Effects of Maillard reaction products from glucose-lysine model systems on oxidative stress markers and against oxidative induction by hydrogen peroxide in Caco-2 cells

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Summary

The ability of Maillard reaction products (MRP) from glucose-lysine (GL) model systems to counteract oxidative stress induced by H_2O_2 was studied in Caco-2 cells, evaluating the effect of heating time. GL mixtures were heated at 100 °C for 15, 60 or 90 min and partly characterized by measuring pH, free lysine and UV-visible spectra. The well-known antioxidant properties of MRP generated were shown in vitro and also ex-vivo, by incubating the Caco-2 cells for 3 h with the isolate samples (1 mg·ml^{-1}). Afterwards, the effects of the combined presence of samples and H_2O_2 (0.5 mmol·l^{-1}) were tested. Changes in cell viability, lipid peroxidation and antioxidant enzymes activity (catalase, superoxide dismutase, glutathione peroxidase) were evaluated. The GL samples did not exert cytotoxic effects, and also significant reductions of the lipid peroxide levels were observed, with a positive influence of heating time. Significant inverse correlations were observed between cellular lipid peroxidation and antioxidant enzyme activities. Incubation of cells with GL mixtures together with H_2O_2 also lowered lipid peroxides and maintained antioxidant enzyme activity levels, although cell damage evoked by H_2O_2 was only partially restored. The ability of MRP to reduce the lipid peroxidation is for the first time shown to be related with their capability to activate the antioxidant enzyme activity.

Keywords

Maillard reaction products; oxidative stress; antioxidant defense; intestinal cells

Oxidative stress appears when the balance between the production of free radicals and antioxidant defense breaks down [1] and it is involved in the development of many degenerative diseases such as cancer, diabetes and cardiovascular diseases, as well as processes related to aging, strenuous physical exercise, etc. Body antioxidant defense systems such as the enzymes catalase (CAT), superoxide dismutase (SOD) or glutathione peroxidase (GPx) and dietary antioxidants such as phenols, flavonoids and vitamins can protect cells from free radical attack. During food processing and storage, natural antioxidants are considerably degraded, but chemical reactions among food components may lead to the formation of secondary antioxidants such as Maillard reaction products (MRP) [2]. These originate from the amino group of an amino acid, peptide or protein and the carbonyl group of a reducing carbohydrate or an oxidized lipid during the Maillard reaction (MR). Interactions between initial compounds and the mechanism for the subsequent generation of different MRP are highly depending on the structure of the direct precursors and the environmental conditions, such as temperature, pH and water content [3]. This reaction occurs frequently in foods during processes such as baking, roasting and frying, and contributes to the aroma, colour and flavour of cooked foods. Thus, considerable amounts of MRP from processed foods are actually consumed on a daily basis [4]. In addition, biological effects have been attributed to MRP, such as decreases in protein digestibility [5], modification of mineral bioavailability [6] and antioxidant activity [7].

The mechanisms by which MRP exert antioxidant activity are not yet fully elucidated; in vitro assays have shown that they may be related to their

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free radical-scavenging activity [8–10] or their ability to chelate pro-oxidant metals such as iron or copper [10–12]. In addition to in vitro studies, it is interesting to study the antioxidant ability of MRP at the cell level, as the activation of cell antioxidant mechanisms is a prerequisite to exert in vivo antioxidant effects. The intestine is the primary site of action for dietary antioxidant agents and this is also where lipid peroxidation is very likely to occur, as it may derive from that present in cell membranes and in dietary fats [13]. Moreover, the formation of lipid hydroperoxides in the intestinal mucosa may also be enhanced by the absorbed oxygen and the presence of metal catalysts that are abundant in foods [14]. Oxidative stress in the intestinal epithelium contributes to colon cancer development and other pathologies such as inflammatory bowel disease and ischemic-reperfusion injury [15]. Therefore, it is important to assess the activity mechanism of antioxidant compounds at the intestinal level and to study whether these compounds protect the intestinal cells from an oxidative insult. In this sense, most studies have been designed considering a pre-treatment with the antioxidant compound followed by an oxidative insult. Such an approach is useful to enhance the effects of the compounds investigated, but not faithfully reproduce the physiologic situation, in which antioxidant compounds and pro-oxidants are simultaneously in the intestinal lumen, interacting with each other and producing cross-effects. To counteract oxidative cellular damage, the intestine has inherent defense mechanisms, mainly mediated by the activity of antioxidant enzymes. Of these enzymes, CAT, SOD and GPx are considered the most important [16].

