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Research Section

Chemical and biochemical properties of casein–sugar Maillard reaction products

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Abstract

The Maillard reaction (MR) involves the condensation reaction between amino acids or proteins with reducing sugars, which occurs commonly in food processing and storage. The Maillard reaction of Glc–, Fru– and Rib–casein was generated at 55 °C, pH 7.0 for up to 28 days. The browning and fluorescence of Glc– and Fru–casein increased with increasing heating time. The temporal development of browning and fluorescence of Rib–casein was relatively faster than Glc– and Fru–casein, respectively. Glc–, Fru– and Rib–casein all exhibited antioxidant activity against Fenton reactant-induced hydroxyl free radicals, while only Rib–casein exhibited a weak DPPH free radical scavenging in addition to preventing Fenton reactant-induced oxidation. It was suggested that casein–sugar MRPs work more efficiently to quench hydrophilic than hydrophobic radicals. All three MRPs showed no toxicity to Caco-2 cell at both low and high concentrations. There was no correlation between the browning and/or fluorescence temporal patterns and biochemical activity of the different sugar-casein generated MRPs. © 2002 Published by Elsevier Science Ltd.

Keywords: Maillard reaction; Glucose; Fructose; Ribose; Casein; 2-Deoxy-ribose; DNA nicking; Caco-2 cells; DPPH; Toxicity; Antioxidant activity

1. Introduction

The Maillard reaction (MR) occurs during food processing and storage (Mlotkiewicz, 1998). The reaction is classified as non-enzymatic browning, which involves sugars, amino acids or proteins that condense and progress into a complex network of reaction products that are collectively known as Maillard reaction products (MRPs; Baxter, 1995; Ho, 1996; Weenen, 1998; Lederer and Buhler, 1999). The MR is influenced by many factors such as temperature, time, pH, water activity (a_w) and reactant source and concentration (Lingnert, 1990; Wijewickreme et al., 1997). At an early stage of the reaction, the protein containing free amino groups, such as the ε-NH_2 groups of lysine and arginine, react with carbonyl groups of sugars to form a reversible Schiff base, which rearranges to stable, covalently bonded Amadori products. In the advanced phase of the reaction, Amadori products undergo further transformation to fluorescent, colored substances, and cross-linked polymers (Mauron, 1990; Morales et al., 1996; van Boekel, 1998). Fluorescence and browning development in MR are generally used as an indicator of the reaction rate and MRPs formation (Yeboah et al., 1999; Leong and Wedzicha, 2000).

The antioxidant activity of MRPs produced from amino acid–sugar model systems has been studied by a number of investigators (Park and Kim, 1983; Alaiz et al., 1996; Chuyen et al, 1998). MRPs have metal chelating and free radical scavenging affinities in different model systems (Hayase, 1996; Wijewickreme and Kitts, 1997; Yen and Liu, 1998). Similar studies have been conducted on the antioxidant activities of MRPs derived from peptide–protein–sugar systems (Vandewalle and Huyghebaert, 1980; Okamoto et al., 1992; Hayase, 1996), where the antioxidant property was considerably increased when protein was heated in the presence of reducing sugar. Although MRPs derived from protein–sugar model systems have lower antioxidant activity than amino acid–sugar model systems (Lingnert and Eriksson, 1980), there are only a few studies that have reported an antioxidant activity of casein–sugar system in emulsion

Abbreviations: BREC, bovine retinal capillary endothelial cells; BRP, bovine retinal capillary pericytes; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FCMP, full-cream milk powders; HCG, heated casein–glucose mixtures; HMW, high molecular weight; MR, Maillard reaction; MRPs, Maillard reaction products; MWCO, molecular weight cut-off; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

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or food model systems (McGookin and Augustin, 1991, 1997). In these studies, heated casein–glucose mixtures (HCG) decreased lipid peroxidation in an emulsified linoleic acid model (McGookin and Augustin, 1991) and increased the shelf-life of the full-cream milk powders (FCMP; McGookin and Augustin, 1997).

