/v) thiobarbituric acid in 0.05 M NaOH and 1 ml of 0.28% (w/v) trichloroacetic acid, followed by heating at 80°C for 15 min. After cooling in an ice bath, the absorbance was measured at 532 nm (Aruoma, 1994; Aruoma, Halliwell, Gajewski & Dizdaroglu, 1989). All readings were corrected for any interference from brown color of the MRPs.

2.3. DNA nicking assay

PBR322 plasmid DNA was purchased from Sigma Chemical Co. (St. Louis, MO). A Fenton-reagent system was used as follows: 2 μl of 20 μM $FeCl_3 \cdot 6H_2O$, 2 μl 10 μM EDTA, 2 μl of 2 μM Ascorbic acid, 2 μl of 9 mM H_2O_2, 2 μl of 10 mg/ml MRPs, and 2 μl of DNA. This system represents a site-specific reaction (Buettner, 1987). A non-site-specific reaction was produced in the presence of EDTA in the system. The reaction system was incubated in a 37°C water bath for 1 h. The reaction mixtures were loaded on 0.7% (w/v) agarose gel and run at a voltage of 46 for 1 h. The gel was stained with ethidium bromide (0.5 μg/ml). The DNA bands were visualized under UV light and photographed (Wijewickreme & Kitts, 1997).

2.4. Cell toxicity test

Caco-2 cells (a human colon adenocarcinoma cell line) were purchased from the American Type Culture Collection (Manassas, VA). The cells were plated at 0.75×10^3 cells/200 μl/well in 96-well cell culture plate with DMEM medium supplemented with 10% fetal bovine serum and maintained two days at 37°C in a CO_2 incubator. The medium was changed with fresh medium and maintained overnight, and MRP solutions at different concentrations were then added into cell cultures. After 48 h incubation, the numbers of cells in each well were counted using a hemacytometer.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey multiple-range test (Systat Inc., Evanston, IL) was used in data analysis. The level of confidence required for significance was selected as $P \leqslant 0.05$. Each experiment was replicated three times.

3. Results

3.1. Assessment of brown color development at 420 nm

The 420 nm absorbance spectra obtained for different Glc-Lys and Fru-Lys MRP models after various heating times are shown in Fig. 1. With an increase in heating time, absorbances at 420 nm also increased in both

Table 1
Inhibition effect of MRPs on oxidative degradation of deoxyribose[a]

MRPs[b]	Concentration (mg/ml)	A_{532}[c]	Inhibition (%)[d]
Control[e]	1	0.751±0.005	0.00
G-Lw	1	0.663±0.004	11.70
G-Lp	1	0.418±0.106[f]	44.30
G-Le	1	0.694±0.005	7.60
F-Lw	1	0.657±0.011	12.50
F-Lp	1	0.469±0.006[f]	37.50
F-Le	1	0.583±0.010[f]	22.30

[a] Values represent mean±S.D. ($n = 3$).

[b] Glucose–lysine whole mixture (G-Lw), ethanol precipitate (G-Lp), and ethanol extract (G-Le); fructose-lysine whole mixture (F-Lw), ethanol precipitate (F-Lp), and ethanol extract (F-Le).

[c] A_{532} = absorbance at 532 nm.

[d] % inhibition = $\frac{[A_{532}(\text{control}) - A_{532}(\text{withMRPs})] \times 100}{A_{532}(\text{control})}$.

[e] Control = 1.2 ml of the control consisted of $FeCl_3$ (25 mM), EDTA (100 μM), H_2O_2 (2.8 mM), deoxy-ribose (2.8 mM), and L-ascorbic acid (100 μM) in potassium phosphate buffer (pH 7.5).

[f] $P < 0.05$ (in comparison with the control).