Among the different cell culture models used to assess antioxidant activity, the Caco-2 cells, which in culture differentiate in mature enterocytes, are an ideal tool to evaluate the cellular antioxidant response to different compounds [17–19]. However, very few data are available on the effects of MRP on oxidative stress markers in the Caco-2 cell line [20, 21], and relationship of parameters such as cellular lipid peroxidation and antioxidant enzyme activity have not been investigated.

The objective of the present work was to assess if the combined presence of MRP from glucose-lysine model systems and a stressor agent, hydrogen peroxide (H_2O_2), in the same way as they are present in the intestinal lumen, is able to eliminate the oxidative stress induced by H_2O_2. With this purpose, changes in cell viability, lipid peroxidation and antioxidant enzymes activity (CAT, SOD and GPx) were evaluated. As heating conditions influence the MR rate and the formation and behaviour of individual MRP [20], the effect of the time of heating the samples was also considered.

MATERIAL AND METHODS

Chemicals

All chemical products and solvents were of the highest available grade and were acquired from Sigma Chemical (St. Louis, Missouri, USA) or Merck (Darmstadt, Germany).

Preparation and characterization of samples

Glucose (G) and lysine (L) were used to prepare the samples. Equimolar mixtures of GL monohydrate, 40% moisture in unbuffered systems, were heated in an oven Selecta 2000210 (JP Selecta, Barcelona, Spain) at 100 °C for 15, 60 and 90 min, using open recipients. After the samples were heated, the reaction was stopped by cooling in an ice bath. The mixtures were removed with spatula and recipients were rinsed with demineralized water (Milli-Q Ultrapure Water System; Millipore, Bedford, Massachusetts, USA) to ensure complete collection. Samples were frozen, lyophilized (TDS-3; FTS System, Stone Ridge, New York, USA), and stored in polyethylene bags at –80 °C until they were used. The samples were characterized by means of weight loss, pH, spectroscopic absorbance measurements and residual free lysine. The samples (50 g) were weighed (AB420; Mettler Toledo, Madrid, Spain) before and after heating to determine the percentage of weight loss. All of the mixtures, unheated (GL0) and heated (GL15, GL60, and GL90), were suspended in demineralized water (1 mg·ml^{-1}), vigorously shaken for 10 min in a vortex, and centrifuged at 3080 ×*g* for 45 min. In these conditions, all of the GL samples were totally soluble. The sample solutions were used for pH measurements (pHM250 ion analyzer MeterLab; Radiometer, Copenhagen, Denmark). Complete absorbance spectra between 200 nm and 700 nm were measured in the samples diluted (1 mg·ml^{-1}) in phosphate-buffered saline (PBS, 50 mmol·l^{-1}, pH 7.4) using a Shimadzu spectrophotometer UV-1700 (TCC-240A; Shimadzu, Duisburg, Germany). The free lysine content in GL mixtures was determined by high performance liquid chromatography (HPLC) according to the Waters Pico Tag method with pre-column derivatization with phenylisothiocyanate using a Waters 2695 separation module (Waters Cromatografía, Madrid, Spain), without the hydrolysis step [22]. A Millennium 32 chromatography manager system (Waters Cromatografía)

was used for gradient control and data processing.

In order to better understand the later ex-vivo effects of samples, their in vitro antioxidant activity was previously tested by measuring the capacity to reduce lipid oxidation in a lipid emulsion (measuring the thiobarbituric acid reactive substances, TBARS), and the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging properties of the samples, as describes below.