Since research considering the antioxidant activity of MRPs has been performed mostly with sugar–amino acid models, relatively less is known about the antioxidant activity potential of sugar–protein models. However, further studies are needed to investigate a correlation between the antioxidant activity of heated products and intensity of generated color/fluorescence in sugar–protein models. Moreover, there is a need to ascertain whether a correlation between the antioxidant activity and cytotoxic effect of the related MR products exists. It has been considered that different sugars have characteristic reaction rates resulting in the formation of MRPs in MR, which might influence functionality (Wijewickreme et al., 1999; Brands et al., 2000). The purpose of this study was to determine the physiochemical characteristics of glucose–, fructose– and ribose–casein models and the associated antioxidant and cytotoxic activities. The cytotoxic effect of the different sugar–casein model products was evaluated in an intestinal model of human origin, the Caco-2 cell culture system.

2. Materials and methods

2.1. Chemicals

Casein, D-glucose (Glc), D-fructose (Fru), D-ribose (Rib), 2-deoxy-ribose, L-ascorbic acid, 2-thiobarbituric acid, PBR322 plasmid DNA, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St Louis, MO, USA); ferric chloride come from BDH Inc. (Toronto, Canada); hydrogen peroxide was purchased from Fisher Scientific (Fair Lawn, NJ, USA); 75 cm^2 plastic flasks and 96-well tissue culture plates were purchased from Sarstedt, Inc. (NC, USA); (4.5 mg/l) Dulbecco's modified Eagle's medium (high glucose) (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen life technology (NY, USA). (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox^{TM}C) was supplied by Fluka Chmie AG (Switzerland).

2.2. Preparation of MRPs from a sugar–casein model system

A MRP model reaction system was modified from the procedure reported by Clark and Tannenbaum (1970). The model consisted of casein (10 g), sugar (5 g) and distilled deionized water (ddH_2O) 100 ml with a pH adjusted to 7.0 using 10 M NaOH. The solution was kept in a 50-ml polypropylene tube that was capped and sealed by Parafilm to prevent the sample from evaporating during heating. Samples were placed in a water-bath at 55±1 °C for up to 28 days under an aluminium foil covering to shield light from the samples. The samples were removed at 2-day intervals and placed in an ice-bath to cool before being stored at 4 °C prior to measuring spectroscopic parameters. Samples were diluted with ddH_2O to a final protein concentration of 1 mg/ml for spectroscopic measurement and to a final protein concentration of 0.1 mg/ml for fluorescence spectra analysis of MRPs (Pongor et al., 1984; Shaw and Crabbe, 1994; Wu et al., 1996). The samples at day 19 of heating time were freeze-dried and used for further experiments. To enrich for high molecular weight (HMW) intermediates, the heated sugar–casein mixtures were dialyzed or produced under very severe conditions (e.g. autoclave for 1 h) (Kitts et al., 1993). In brief, the heated sugar–casein mixtures were dialyzed repeatedly against ddH_2O until dialysate appeared colorless, using dialysis tubing with molecular weight cut-off (MWCO) of 3500. The retained portions of the heated sugar–casein mixtures from the dialysis and the autoclaved sugar–casein mixtures were freeze-dried for the cell toxicity test.

2.3. Assay of 2-deoxy-ribose oxidation degradation

The reaction mixtures for the oxidation assay of 2-deoxy-ribose contained the following reagents: 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), formulated in a total volume of 1.2 ml. The mixtures were incubated at 37 °C for 1 h. The degradation of 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 at al., 1989; Aruoma, 1994). MRP color was controlled by adding MRPs in 1.2 ml of a KH_2PO_4/KOH buffer (pH 7.4) and deducted from the reacting sample absorbance. All readings were corrected for interference from brown color of the MRPs. Trolox (5 μg/ml), a hydrophilic form of α-tocopherol, was used as the positive control.

2.4. DNA nicking assay

PBR322 plasmid DNA was reacted with Fenton reagents that consisted of: 2 μl of 20 μM $FeCl_3 \times 6H_2O$, 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. The Fenton reaction was designed for both non-site-specific DNA nicking and site-specific DNA nicking. In the latter example, EDTA was absent from the reaction and

therefore subsequent hydroxyl radicals were generated from the reduced ferric directly on the DNA molecules (Buettner, 1987; Halliwell, 1990; Wijewickreme et al., 1999). All reactions were incubated in a 37 °C water-bath for 1 h. Samples were loaded onto a 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), and DNA bands were visualized under UV light and photographed (Wijewickreme and Kitts, 1997).

2.5. Free radical scavenging activity

The stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect of the generated MRPs was measured according to the method of Hu and Kitts (2000). The test compounds were solubilized with 0.1 M sodium phosphate buffer, pH 7.0. Solutions containing MRPs (20 mg/ml) were mixed with 0.1 mM of DPPH in ethanol. The mixture was shaken vigorously and left for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm. Trolox (5 μg/ml) was used as the positive control.