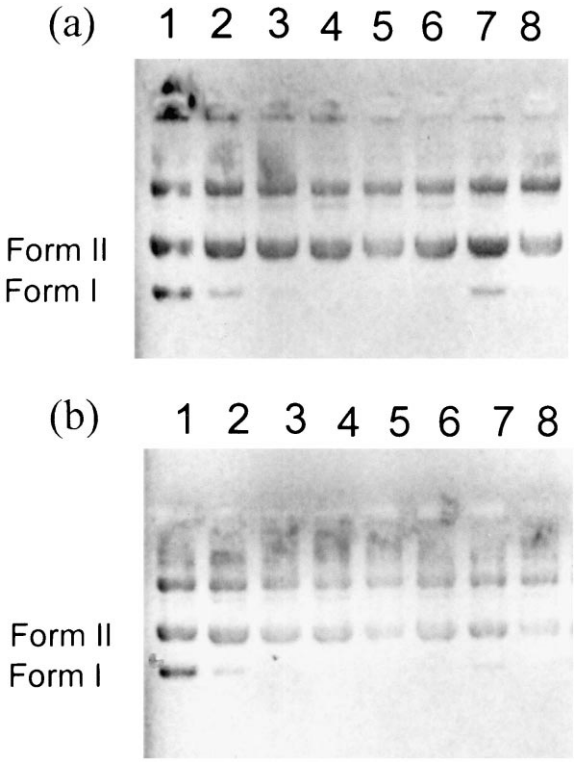


Fig. 2. Modulation of site-specific (a) and non site-specific (b) DNA strand cleavage by Glc-Lys and Fru-Lys MRPs. Lane 1 represents original DNA; lane 2 (a) = DNA (0.2 μg/ml) + $FeCl_3$ (3.3 μM) + ascorbic acid (0.33 μM) + H_2O_2 (1.5 mM), (b) = 2 (a) + EDTA (1.67 μM). Lanes 3–5 are DNA + Glc-Lys MRPs (1.6 mg/ml); lanes 6–8 are Fru-Lys MRPs (1.6 mg/ml). Lanes 3–5 are G-Lp, G-Le, $G\text{-}L_{15\,000}$; lanes 6–8 are F-Lp, F-Le, $F\text{-}L_{15\,000}$. Form I = circular DNA, form II = nicked circular DNA. 15 000 = molecular weight cut-off.

solutions of Glc-Lys and Fru-Lys. Higher absorbance readings were detected for the Glc-Lys model than that for the Fru-Lys model at 420 nm. In addition, large differences in the 420 nm absorbance readings between two Glc-lys and Fru-lys model systems were observed at 1 and 2 h of heating time.

3.2. Assessment of oxidative degradation of deoxyribose

The protective effect of MRPs assessed using oxidative degradation of deoxyribose is shown in Table 1. Inhibition of the oxidative degradation was seen in all MRP fractions at a concentration of 1 mg/ml. The fractions of Glc-lys and Fru-lys precipitates (G-Lp and F-Lp) exhibited higher inhibition percentages, compared to other fractions of MRP derived from Glc-Lys and Fru-Lys model systems. A significant difference ($P < 0.05$) in reducing deoxyribose oxidative degradation was present for G-Lp, F-Lp and F-Le.

3.3. DNA nicking assays

Fig. 2 shows the separation of form I circular DNA from form II nicked circular DNA using agarose gel electrophoresis. Form I DNA migrated faster than form II DNA on agarose gel, denoting a difference in DNA structure. The results of this study demonstrated that the Fenton reaction system effectively initiated DNA strand breakage (lane 2) as visualized by a loss in form I, and by an increase in the extent of form II. The addition of MRPs did not prevent the loss in form II nicked circular DNA, caused by the Fenton reaction system (lanes 3–8). The electrophoresis gel patterns with MRP treatment are similar to that of the Fenton–reagent mixtures. These patterns could not be changed by substituting with the molecular (MWCO $\geqslant$ 15 000) polymers of MRPs in this assay.

