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ORIGINAL ARTICLE

Testing biological activity of model Maillard reaction products: studies on gastric smooth muscle tissues

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Abstract Water-soluble Maillard reaction products obtained from five different model systems were investigated for their effects upon the mechanical activity of rat gastric smooth muscle. Most of the total Maillard reaction products applied at concentration of 1.5 mg/ml evoked contractions; among them the product obtained from arginine and glucose (Arg-Glc) produced the most powerful contractions. The product obtained from glycine and ascorbic acid (Gly-AsA) was the only one that brought about relaxation response. The high molecular weight fractions (>3,500 Da) isolated from the reaction systems Arg-Glc and Gly-AsA demonstrated effects similar in type and amplitude to those evoked by non-fractioned reaction products. The results obtained suggest that moieties of molecules acting upon the muscle tonus originate mainly from lysine and arginine residues; that these structures are available in both low and high molecular pools in similar concentrations, and most likely these fragments act upon membrane-located cellular structures involved in calcium transport.

Keywords Maillard reaction · Melanoidins · Ascorbic acid · Arginine · Gastric smooth muscle

Abbreviations

MRP	Maillard reaction product
SMP	Smooth muscle preparation
FTIR	Fourier-transform infrared spectroscopy

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Introduction

The Maillard reaction is a multi-step interaction between reducing sugars and amino acids, proteins or other compounds bearing free amino groups. When the reaction takes place under physiological conditions it is called glycation. Glucose-derived advanced glycation products formed by proteins are well-known factors contributing to the human pathology: diabetes-related complications (Brownlee 2001), Alzheimer disease (Vitek et al. 1994), and aging (Thorpe and Baynes 1996).

The Maillard reaction is also largely responsible for the production of specific aroma, color, and texture during thermal processing of foods. The research on the Maillard reaction products (MRPs) formed during food heating and cooking is very complicated because of the variety of conditions used and the resultant large number of substances formed. The investigations on food-derived MRPs have been mostly performed with sugar–amino acid models and focused on the elucidation of chemical structures of the products and their importance for food quality. Only recently the metabolism of reaction products and the physiological effects of ingested MRPs started to provoke considerable interest. Some of the low molecular weight MRPs such as N_{ε}-(carboxymethyl)lysine (Bergmann et al. 2001), pyrraline, and pentosidine (Forster et al. 2005) represent non-reactive products that are readily excreted in the urine, while 3-deoxyglucosone, methylglyoxal, and supposedly many others, are reactive intermediates called glycotoxins that may attach onto circulating and tissue proteins (Koschinsky et al. 1997).

Still little is known about the biological effects of the higher molecular weight MRPs, classified as melanoidin precursors, premelanoidins and melanoidins (Finot 1990). It has been generally assumed that melanoidins have

effects similar to dietary fiber because these products are indigestible and excreted slightly modified or unmodified in the feces (Finot and Magnenat 1981). Nevertheless, some specific biochemical and physiological effects of the advanced MRPs have been reported. Products from model Maillard reaction system glutamate-glucose caused severe diarrhea in rats fed a 10% MRPs diet (O'Brien and Walker 1988). A study conducted with dietary melanoidins suppressed the elevation of cholesterol level of plasma and liver of rats and effected intestinal metabolism of cholesterol (Miura and Gomyo 1990). Ames et al. (1999) demonstrated that melanoidins obtained from glucose and glycine could stimulate the growth of intestinal anaerobic bacteria during their gastro-intestinal transit. Several reports have shown that the advanced brown products of the Maillard reaction could influence the activity of specific xenobiotic enzymes (Kitts et al. 1993; Hofmann et al. 2001; Faist et al. 2002; Borrelli et al. 2003).