2.6. Cell toxicity test

Caco-2 cells were cultured in 75 cm^2 plastic flasks. DMEM containing 10% FBS (DMEM10) was used as a basal growth medium. The cells were cultured in an incubator (37 °C) under an atmosphere of 5% CO_2 with 90% humidity. All experiments were performed with cells between 20 and 40 of passage generation. The culture medium was changed every 2–3 days. Prior to reaching confluence, the monolayer cells were detached and separated with trypsin (0.25%)–EDTA (1 mM) and aspirated to make a single cell suspension. The cells were plated at 0.75×10^3 cells/ml (100 μl/well) in 96-well cell culture plate with DMEM10 and maintained overnight at 37 °C in the CO_2 incubator. The cell culture medium was changed with fresh DMEM10, DMEM or PBS, respectively, and then the MRPs were added to the cell cultures. After 3 h of incubation with treatment, DMEM and PBS media were replaced with basal growth media, and the treated cell cultures were carried on for further 72 h before assessing the cell viability with MTT.

2.7. MTT assay

The MTT assay was performed according to Mosmann (1983). Culture media of monolayer cells were removed from the cell culture plates, and 70 μl of MTT solution (0.5 mg/ml) was added to each well. Cultures were incubated at 37 °C for 3 h and then the untransformed MTT was removed before adding 90 μl of 0.04 N HCl–isopropanol to each well. The plate was vigorously shaken for 5 min on a vortex and the optical density of each well was measured at an absorbance of 570 nm, using a Microplate reader (BIO-RAD model 550). Absorbance values measured at 570 nm were corrected for background absorbance with wells containing only 90 μl of the 0.04 N HCl–isopropanol.

2.8. Statistical analysis

A one-way analysis of variance (ANOVA), followed by Tukey multiple-range test (Minitab Inc., State College, PA, USA) was used for 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. The browning and fluorescence of the MRPs

The fluorescence of Glc– and Fru–casein models both increased gradually during the 28-day heating period. Fluorescence reading of the Rib–casein mixture was characteristically different from that of Glc– and Fru–casein models. For example, fluorescence intensity quickly reached a maximum within 4 days of heating before decreasing to a plateau phase (Fig. 1).

An increase in browning occurred for the Glc–, Fru– and Rib–casein mixtures over time when heated at 56 °C for up to 28 days. The Rib–casein mixture displayed a 10-fold greater browning development than the Glc– and Fru–casein mixtures, and reached a maximum absorbance and then plateaued after 10 days of heating. In contrast, browning increased gradually throughout the 28-day heating period for both Glc– and Fru–casein systems. The browning development was higher ($P < 0.05$) for the Glc–casein mixture than that the Fru–casein model after 19 days (Fig. 2).

Both fluorescence and browning intensity of Glc– and Fru–casein were increased as a result of increasing heating time. A correlation between fluorescence and browning intensity for both Glc– and Fru–casein was observed ($r^2 = 0.985$ and 0.950, respectively); however, a similar correlation was not seen in the Rib–casein model (Figs 1 and 2).

3.2. Hydroxyl radical scavenging

The scavenging affinity of Glc–casein, Fru–casein and Rib–casein for Fenton reactant-induced hydroxyl radicals was assessed using the oxidative degradation of deoxyribose (Table 1). Inhibition of the oxidative degradation was evident for all three sugar–casein models at a concentration of 1 mg/ml. Glc–casein and Fru–casein produced a similar inhibition of oxidative degradation of deoxyribose, which was 28 and 30%, respectively. Rib–casein had a lower ($P > 0.05$) inhibition (e.g. 15%) of hydroxyl radical damaged deoxyribose.