3.4. Assessment of cytotoxicity

Figs. 3 and 4 show the relative toxicity of Glc-Lys and Fru-Lys MRPs derived from both whole mixtures (w) and ethanol extracts (e) on Caco-2 cells after 48 h. At a concentration of 1 mg/ml or less, no significant toxicity was observed for either whole mixtures (w) or ethanol extracts (e) of both Glc-Lys and Fru-Lys MRPs.

Fig. 5a and b show the toxicity potential of ethanol fractionated MRP precipitates (p) derived from Glc-Lys and Fru-Lys, respectively, on Caco-2 cells over different concentration ranges (0.02–2 mg/ml). The precipitate fractions (e.g. high molecular weight MRP polymers) exhibited the greatest ($P < 0.05$) toxicity for the Fru-Lys

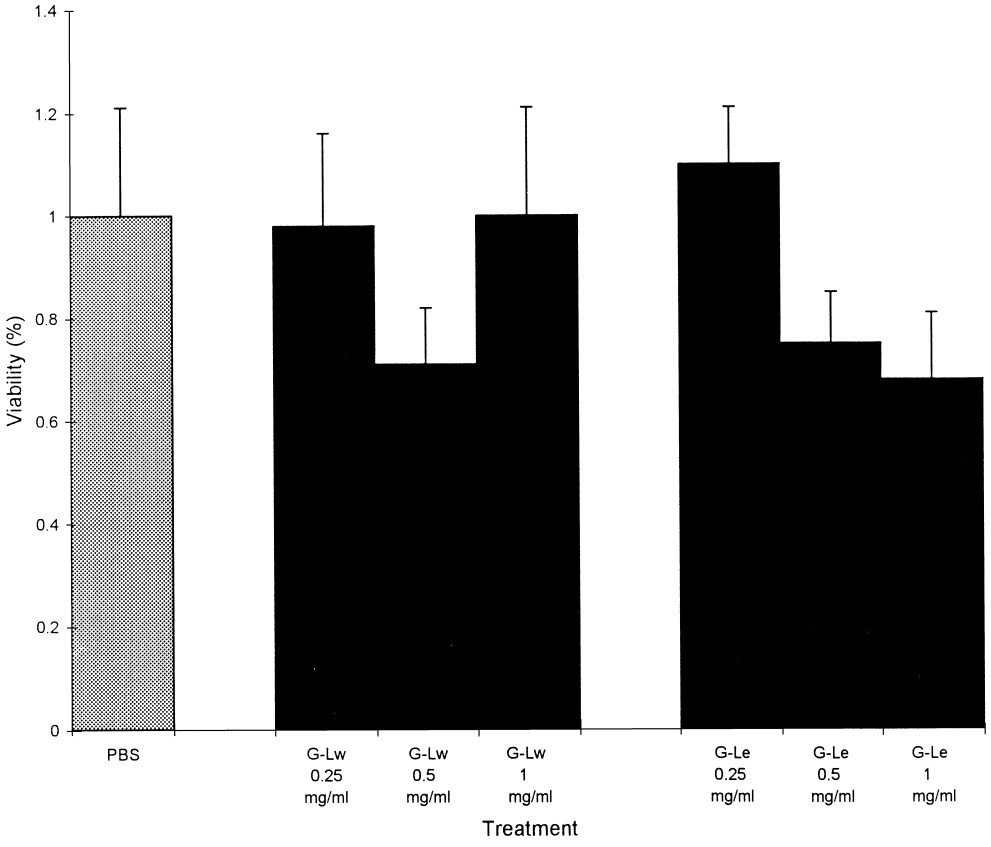


Fig. 3. Comparison of the relative cytotoxic potential of different fractions of Glc-Lys MRPs on Caco-2 cells. MRPs were incubated with Caco-2 cells at 37°C, 5% CO_2 for 48 h. Value represents mean±S.E.M. ▒ = Control, ■ = Glc-Lys.