In vitro TBARS assay

The inhibitory effect on lipid peroxidation of the samples was measured in a linoleic acid emulsion system, following the procedure described by Wijewickreme and Kitts [23] with some modifications. First, the linoleic acid emulsion was prepared by adding linoleic acid (1.5 g) to a mixture of 0.1 mol·l^{-1} phosphate-buffered saline (PBS), pH 6.8 (200 ml), and Tween 80 (0.4 g). Then, the samples (4 mg) were incubated with 5 ml of linoleic acid emulsion and 5 ml of PBS (0.1 mol·l^{-1} sodium phosphate buffer, 0.15 mol·l^{-1} NaCl, pH 7.4) at 45 °C for 24 h under dark conditions. Following incubation, the solution was diluted (1:1) with 25 mmol·l^{-1} Tris buffer (pH 7.4) containing 0.02% sodium azide, vigorously shaken, and used to measure TBARS. One milliliter of the mixture containing the sample was added to 0.8% thiobarbituric acid (TBA; 1 ml) in test tubes with marble caps and incubated in a water bath (100 °C) for 15 min. To lower the metal-catalysed autoxidation of lipids, butylated hydroxytoluene (BHT; 0.02%) was added to the TBA reagent. After the mixture was cooled, 1.5 ml of *n*-butanol was added and the mixture was shaken vigorously. The samples were centrifuged (15 min, 1 700 ×*g*, 4 °C), and the absorbance of the upper layer was measured at 532 nm using a Shimadzu 1700 UV-visible spectrophotometer. A standard curve prepared from 1,1,3,3-tetraethoxypropane in 1% sulfuric acid was used. Controls without samples were prepared in the same manner. Results were expressed as the percentage of lipid peroxidation inhibition (*LPI*), calculated as

$$LPI = \frac{\left(TBARS_{control} - TBARS_{sample}\right)}{TBARS_{control}} \times 100 \quad (1)$$

In vitro DPPH assay

The DPPH radical-scavenging activity of different samples was estimated according to the method reported by Jing and Kitts [20] with slight modification. One milliliter of the samples dissolved in PBS (0.1 mg·ml^{-1}) was mixed with 1 ml of 0.1 mmol·l^{-1} DPPH in ethanol. The mixture was shaken vigorously and kept at room temperature for 30 min under dark conditions. The absorbance of the resulting solution was measured at 517 nm using a UV-visible spectrophotometer (UV-1700, Shimadzu). The control was prepared in the same manner, using PBS instead of the samples. The capability to scavenge the DPPH free radical of the sample (scavenging activity, *SA*) was expressed as the percentage of disappearance of the initial purple colour and was calculated according to the following equation,

$$SA = \frac{\left(A_{control} - A_{sample}\right)}{A_{control}} \times 100 \quad (2)$$

where A is absorbance.

Cell culture

Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC) through the Cell Bank of Granada University (Spain) at passage 20, and used in experiments at passages 28–32. All cell culture media and cell culture-grade chemicals were obtained from Sigma Chemical. Culture flasks were purchased from Corning Costar (Cambridge, Massachusetts, USA). Cells were grown in 75 cm^2 plastic flasks at a density of cells 5×10^4 cm^{-2} containing high glucose Dulbecco's modified minimal essential medium (DMEM), supplemented with heat-inactivated fetal bovine serum (10%), sodium bicarbonate (3.7 g·l^{-1}), non-essential amino acids (1%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 15 mmol·l^{-1}), bovine insulin (0.1 UI·ml^{-1}) and 1% antibiotic-antimycotic solution (A-5955, Sigma). The cells were maintained at 37 °C in an incubator in a 95:5 atmosphere of air:CO_2 at 90% humidity, and given fresh medium every 2 or 3 days.