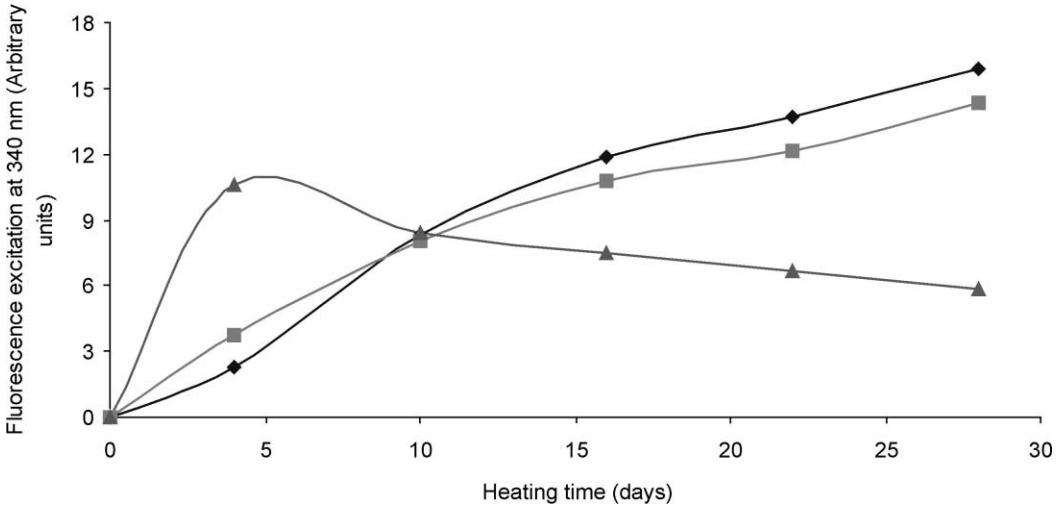

Fig. 1. Temporal development of fluorescence in Glc–, Fru– and Rib–casein models.(◆) Glc–casein; (■) Fru–casein; (▲) Rib–casein.

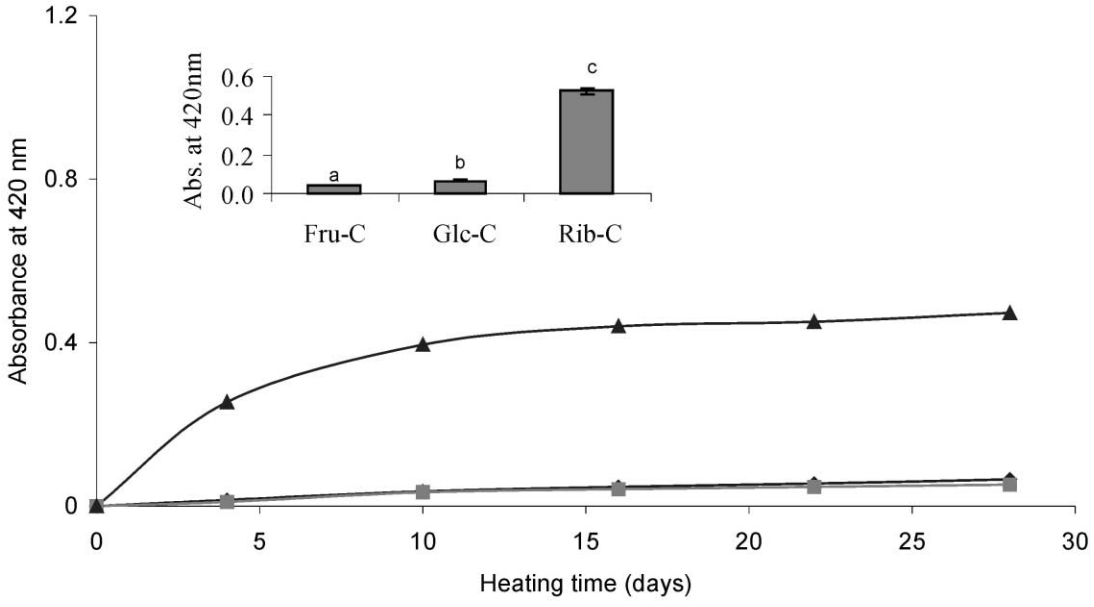

Fig. 2. Temporal development of browning in Glc–, Fru– and Rib–casein models. (◆) Glc–casein; (■) Fru–casein; (▲) Rib–casein. Inserted graph represents the browning intensity of Glc–, Fru– and Rib–casein solutions after 19 days of heating at 55 °C. [abc]Different letters are significantly different ($P < 0.05$).

3.3. DNA nicking prevention

Figure 3 shows the separation of Form I circular DNA from form II nicked circular DNA, using agarose gel electrophoresis denoting DNA stand breakage (e.g. lane 5) by hydroxyl radicals generated from the Fenton reaction system and visualizing by a loss in the Form I DNA and resultant increase in the extent of Form II nicked circular DNA. The addition of Glc–casein, Fru–casein and Rib–casein to the mixture retained Form I circular DNA and prevented generation of Form II nicked circular DNA, caused by the Fenton reaction system (lanes 2–4). The Glc–casein, Fru–casein and Rib–casein model products produced similar electrophoresis gel patterns, denoting little difference in source of MRPs in protecting Fenton-induced hydroxyl radical formation.