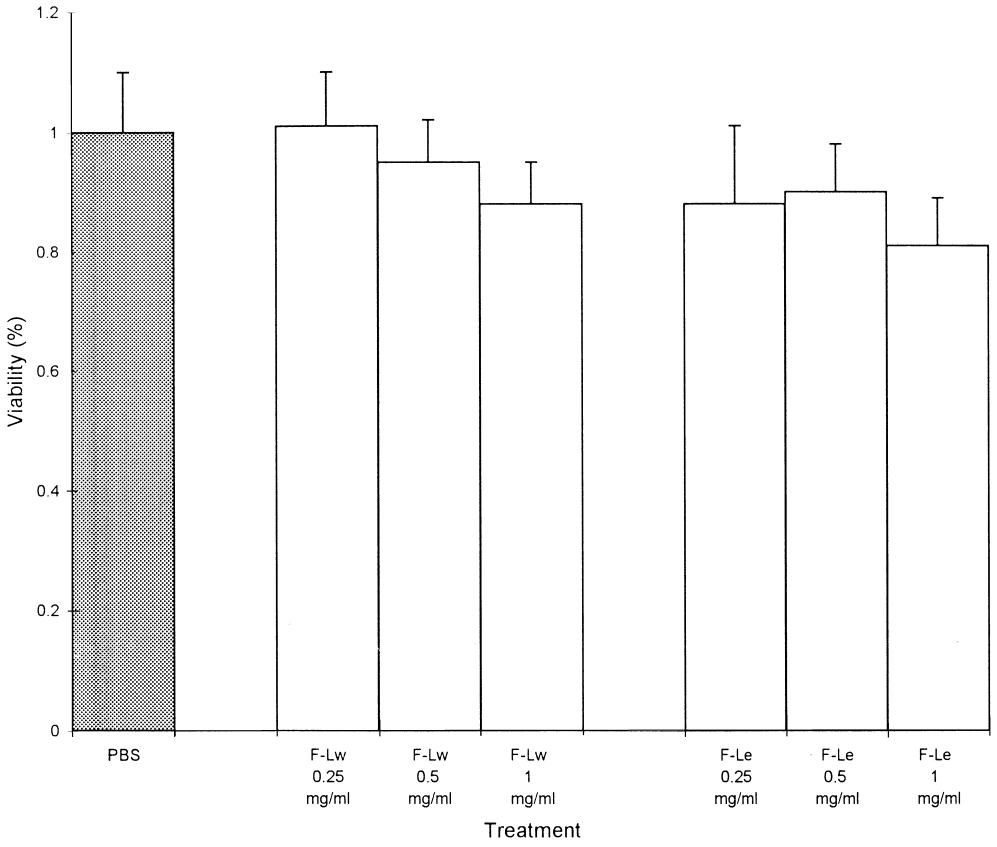


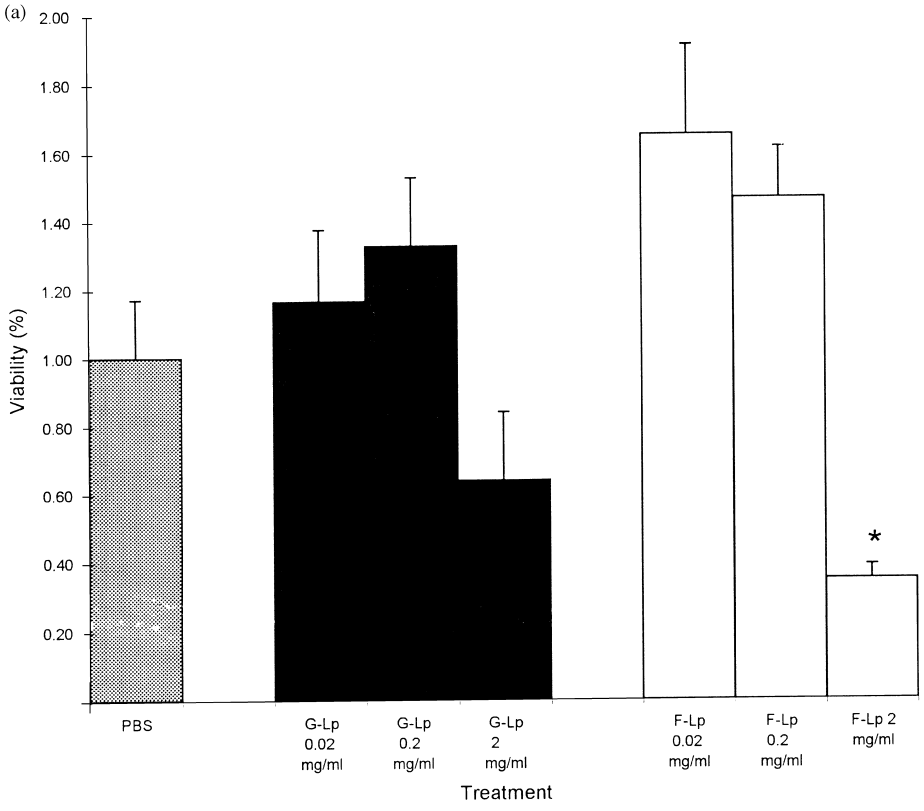
Fig. 4. Comparison of the relative cytotoxic potential of different fractions of Fru-Lys MRPs on Caco-2 cells. MRPs were incubated with Caco-2 cells at 37°C, 5% CO_2 for 48 h. Value represents mean±S.E.M. ▒ = Control, □ = Fru-Lys.

MRPs, compared to the Glc-Lys MRP after 24 h incubation time. Only the F-Lp exhibited significant ($P < 0.05$) toxicity at 2 mg/ml (Fig. 5a), with no corresponding toxicity observed for the G-Lp. Following 48 h incubation, the cytotoxicity for F-Lp was obtained at both 0.2 and 2 mg/ml concentrations, while G-Lp produced cytotoxicity at only 2 mg/ml (Fig. 5b).

4. Discussion

Our previous study showed that higher absorbance readings for Glc-Lys compared to Fru-Lys at 420 nm occurred after oven heating conditions (Wijewickreme et al., 1997). The present study further confirms that the products of Glc-Lys and Fru-Lys model MRPs differ as evidenced by the higher absorbance readings at 420 nm for Glc-Lys compared to Fru-Lys model MRP, following autoclave-heating. Glucose and fructose were selected as sugar reactants due to the difference in aldose and ketose structure and the fact that each sugar produces different amounts and types of MRP compounds (Wijewickreme et al., 1997; Wu, Russell & Powrie, 1987). MRP made from the Glc-Lys model produced greater yields than that of the Fru-Lys model, which is partially explained by the more reactive nature of the aldehyde carbonyl group of glucose compared with the ketone carbonyl group of fructose (Yeboah, Ali & Yaylayan, 1999). These differences also reflect variations in average empirical formulas; Fru-Lys MRP having one carbon atom less but a higher C to N ratios than Glc-Lys counterpart (Wijewickreme et al., 1997). The reaction temperature of 121°C in the present study was selected to represent the sterilization temperature of canned food (Powrie, Wu, Rosin & Stich, 1981). The selection of heating at 121°C for 1 h was based on the observation that a high browning and maximum yield of melanoidins from the Glc-Lys model system could be reached after heating at 121°C for 1 h (Wu et al., 1987).

The Fenton reaction system was used in both deoxyribose degradation and DNA nicking assays to generate hydroxyl radicals. The Fenton reaction produces a high yield of DNA base hydrolysis products and also causes damage to deoxyribose. The Fenton reaction system used in this experiment contained ferric ions that will bind deoxyribose and produce a site-specific effect, versus the presence of a EDTA-Fe^{3+} complex which produces a non-site specific effect. The treatment of deoxyribose with the Fenton reaction reagents used in this experiment resulted in a high rate of deoxyribose degradation,