Cell treatment

Aimed to determine if the combined presence of MRP and H_2O_2 could palliate the effects promoted by this pro-oxidant in the cells, it was firstly necessary to check the basal antioxidant activity of the isolate MRP in Caco-2 cells, previously appointed by other authors [21]. Thus, the Caco-2 cells were incubated with the GL samples and different oxidative stress markers were analysed (experiment 1). Once the antioxidant ability was measured, another experiment (experiment 2) was conducted to elucidate if such effect was able to counteract the oxidative stress induced by H_2O_2. In this experimental design, the GL mixtures and the pro-oxidant were simultaneously administered to the intestinal cells, trying to mimic the physiological situation after food intake, and the same oxidative stress markers were again analysed. In both experiments, the effects on cell damage, li-

pid peroxidation and antioxidant enzyme activity (CAT, SOD, GPx) were assessed. Cells were trypsinized using trypsin/EDTA (0.25% trypsin, 2 mmol·l^{-1} EDTA-4Na) and seeded in 96-well cell culture plates (for determination of cell damage, see below) and in 25 cm^2 plastic flasks at density of cells 5×10^4 cm^{-2} (for lipid peroxidation and antioxidant enzyme activity assays). The samples and pro-oxidant were dissolved in a test buffer (130 mmol·l^{-1} NaCl, 10 mmol·l^{-1} KCl, 1 mmol·l^{-1} $MgSO_4$, 5 mmol·l^{-1} glucose, and 50 mmol·l^{-1} HEPES, pH 7) before being added to the cells. To study the basal antioxidant effect of GL mixtures, samples at a concentration of 1 mg·ml^{-1}, dissolved in test buffer, were added to the cells and incubated for 3 h (experiment 1). In experiments to assess the ability of assayed MRP to counteract the effects of the stressor agent, cells were incubated with the samples plus H_2O_2 (0.5 mmol·l^{-1}) in test buffer (3 h; experiment 2).

Cells seeded in 25 cm^2 plastic flasks were used for experiments after 7 days in culture. Cell viability prior to experiments was assessed by trypan blue exclusion and it was never < 90–95%. After incubation with samples and/or H_2O_2, cells were harvested by a brief (5 min) trypsinization with trypsin/EDTA and then centrifuged at 20 °C at 100 ×*g* for 5 min (Eppendorf Centrifuge 5810R; Eppendorf, Minnesota, USA). Supernatants were discarded and cell sediments were exposed three times to a freeze (–80 °C, 2 h) and thaw (37 °C, 5 min) cycle to release cytosol [19]. Then they were resuspended in ice-cold PBS and centrifuged at 4 °C at 12300 ×*g* for 10 min. The supernatants were kept at –80 °C for lipid peroxidation determination and antioxidant enzyme activity assays.

Cell damage

Cell damage of human colorectal adenocarcinoma Caco-2 cells was assessed by a neutral red (NR) cytotoxicity assay procedure, based on the ability of viable uninjured cells to actively incorporate NR, a supravital dye, into lysosomes [24]. Cells were seeded in 96-well microtitre plates at a density of 7.5×10^4 cells per well in 100 μl of culture media and maintained overnight under 5% CO_2, at 37 °C in humidified air to allow them to adhere to the wells. Growth medium was removed and test buffer containing samples (GL, 1 mg·ml^{-1}) and/or H_2O_2 (0.5 mmol·l^{-1}) was added to the cells. Control wells received test buffer only. After 3 h of incubation (37 °C, 5% CO_2, 95% humidity), the solutions were aspired and cell viability was assessed by staining with NR (2 h at 37 °C), followed by cell fixation (0.5% formaldehyde, 0.1% $CaCl_2$ for 30 s at room temperature). Microtitre plates were washed by three brief immersions in PBS and the cells were lysed (50% ethanol containing 1% acetic acid overnight at 4 °C). The absorbance of the resulting solutions was measured at 550 nm using a BioRad model 550 microplate reader (BioRad, Hercules, California, USA). Cell viability data were expressed as a percentage referred to control data.

Lipid peroxidation

Lipid peroxidation was assessed by measuring conjugated dienes in cytosol of the cell lysates. Conjugated dienes were quantified according to the method described by Buege and Aust [25] with slight modification. A volume of 0.5 ml of cell cytosol in PBS was mixed thoroughly with 5 ml of chloroform/methanol (2:1) solution, followed by centrifugation at 1000 ×*g* for 15 min until phase separation was achieved. The upper layer was removed by suction, and 3 ml of the lower chloroform layer was transferred to a test tube. Chloroform was removed in a centrifugal vacuum evaporator (Gyrovap; V. A. Howe, Banbury, Oxon, United Kingdom) for 2 h, and the lipid residue was dissolved in 1.5 ml of cyclohexane. The absorbance of the solution at 233 nm was measured (Shimazdu UV-1700 spectrophotometer) against a cyclohexane blank at 233 nm. Conjugated dienes were reported as absorbance at 233 nm.