3.4. DPPH radical scavenging

A range of concentrations of 0.1–0.5 mg/ml of the sugar–casein MRPs was used for the DPPH radical inhibition assay. Both Glc–casein and Fru–casein showed no DPPH scavenging activity at the concentrations tested. A very low DPPH radical scavenging activity (3–7% inhibition, data not show) was obtained for Rib–casein. The presence of Trolox resulted in 68.3 ± 2.1% inhibition.

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

MRPs	Oxidation of D-ribose	Inhibition (%)
Control	1.00±0.07	–
Trolox	0.08±0.00	92
Glc–casein	0.72±0.02*	28
Fru–casein	0.70±0.04*	30
Rib–casein	0.85±0.02*	15
Casein	1.03±0.24	–
Glucose	0.90±0.11	–
Fructose	0.93±0.21	–
Ribose	1.10±0.20	–
Glc/casein	1.07±0.15	–
Fru/casein	1.09±0.09	–
Rib/casein	1.05±0.13	–

Values represent mean±S.D. ($n=3$), and control was set as 1.0. 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). Casein, glucose (Glc), fructose (Fru) and ribose (Rib) are tested at a concentration of 1 mg/ml; Trolox is 5 μg/ml; Glc–casein, Fru–casein, Rib–casein = heated mixtures of 1 mg/ml; Glc–casein, Fru–casein, Rib–casein = unheated mixture containing 0.5 mg/ml of sugar and 1 mg/ml of casein.

[a] $\%\text{inhibition} = \dfrac{[\text{Control-MRPs} \times 100]}{\text{Control}}$

* $P < 0.05$ in comparison with control.

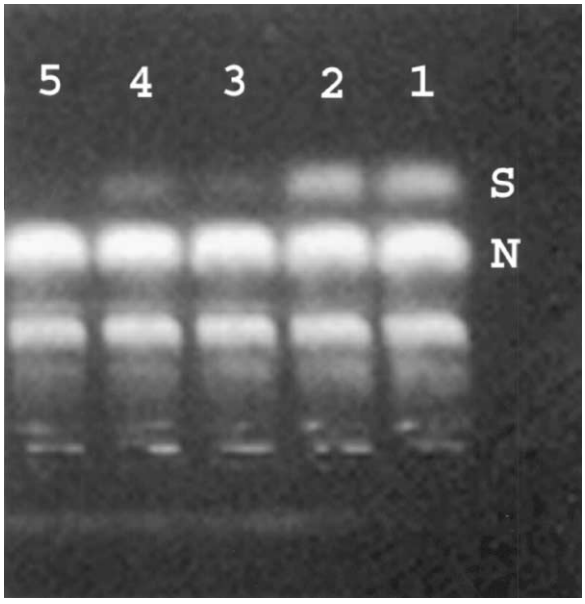

Fig. 3. Modulation of DNA strand cleavage by sugar–casein model reaction products. Lane 1 = DNA (native); Lanes 2–4 = DNA + -Fenton reactants + Glc–casein (lane 2), Fru–casein (lane 3) and Rib–casein (lane 4); lane 5 = DNA + Fenton reactants ($FeCl_3$ + ascorbic acid + H_2O_2); S = supercoiled DNA; N = nicked circular DNA.

3.5. MRPs and Caco-2 cell viability

Caco-2 cell culture was performed in 10% FBS medium, and sugar–caseins were added to the cell cultures for 4–5 days before determining cell viability with the MTT assay. Similar experiments were carried out in cultures containing DMEM or PBS. The cells were incubated with the samples in DMEM or PBS for 3 h, which were then replaced with 10% FBS medium and incubated for 3 days before performing the MTT assay. In all cases, sugar–casein MRPs displayed minimal, or no, toxicity on cultured Caco-2 cells (Table 2). Moreover, heated sugar–casein MRPs were also dialyzed to remove free sugars and early-stage MRPs, thus yielding the higher molecular weight MRPs. Furthermore, the sugar–casein models were also processed under harsh condition (autoclave for 1 h) to obtain high molecular weight or advanced-stage MRPs. There was slight, but no significant toxicity on Caco-2 cells exhibited from high molecular weight or advanced-stage MRPs (Table 3).