The demonstrated biological effects are most likely limited to interactions of MRPs with membrane-located cellular structures that trigger a release of secondary messengers and evoke a subsequent cellular response. In the present work, we tested this hypothesis by using MRPs obtained from amino acids modeling different sites for modifications via Maillard reaction in real foods: glycine (containing only α-amino group), lysine (having a second amino group in its side chain), and arginine (containing guanidine moiety). We also used different carbohydrates to clarify whether the carbohydrate nature is of importance for the biological properties of the MRPs. The biological activity of these model products was evaluated by a registration of mechanical reactions of gastric smooth muscle tissue in the presence of MRPs. Smooth muscle cells are abundant in membrane receptors whose activation most often results in changes in the muscle tonus, namely muscle contraction or relaxation.

Materials and methods

Chemicals and instruments

Glycine (Gly), L-lysine (Lys), L-arginine (Arg), D-glucose (Glc), D-xylose (Xyl), L-ascorbic acid (AsA), ninhydrin, 2,6-dichloroindophenol, acetylcholine, and cytochrome *c* were obtained from Sigma (St. Louis, MO, USA). Insulin was a product of Novo Nordisk (Copenhagen, Denmark). Dialysis membranes Spectra/Por®3 (cut-off 3,500 Da) were purchased from Spectrum B·V.Breda (The Netherlands) and pre-treated as recommended by the manufacturer.

Non-reacted carbohydrates were quantified by Glucose liquicolor test, (Human Gesellschaft fuer Biochemica und Diagnostica mbH, Wiesbaden, Germany) according to the manufacturer's recommendations. The amounts of residual amino acids were measured spectrophotometrically by ninhydrin reaction (Friedman 2004). Free ascorbic acid content in the total Gly-AsA MRP was determined by redox titration using the reaction between ascorbic acid and 2,6-dichloroindophenol (Boyer 1986).

Krebs solution had pH 7.4 and composition (in mM): NaCl 120, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 15.4, and glucose 11.5. All ingredients used for this solution had analytical grade and were obtained from Merck (Darmstadt, Germany). Deionized water (18.2 mΩ/cm^2) was used thoroughly.

Fourier-transform infrared (FTIR) spectra were collected in KBr pellets on a Nicolet Avatar 330 spectrometer (Thermo Electron Corporation, Madison WI, USA). Cary 1 spectrometer (Varian, Australia) was used for the UV spectra and absorbance readings.

Preparation of Maillard reaction products

Three amino acids (Gly, Lys, and Arg) and three carbonyl components (Glc, Xyl, and AsA) were used as starting compounds. Model MRPs were prepared according to the COST 919 action standard protocol (Obretenov et al. 2002). Briefly, 0.05 mol of amino acid and carbohydrate were dissolved in small volume of water and then freeze-dried. The solid mixture obtained was heated at 125°C for 2 h in an open beaker. Thereafter, the reaction product was dissolved in 25–30 ml of water; the water-soluble fraction of MRPs was separated by filtration through Whatmann No. 4 filter, and assigned as total MRP. The quantity of solid matter in the total MRPs was determined gravimetrically after freeze-drying of 3-ml aliquots. Stock solutions with concentration 200 mg/ml were prepared after subsequent dilution with Krebs solution.

Melanoidin separation and molecular weight evaluation

Separation of the melanoidins Arg-Glc and Gly-AsA from low molecular weight products and starting compounds was done by dialysis against deionized water for 72 h at 4°C. The product obtained after heating was dissolved in 25 ml of distilled water and packed in a 3,500-Da cut-off dialysis tubing. Dialysis fluid (1 l) was changed at least four times a day. Melanoidins were obtained after freeze-drying of the retained solution and used further for preparation of stock solutions (200 mg/ml in Krebs solution). The molecular weight of the melanoidins above was evaluated by gel-permeation chromatography on a column (16 × 300 mm) packed with Sephadex G-50 medium. Sodium phosphate buffer

(20 mM pH 7.0) was used as a mobile phase and was delivered by peristaltic pump at a flow rate of 1 ml/min. Fractions, 2 ml each, were collected and the absorbance of each fraction was read at 300 nm. Cytochrome *c* (12,384 Da) and insulin (5,780 Da) were used as molecular weight markers. They were detected by absorbance at 280 nm.