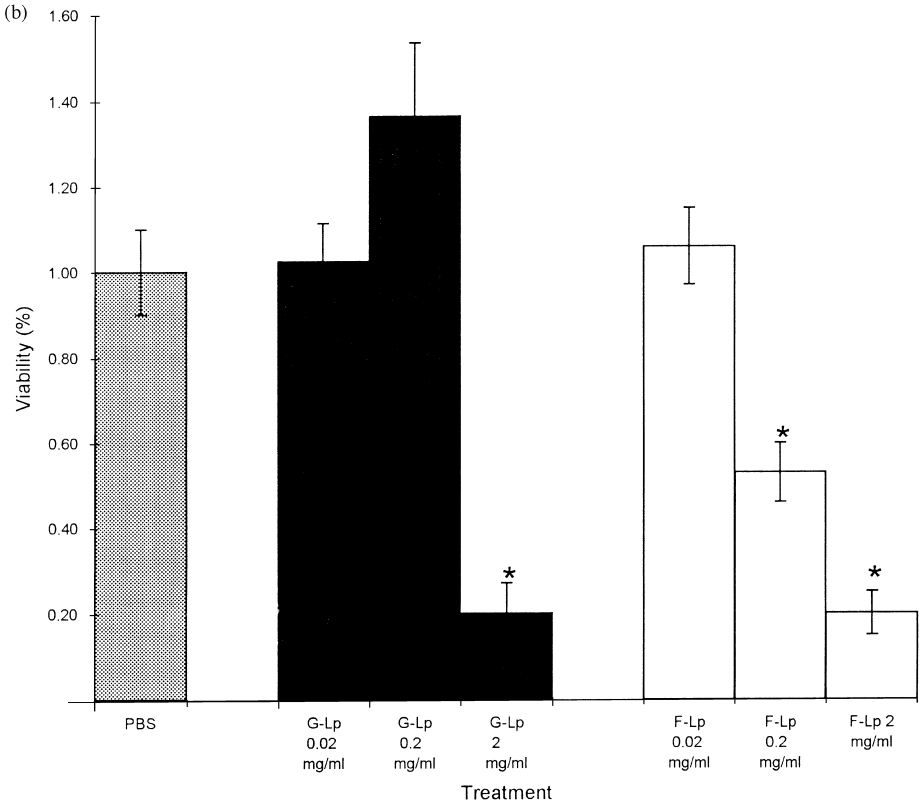


Fig. 5. (a) The cytotoxic effect of the ethanol precipitates derived from Glc-Lys and Fru-Lys MRPs. MRPs were incubated with Caco-2 cells at 37°C, 5% CO_2 for 24 h. Value represents mean±SEM. $^*P < 0.05$ in comparison with control (PBS buffer). ▒ = Control, ■ = Glc-Lys, □ = Fru-Lys (b) The cytotoxic effect of the ethanol precipitates derived from Glu-Lys and Fru-Lys MRPs. MRPs were incubated with Caco-2 cells at 37°C, 5% CO_2 for 48 h. Value represents mean±S.E.M. $^*P < 0.05$ in comparison with control (PBS buffer). ▒ = Control, ■ = Glc-Lys, □ = Fru-Lys.

as demonstrated by Aruoma (1994) and Wijewickreme, Krejpcio and Kitts (1999). The results of this study show that the fractions of the Glc-Lys and Fru-Lys ethanol precipitates produced the highest inhibition of deoxyribose degradation, which is in support of other studies that have attributed antioxidant activity to reside primarily with melanoidin fractions (Kitts, Wu & Powrie, 1993; Namiki, 1988; Wijewickreme & Kitts, 1998). Furthermore, Monti et al. (1999) also suggested that high molecular weight MRP fraction compounds (HMW $\geqslant$ 10 kDa) were responsible for the antioxidative efficiency of MRP solutions. It is of interest to see that significant antioxidative activity also existed with the ethanol extract of Fru-Lys model system, but not with the Glc-Lys counterpart. The precise chemical makeup of the complex mixture of compounds produced in Fru-Lys and Glc-Lys model systems, although presently unknown, is required for determining structure–activity relationships that ascertain the basis for differences in antioxidative activity between the ethanol extracts of both model systems.