4. Discussion

In the present study, casein was used as a representative food protein, along with three common sugars: glucose, an aldose sugar; fructose, a ketose isomer of glucose; and ribose, an aldopentose sugar. Glucose, fructose and ribose were selected as sugar reactants due to differences in the reaction rates and products of MRPs that have been associated with antioxidant substances (Reynolds, 1965; Hollnagel and Kroh, 1998; Brands et al., 2000). The reaction mechanism of fructose is known to be considerably different from that of glucose (Reynolds, 1965). The temperature of 55 °C was chosen for the casein-sugar model systems because this temperature is often used in accelerated storage trials (Leong and Wedzicha, 2000). Maillard reactions occur slowly at 35 °C, but are accelerated at a temperature 55 °C or greater (Cerrutti et al., 1985). There are also marked differences in the chemical changes that modify casein in the casein and reducing sugar model systems, when the mixtures are heated within a range of 50–60 °C. The MRPs formed at this range of temperature have also been reported to have biological effects, such as the increased urinary Zn excretion (Hurrell et al., 1983; Furniss et al., 1989). To date, there remains very little information concerning the absolute chemical composition of MRPs constituents derived from these reaction conditions.

The Maillard reaction is associated with the development of fluorescence compounds formed prior to the generation of brown pigments (Pongor et al., 1984; Morales et al., 1996). The brown color development is largely due to the formation of chromophores, which have been widely studied in different model systems (Rizzi, 1997; Tressl et al., 1998; Monti et al., 2000). For example, it has been established that color substances

Table 2
Effect of MRPs on viability of Caco-2 cells at different conditions[a]

Treatment	(mg /ml)	Incubation media		
		DMEM10[b]	DMEM[c]	PBS[c]
Control		100±3	100±19	100±9
Glc–casein	0.5	104±13	105±18	98±8
	1.0	104±6	90±11	95±8
	2.0	109±4	96±12	95±9
Fru–casein	0.5	90±7	113±14	91±7
	1.0	104±8	111±10	90±9
	2.0	102±5	106±7	92±6
Rib–casein	0.5	106±10	111±11	109±7
	1.0	105±9	106±21	99±9
	2.0	108±8	94±22	108±9

[a] The viability was expressed as percentage of control viability (controls as 100%), and the values are presented as mean±S.D.
[b] The cells were incubated with the heated sugar–caseins in DMEM10 for 3 days.
[c] The cells were incubated with the heated sugar–caseins for 3 h in DMEM and PBS, respectively.

Table 3
Effect of different MRPs on viability of Caco-2 cells[ab]

Treatment	(mg/ml)	MRP types		
		Undialyzed[c]	Dialyzed[d]	Autoclaved[e]
Control		100±9	100±8	100±9
Glc–casein	0.5	98±8	91±9	104±9
	1.0	95±8	108±12	102±10
	2.0	95±9	100±10	110±11
Fru–casein	0.5	91±7	91±14	94±12
	1.0	90±9	92±16	101±6
	2.0	92±6	95±9	97±11
Rib–casein	0.5	109±7	95±11	108±16
	1.0	99±9	95±12	88±10
	2.0	108±9	90±12	88±13

[a] The viability was expressed as percentage of control viability (control as 100%), and the values are presented as mean±S.E.
[b] The cells were incubated with the heated sugar–caseins in PBS for 3 h.
[c] The heated sugar–casein mixtures (55 °C, 19 days).
[d] The retained portion of the heated sugar–casein mixtures from the dialysis (MWCO 3500).
[e] Glc–casein, Fru–casein and Rib–casein were autoclaved for 1 h.

generated from casein–sugar model systems are due mainly to the formation of protein oligomers that are mediated by chromophoric substructures derived from carbohydrates (Hofmann, 1998). Fluorescent compounds are possible precursors of brown pigments (Labuza and Baisier, 1992), and remain to be fully characterized (Morales et al., 1997). Cerrutti et al. (1985) reported a linear increase of fluorescence with increasing heating time of a lysine–glucose system, while Ananth-Narayan and Andreotti (1989) did not find a linear increase of fluorescence with increasing heating time using a lysine/glucose system. Other workers have detected a reduction of fluorescence in a lactulose/β-lactoglobulin model during storage (Matsuda et al., 1991). In the present study, Glc– and Fru–casein models showed a similar fluorescence pattern, which was characterized by a gradual increase of fluorescence with increasing heating time. A different fluorescence pattern was observed for the Rib–casein model, which reached maximum fluorescence rapidly before decreasing to a plateau phase. This corresponded to an increase with the browning intensity in the Rib–casein model, followed by a plateau phase after 15 days, which was approximately 10 times greater than that of Glc– and Fru–casein models. Similar to the fluorescence pattern, the Glc– and Fru–casein models had a gradual increase in browning with increasing heating time, while the browning intensity of Glc–casein was markedly greater than that of Fru–casein model after 19 days.