Registration of gastric corpus smooth muscle mechanical activity

Gastric corpus smooth muscle preparations (SMPs) were obtained from adult male Wistar rats, weighing between 280 and 320 g. All experimental procedures were carried out in strict accordance with the current European regulations (86/609/EEC) regarding the protection of animals used for experimental purposes. Total number of animals used in this study was 36. The animals were housed under standard conditions: 20–22°C temperature, free access to food, and 12-h light/dark cycle. They were decapitated under ether anesthesia; the stomach was immediately excised, and the circular gastric corpus SMPs 12–13-mm long and 1.0–1.1-mm wide were dissected. Two or three muscle strips were taken from one rat stomach. The strips were mounted on a glass holder at one end and attached to a Swema tensodetector (Sweden) at the other under 7.5-mN initial tension. The mechanical activity of SMP was registered isometrically with a Microtechna amplifier (Czech Republic) and recorded by a Linsseis polygraph (Germany). Krebs solution aerated with a mixture of 5% CO_2 and 95% O_2 at 37°C was used in the tissue bath. All preparations were equilibrated prior to the experiments for a period of 60 min; during this period the Krebs bathing solution was refreshed several times. The MRPs and melanoidins were added to the tissue bath as aliquots of concentrated stock solutions. Between each treatment with MRP the intactness of the contractile apparatus of the preparations was checked by 1×10^{-6} M acetylcholine.

Statistical analysis

The amplitude values of both, contractile and relaxant responses refer to the maximum peaks obtained. All data were expressed as mean values ± standard error of the mean (mean ± SEM); the number of muscle preparations used for each data point was indicated by *n*. The results were analyzed by analysis of variance (ANOVA) to find the values that showed significant difference ($P < 0.05$). INSTAT computer program (GraphPad Software, Inc., San Diego, CA, USA) was applied for the analysis of experimental data.

Results

Characteristics of the model Maillard reaction products and melanoidins

The amino acids and carbohydrates selected for this study gave high yields of water-soluble brown products. For most of the MRPs the loss of dry matter after heating was less than 4%. Only the reaction system Arg-Glc lost 17.8 ± 0.8% ($n = 5$) of the initial weight of reagents; the intensive rising of baked product suggested a lavish release of carbon dioxide presumably through Strecker degradation. Typically, the amounts of both, non-reacted amino acid and carbohydrate did not exceed 3% of the total MRPs (calculated as dry matter). The yield of melanoidins isolated after dialysis through 3,500 Da cut-off membranes was 27.7 ± 1.2% and 21.6 ± 3.1% for Arg-Glc and Gly-AsA, respectively ($n = 5$). The brown polymers demonstrated relatively narrow band of molecular masses as evidenced by gel permeation chromatography (data not shown). The bulk melanoidins from both reaction systems were eluted between the two markers of molecular weight: insulin and cytochrome *c*. Due to the dark brown color of melanoidins it can be concluded that conjugated double bonds are abundant in their molecules. Brown indices (absorption at 420 nm) of the melanoidins Arg-Glc and Gly-AsA at concentration 0.1 mg/ml in water were very similar: 0.263 ± 0.031 ($n = 3$) and 0.257 ± 0.053 ($n = 3$), respectively.