Catalase assay

Catalase was assayed spectrophotometrically by following the absorbance of H_2O_2 at 240 nm according to the method of Aebi [26] with slight modification. The catalase activity was expressed as units per milligram of protein (U·mg^{-1}), determined by the Lowry method [27].

Superoxide dismutase assay

The procedure of McCord and Fridovich [28] was used to detect SOD activity in cell lysates. In this method, superoxide radicals were generated using a xanthine/xanthine oxidase system. In the presence of superoxide radical, a reduction of cytochrome C occurs, which is monitored at 550 nm. The activity of SOD is expressed as units per milligram of protein (U·mg^{-1}).

Glutathione peroxidase assay

GPx activity was measured according to the method described by Flohé and Günzler [29] with slightly modification. This method is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalysed by GPx, which is then coupled with recycling GSSG back to GSH utilizing glutathione reductase (GR) and reduced

form of nicotinamide adenine dinucleotide phosphate (NADPH). The decrease in NADPH at 340 nm during oxidation of NADPH to nicotinamide adenine dinucleotide phosphate (NADP) is indicative of GPx activity. The activity of GPx is expressed as milliunits per milligram of protein ($mU \cdot mg^{-1}$).

Statistics

One-way analysis of variance (ANOVA) was performed between data from each experiment, followed by Duncan's multiple-range test to compare significant variations between means ($p < 0.05$). The results for GL mixtures were compared with control data (obtained in basal cellular conditions) in experiment 1, or with oxidative data (resulting from incubation with H_2O_2) in experiment 2. In addition, the effect of adding H_2O_2 to cell cultures was also compared to basal conditions by means of one-way analysis of variance. The relationship between the different variables were evaluated by computing the relevant correlation coefficient (Pearson linear correlation) at $p < 0.05$ confidence level. Analyses were performed using Statgraphics Plus, version 5.1, 2001 (Statistical Graphics, Rockville, Maryland, USA).

RESULTS AND DISCUSSION

Characterization of samples

Tab. 1 shows pH values, free lysine and weight loss of the samples. Fig. 1 depicts the absorption spectra (λ = 200–700 nm) of samples. All variables measured confirmed the progression of the MR associated with the time of heating.

In our experimental conditions, the system was not buffered and so MR proceeded without external pH control. The pH value decreased progressively as the time of heating increased, varying from 9.59 (GL0) to 7.65 (GL90). The pH drop with extended heating time has been previously shown in model systems from lactose or glucose-amino acids [9, 10] and glucose, fructose or galactose-proteins [30], being associated with the MR rate in glucose-lysine unbuffered systems [9, 31]. The pH decrease induced by heating has also been observed in foods such as milk [32] and coffee [33]. The decline may be attributed to the formation of organic acids [34] and the decrease of basic lysine-amino groups, as a correlation between pH values and free lysine was observed in the present assay ($r = 0.81, p = 0.0014$).

It is known that in many foods, the ε-amino groups of lysine are the most important source of reactive amino groups. Due to the blockage in Amadori products, this lysine is not available for digestion, and therefore its nutritive value is decreased. Thus, determining free residual lysine is a useful method to assess the progress of MR. A decrease in the residual free lysine percentage

Tab. 1. Characterization of glucose-lysine mixtures.

Sample	pH	Free lysine [%]	Weight loss [%]
GL0	9.59 ± 0.03 [a]	44.8 ± 0.84 [a]	–
GL15	9.34 ± 0.01 [b]	24.6 ± 0.21 [b]	1.68 ± 0.05 [a]
GL60	8.77 ± 0.04 [c]	12.9 ± 0.18 [c]	12.93 ± 0.06 [b]
GL90	7.65 ± 0.01 [d]	11.2 ± 0.08 [d]	20.96 ± 0.07 [c]

GL0 – unheated mixture, GL15, GL60, GL90 – mixtures heated at 100 °C for 15, 60 and 90 min.
Values are mean ± standard deviation, (n = 3).
Different letters in the same column indicate significant differences (one way ANOVA followed by Duncan's test; $p < 0.05$).