Our findings also indicate that the source of sugar composing the MRPs will influenced the fluorescent and browning development patterns in the sugar–casein model systems. Although it is generally accepted that aldoses are intrinsically more reactive than ketoses (O'Brien and Morrissey, 1989), there have been conflicting reports on the relative reactivity of glucose compared with fructose. Several researchers have reported that fructose is more reactive than glucose (Ashoor and Zent, 1984; Walton et al., 1989; Suarez et al., 1995; Hollnagel and Kroh, 1998), while other studies have reported that glucose is the more reactive sugar (Spark, 1969; Wijewickreme et al., 1997; Naranjo et al., 1998; Yeboah et al., 1999). In a study using a sugar–casein model, it was shown that the ketose sugar fructose browned more quickly than the aldose isomer, glucose, when heated at 120 °C and at a pH 6.8 for up to 60 min (Brands et al., 2000). Caramelization of ketose has been suggested to contribute to the browning, while caramelization of aldose systems has not been related to a significant browning (Buera et al., 1987a, b). The discrepancies in the literature may be related to the great diversity of conditions used in the heating treatment, the indicators used to measure the reaction, and amine sources such as amino acids or proteins used in the reaction. Generally, pentoses are more reactive than hexoses in the sugar–amino acid models (Ashoor and Zent, 1984; O'Brien and Morrissey, 1989). The results of the present study using the sugar–casein model confirmed that the pentose sugar, ribose, is more reactive than hexoses, such as glucose and fructose in browning intensity. Furthermore, the data shown here support our former study that glucose was relatively more reactive than fructose in the sugar–casein Maillard reactions (Wijewickreme et al., 1997).

Radical scavenging activity of the different MRPs was tested in part using the stable free radical, DPPH, which

has a strong absorption at 517 nm. Our results suggest that only MRPs derived from Rib–casein possessed some, albeit very little DPPH free radical scavenging activity, while Glc–casein and Fru–casein MRPs had no detected affinity for DPPH. Dean et al. (1991) demonstrated that hydrophobic radicals are unable to attack macromolecules such as proteins in solution. Therefore, it is unlikely that DPPH, a relatively hydrophobic radical, would interact with sugar–casein MRPs to a sufficient extent in the organic solvent ethanol used for this assay.

Unlike hydrophobic radicals, hydrophilic radicals, such as hydroxyl radical, can interact with MRP in aqueous solution (Rival et al., 2001). The Fenton reaction system, containing ferric–ascorbate–EDTA–H_2O_2, generates hydroxyl radicals at a rapid rate (Narla and Rao, 1995), which in turn will react with deoxyribose and DNA, as targets of hydroxyl radical-induced peroxidation (Aruoma, 1994; Wijewickreme and Kitts, 1997). All three heated mixtures of Glc–, Fru– and Rib–casein exhibited varying protective effects against hydroxyl radicals in both the deoxyribose oxidation and DNA nicking tests. The Glc– and Fru–casein decreased the deoxyribose oxidation by 28 and 30%, respectively, thus exhibiting a similar efficacy to scavenge hydroxyl radicals. The Rib–casein inhibition rate was relatively lower. The metal-ion binding of MRPs could represent a mechanism for antioxidant activity more than direct free radical scavenging activity (Wijewickreme and Kitts, 1997).

The browning of a sugar–amino acid mixture has been reported to increase almost linearly with increasing heating time, while fluorescence spectra follow a different pattern that reaches a maximum first before plateauing off during the heating period (Morales and Jimenez-Perez, 2001). These workers indicated that the DPPH radical scavenging activity of the heated sugar–amino acid mixture was correlated to the change in fluorescence (Morales and Jimenez-Perez, 2001). This result was not confirmed in the present study with the DPPH assay, although the Rib–casein model did exhibit a small degree of scavenging affinity for the DPPH free radical that was related to relatively greater browning compared to both Glc– and Fru–casein. The latter MRPs had no DPPH scavenging power at the concentrations used and were characteristically different from the Rib–casein complex in browning or fluorescence patterns.