The identity of melanoidins obtained from different synthetic lots was confirmed by their FTIR spectra. Spectra comparison carried out by OMNIC instrument software gave minimum 98.8% match. FTIR spectra of Arg-Glc and Gly-AsA had a typical polymeric low-resolution band spreading (Fig. 1). Some characteristic bands were observed and assigned to group frequencies. The absorption band registered at 1,680 cm^{-1} was characteristic to conjugated C=N or C=C bonds, which was in line with the strong absorption of melanoidins in the visible region of their electronic spectra. Different types of O–H and N–H bonds gave broad bands, the maximums of which were at 3,380 cm^{-1}. Asymmetric and symmetric stretching of C–H bonds gave two characteristic bands at 2,940 and 2,850 cm^{-1}, respectively. Both polymers display bands at about 1,400 cm^{-1}, which are typical of carboxylic groups originating from amino acids.

Effects of the total MRPs on the gastric smooth muscle tonus

Total MRPs obtained from glucose and different amino acids applied at concentration 1.5 mg/ml demonstrated contractile effects on SMPs that were different in intensity

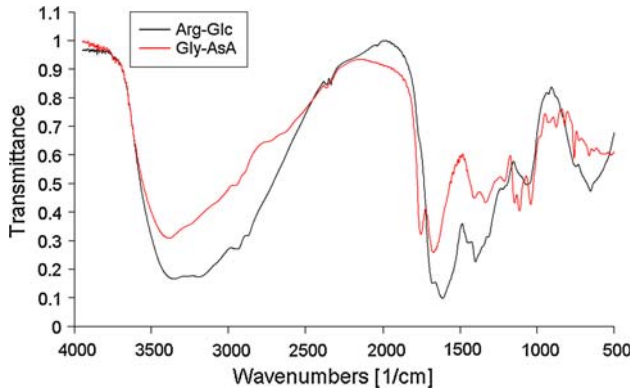

Fig. 1 Fourier-transform infrared spectra of melanoidins obtained from arginine and glucose (Arg-Glc) and glycine and ascorbic acid (Gly-AsA)

(Table 1). Gly-Glc evoked very weak contraction, and Lys-Glc also exerted a weak contractile effect. The strongest contractile effect was manifested by Arg-Glc. Statistical analysis revealed that the intensity of contraction caused by each total MRP containing constant Glc but varying amino acids differed significantly compared to the other two. All contractile effects were reversible, i.e., after replacement of the melanoidin solution with Krebs solution the initial muscle tonus was restored.

Total MRPs (1.5 mg/ml) obtained from glycine as amino component and glucose and xylose as carbohydrates exerted very weak reversible contractile effects (Table 1) without significant difference between their amplitudes. In contrast, MRPs obtained from glycine and ascorbic acid caused an initial peak of relaxation registered 1.65 ± 0.47 min after the treatment.

Table 1 Effects of total Maillard reaction products and some of the high molecular weight melanoidins obtained from different amino acids and carbohydrates, on the tonus of gastric smooth muscle preparations

No.	Maillard reaction product (1.5 mg/ml)	No. of preparations	Changes in mechanical activity (mN)
1	Total Gly-Glc	17	0.25 ± 0.21
2	Total Lys-Glc	10	0.70 ± 0.38*
3	Total Arg-Glc	25	3.46 ± 1.05*
4	Total Gly-Xyl	13	0.21 ± 0.16
5	Total Gly-AsA	21	–2.23 ± 1.31[a]
6	Melanoidin Arg-Glc	8	3.63 ± 1.44
7	Melanoidin Gly-AsA	8	–3.52 ± 0.97[a]

* Statistically significant difference ($P < 0.05$) compared to the effect demonstrated by total Gly-Glc