Fenton-reagents cause a high yield of base scission products formed on DNA (Aruoma et al., 1989). The hydroxyl radicals generated by Fenton reactants attack DNA guanosine residues, resulting in strand breakage and transformation from the native circular DNA form to a nicked circular or linear form. Phage DNA along with Fenton-reagents has been used to assess the potential antioxidative activity of different MRP fractions (Wijewickreme et al., 1999). In this study, both Glc-Lys and Fru-Lys fractions exhibited no antioxidative protection of Fenton-induced DNA scissions. The present result is consistent with our previous report that the Glc-Lys and Fru-Lys model MRP mixtures fail to prevent a Fenton reactant-induced oxidative damage to DNA at low concentrations (Wijewickreme & Kitts, 1998). In another study, Wijewickreme and Kitts (1997) demonstrated that the addition of MRPs at high concentrations were effective at causing DNA breakage. Taking these results together indicates that the genotoxic potential of MRPs is concentration-dependent and relatively ineffective at protecting against hydroxyl radical induced DNA scission.

Caco-2 cells were used in this study because they have been reported to resemble the morphology and function of human intestine epithelial cells (Hidalgo, Raub & Borchardt, 1989). Therefore, this model may reflect closely the potential effects of different food components (e.g. MRPs) on human intestine epithelial cell health and function. MRP whole mixtures produced from Glc-Lys and Fru-Lys model systems have been reported to produce cytotoxic effects on rat glioma cells (Wang et al., 1987). Our finding did not confirm the apparent toxicity with the MRP whole mixtures or extend this conclusion to ethanol extracts derived from both Glc-Lys and Fru-Lys MRPs in Caco-2 cells of human origin. Rather, cytotoxic effects were only seen with the ethanol precipitates derived from Glc-Lys and Fru-Lys. The results of this study demonstrate that high molecular weight polymer of MRP possesses greater potential toxicity in comparison with low molecular MRP, despite apparent antioxidant properties.

Our data confirmed the reports of others (Vagnarelli et al., 1991; Wang et al., 1987), that MRPs have a cytotoxic effect, but clarified this fact by showing that cytotoxicity occurred only after 24 h. No cytotoxicity was observed at 6, 12 and 24 h incubation time (data not shown). Although there is a lag time for developing toxicity in Caco-2 cells by MRP, the underlying mechanism of MRP-induced cytotoxicity is not clear at this time. MRPs have been reported to cause changes in cell morphology and also inhibit RNA synthesis, while no inhibition in DNA and protein synthesis was observed (Wang et al., 1987). These latter events are less likely protected by antioxidant activity. Further studies are needed to investigate the mechanism for the time dependent cytotoxicity of high molecular weight MRPs observed herein.

5. Conclusions

Notable differences in the spectral absorbances between Glc-Lys and Fru-Lys MRP model denoted differences in the chemical components of Glc-Lys and Fru-Lys MRP models. These differences reflected variations in chemical and biochemical activities in reference to both antioxidative and cytotoxic activities. The MRP ethanol precipitates exhibited significant ($P < 0.05$) antioxidative activity in comparison with lower molecular weight fractions of Glc-Lys and Fru-Lys. Further differences in MRP fraction composition were noted as evidenced by the antioxidative properties of F-Le but not G-Le. This assessment of bioactivity was dependent on the assay used to assess antioxidant activity, since no potential was observed in the DNA nicking test. MRP made from Fru-Lys model system were more toxic than MRP derived from Glc-Lys. A comparison of the relative toxicities of the different fractions from MRP, showed that the MRP precipitates denoting high molecular weight polymers had greater toxicity on Caco-2 cells, compared to other fractions recovered from reducing sugar-lysine model systems. Finally, concentration of MRPs and the incubation time used for reacting Caco-2 cells with MRPs were important factors in the expression of toxicity by different MRPs.

Acknowledgements

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