Maillard reaction products generated from sugar–amino acid models were reported to have potentially toxic effects (O'Brien and Morrissey, 1989). Relative cytotoxic effects of brown mixtures obtained by heating a lysine–glucose or lysine–fructose mixtures at 121 °C for 1 h have been demonstrated with mouse C6 glioma cells (Wang et al., 1987). Vagnarelli et al. (1991) also showed that MRPs derived from a Rib–Lys model exhibited marked toxicity on FT cells (HeLa clone). Our previous study further confirmed the toxicity of Glc– and Fru–Lys MRPs on Caco-2 cells of human origin (Jing and Kitts, 2000). There are a few studies that have been conducted on the cytotoxic effect of MRPs produced from protein–sugar models. Chibber et al. (1997) made MRPs from bovine serum albumin (BSA) and glucose at 37 °C for 6 weeks, and observed toxicity to both bovine retinal capillary pericytes (BRP) and bovine retinal capillary endothelial cells (BREC). These studies assessed the cytotoxic effect of MRPs in culture medium containing 10% FBS or PBS. It was suggested that glycated protein could be toxic. Results from our laboratory have determined that BSA has a protective effect against MRPs cytotoxicity (unpublished data). Results observed with Glc–, Fru– and Rib–casein heated mixtures varied little in cytotoxicity whether they were tested in complete media, serum-free media or a phosphate buffer, with an attempt to access cytotoxic effect specific to individual MRP models. In fact, all three MRPs in the present study showed no significant toxicity to cultured Caco-2 cells. Since the temperature of sugar–casein model system was relatively mild, MRPs were also produced under very severe condition (e.g. autoclave for 1 h) and dialyzed to enrich for HMW intermediates. The cytotoxic effects of the HMW fractions were also assessed and showed to be minimal. It can be concluded from these experiments that the glycated casein is relatively inert compared with the sugar–amino acid MRPs from our previous study (Jing and Kitts, 2000). It is also possible that different cell types have different sensitivities towards MRPs, and for that matter, Caco-2 cells of gastrointestinal origin could be more resistant to sugar–casein MRP toxicity due to metabolic compensation mechanisms reported by Gamet-Payrastre et al. (1998). For example, Brands et al. (2000) reported that the sugar–casein systems displayed a weak mutagenic activity, which was absent in the presence of S9 (rat liver homogenate). Kitts et al. (1993) also showed detoxification of the direct mutagenic effect of the MRPs with S9. Similar detoxification enzyme systems exist in Caco-2 cells (Hofmann et al., 2001).

Recently, there has been an attempt to correlate the biological and chemical effects of MRPs with the browning and/or fluorescence rates (Brands et al., 2000; Morales and Jimenez-Perez, 2001). In the present experiment, it appeared that both browning and fluorescence were not clearly correlated to cytotoxicity, DPPH free radical scavenging affinity, or the inhibition of hydroxyl radical damage to deoxyribose or DNA. The MR is a complex reaction, since it is influenced by many factors such as temperature, time, pH, water activity (a_w) and reactants (Nursten, 1986; Lingnert, 1990; Wijewickreme et al., 1997). Changing any one of these factors will alter reaction rate, reaction pathways

and reaction end-products. More studies are required to determine an appropriate reference that will evaluate correlating the products of MRP formulation to specific chemical and biological activity.

In conclusion, the MR of Glc–, Fru– and Rib–casein was performed at 55 °C, pH 7.0 for up to 28 days. The browning and fluorescence of Glc– and Fru–casein increased with duration of heating. The browning and fluorescence of Rib–casein reached a maximum level earlier, indicating a relatively faster reaction rate than Glc– and Fru–casein reactants. Browning intensity followed the order of Rib– > Glc– > Fru–casein. Albeit limited, Rib–casein exhibited both hydroxyl and DPPH free radical scavenging affinity, while the Glc– and Fru–casein MRPs demonstrated only hydroxyl free radical scavenging affinity in the Fenton assay. All three models showed no toxicity to Caco-2 cell at both low and high concentrations. There was no correlation between the temporal patterns of browning and/or fluorescence and biochemical activity of the different MRPs.

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