[a] Negative values refer to a relaxant effect

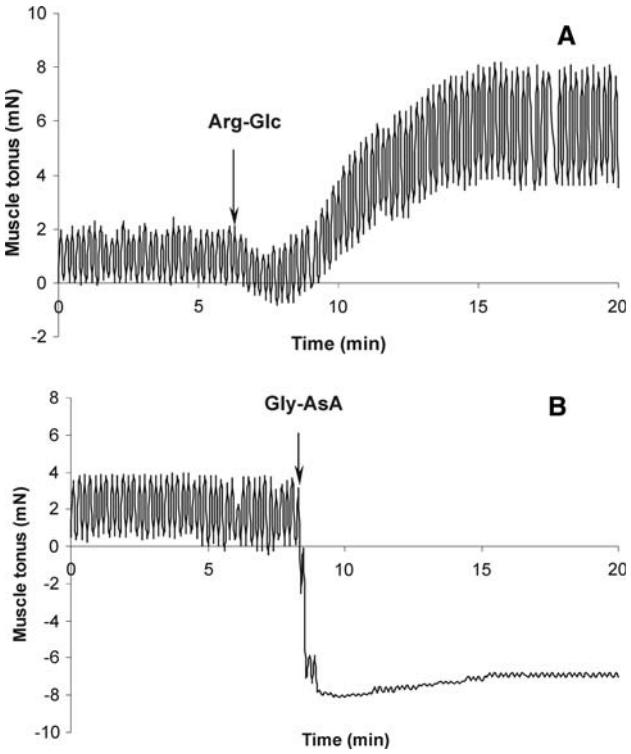

Fig. 2 Records of the mechanical reactions elicited by the melanoidins Arg-Glc and Gly-AsA applied at concentration 1.5 mg/ml

Effects of the initial amino acids and carbonyl compounds on the mechanical activity of gastric smooth muscle

Possible effects of the residual non-reacted amino acids and carbohydrates on the changes in muscle tonus were also tested. When applied in concentrations equal to those found in 1.5 mg/ml of the total MRP (22.2 μg/ml Glc, 0.66 μg/ml Xyl, 20 μg/ml Gly, 32 μg/ml Lys, and 43 μg/ml Arg), none of the starting amino acids or carbohydrates demonstrated measurable effects on the contractility of SMPs. Ascorbic acid was also tested for a possible effect upon the SMP tonus. When applied at a concentration equal to that of non-reacted AsA in the total Gly-AsA (42.5 μg/ml), a weak contraction (0.43 ± 0.28 mN, $n = 16$) was registered after the addition of AsA to the tissue bath.

Effects of melanoidins on gastric corpus smooth muscle tonus

Melanoidins of molecular mass higher than 3,500 Da obtained from two of the reaction systems were tested for their effects upon the contractility of SMPs (Fig. 2). The melanoidin Arg-Glc applied at a concentration 1.5 mg/ml evoked a contraction, whose amplitude did not differ significantly from that of the total Arg-Glc product (Table 1).

The relaxation effect caused by the melanoidin Gly-AsA applied at a concentration 1.5 mg/ml was not statistically different from that caused by the total Gly-AsA (Table 1) and remained stable over more than 20 min.

Discussion

The results obtained in this study showed that MRPs, the ubiquitous components of daily human diet, were capable of acting upon the mechanical activity of gastric smooth muscle, whereas their amino acid and carbohydrate precursors were not. However, the chemical composition of these products had an impact on the type and magnitude of the mechanical reactions. The initial screening aimed to select the most active model melanoidins and was carried out with total MRPs. They have the advantage of resembling real foods, in which brown melanoidins with different molecular masses co-exist with low molecular weight precursors, breakdown products, non-reacted amino acids, and carbohydrates. The majority of tested reaction systems caused contractile response of SMPs ranging from 0.25 mN for Gly-Glc to 3.46 mN for Arg-Glc. For comparison, the amplitude of contractions was of the same order of magnitude as that caused by 1×10^{-6} M acetylcholine, a well-known neurotransmitter (5.53 ± 2.77 mN). The variance of amino acid component and one and the same carbohydrate used to obtain MRPs showed that structures formed during the reaction with participation of an α-amino group and modeled by Gly were not as good effectors of the SMP mechanical activity as those formed with participation of Lys side chain. It has been shown (Argirova et al. 1999) that at neutral pH values the α-amino group is a more reactive site for interaction with Glc than the ε-amino group of Lys. Therefore, the higher contractile activity demonstrated by Lys-Glc cannot be attributed to the higher quantity of one and the same structure affecting the muscle tonus but rather to different chemical structures formed by Lys and Gly. Guanidine moiety of Arg produces different structures as compared to primary amines, often with imidazolone- or pyrimidine-like structures (Westwood et al. 1997; Glomb et al. 2001).

Glucose is the least reactive carbohydrate in the Maillard-type reactions. The similar amplitude of contraction brought about by the total MRPs Gly-Glc and Gly-Xyl suggests that the substitution of the six-carbon Glc with the more reactive five-carbon Xyl does not basically change the chemical structure(s) provoking the contractions registered. Studies on the participation of AsA in the Maillard reaction have shown that both the primary amino group (Argirov et al. 2003) and the guanidinium group (Pischetsrieder 1996) are possible sites for ascorbylation. Cross-link structures involving Lys and Arg side-chains also were characterized (Reihl et al. 2004). The finding that amino acids side chains are involved in the formation of biologically active structures suggest that food proteins modified during thermal processing could also demonstrate similar biological properties.

Although substantial effort has been put in the investigation of structural features of melanoidins, they remain largely unknown because a heterogeneous polymeric structure is a very complex product to analyze. Moreover, several reports have pointed out that the structure and chemical composition of melanoidins obtained from one and the same constituents are strongly dependent on the reaction conditions: temperature, moisture, pH, etc. (Cämmerer and Kroh 1995). The present work shows that the difference in the chemical composition of MRPs leads to differences in the nature and amplitude of the mechanical effects. The relaxation effect evoked by Gly-AsA is most likely due to specific structures formed by AsA that act upon cellular mechanisms involved in muscle relaxation.

The studies carried out with melanoidins with molecular weight above 3,500 Da and isolated from the most active of total MRPs tested, Arg-Glc and Gly-AsA, showed that their effects on the muscle tonus did not differ significantly from those evoked by the respective total MRPs. Therefore, the concentration of structure(s) activating the smooth muscle tissue remains practically one and the same in both total and high molecular weight fractions. It may be speculated that these melanoidins obtained under conditions that simulate low-moisture baking have regular structure repeating the structure of building blocks comprising the low molecular weight pool.

A third major conclusion from this study is based on the finding that the high molecular pool of MRPs was capable of evoking mechanical reactions of smooth muscles. The Rule of Five, a model developed for prediction of structure–bioavailability relationship forecasts very poor cellular bioavailability for compounds with molecular weight higher than 500 Da (Lipinski et al. 1997). From an evolutionary perspective humans have been used thermally processed food for a relatively short period (Somoza 2005) and a presence of specific transmembrane mechanisms that could allow penetration of MRPs into the cells of gastrointestinal tract is unlikely. Therefore, the melanoidin-caused effects should be limited to interactions with the cellular membranes. One of the most ubiquitous pathways for modulation of smooth muscle excitability is the change of intracellular calcium level (Akbarali 2005). The most plausible hypothesis explaining the contraction/relaxation registered may be an influence of the tested MRPs and melanoidins over the intra- or/and extracellular Ca^{2+} concentration. In fact, a recent study reported a disrupted calcium metabolism in

animals fed with a MRP diet (Delgado-Andrade et al. 2005). This finding may be related to the previously reported ability of MRPs to bind metal ions, including Ca^{2+} (O'Brien and Morrissey 1997). A scenario in which depletion in extracellular calcium as a result of its chelation by melanoidin provokes a subsequent decrease in the intracellular calcium could explain only the relaxation response but not the contractile one. Most likely the products of the Maillard reaction facilitate Ca^{2+} influx into smooth muscle cells via activation of membrane-located receptors, channels or other cellular structures responsible for the Ca^{2+} transport but the exact mechanisms involved in the mechanical reactions and their interplay remain to be studied in detail.

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