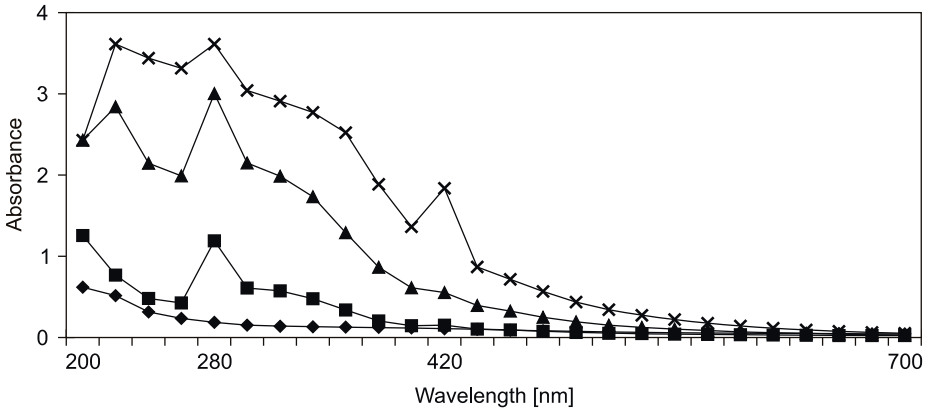

Fig. 1. Absorption spectra (λ = 200–700 nm) of glucose-lysine mixtures.

GL0 – unheated mixture, GL15, GL60, GL90 – mixtures heated at 100 °C for 15, 60 and 90 min, respectively.

associated with heat treatment was observed in the samples, being quantitatively more important in the early stages of heating (15 min).

Concerning the weight loss of samples, it increased in parallel with the heating time, significantly in all cases. Only 1.68% of weight was lost at the first stage of heating, but up to 21% was lost after heating for 90 min, due to a significant water loss and the formation of volatile compounds. A similar behaviour was observed in previous studies of our research group working with the same model system heated at similar temperatures [35].

The full range absorbance spectra (λ = 200–700 nm) showed a specific peak at 280 nm for the three heated mixtures, whereas only the GL90 sample displayed a significant peak at 420 nm. In this sense at initial stages of MR, glucose reacts with amino acid forming colourless compounds that do not absorb visible light [36]. Therefore, measurement of absorbance in the range 280–294 nm is associated with the early stage of the reaction and with low-molecular-weight complexes [20]. The spectral pattern found for the GL mixtures showed that higher amounts of early MRP were progressively formed as the time of heating of the samples was extended, reaching maximum values for GL60 and GL90. Coloured compounds, formed at later stages of MR and measured by absorbance at 420 nm [9, 20], were in ascending order of GL0 < GL15 < GL30 < GL90 samples, but without reaching 280 nm values in any case. Different authors have demonstrated that the possible compounds that are contained in the brown products include furans, pyrrols, pyridines and pyrazines [20]. On the other hand, JING and KITTS [20] were able to separate the HMW-MRP fraction present in a GL model system heated at 121 °C 60 min and, based on the carbon and nitrogen content, they concluded that a great extent of polymerization occurred in the isolated fraction. Unfortunately, they were unsuccessful to ascertain its molecular mass.

Thus, the experimental conditions used in the present assay seem to have promoted the formation of the early MRP in higher proportions than that of the more advanced ones, which is in accordance with the findings of other authors who assayed MRP model systems at similar temperatures [20, 32].

The mentioned study of JING and KITTS [20] evidenced the suitability of fluorescence and absorbance spectra to unspecifically characterize MRP. Although the fluorescence spectra were not measured in the present work, based on results of JING and KITTS [19] for the same GL model system, it can be hypothesized that fluorescence intensity would decrease in our samples as heating time progressed, supporting a parallel increase in browning intensity. This statement is also based on the well-established fact that fluorescence products are usually precursors of the brown pigment [37].

In vitro antioxidant activity

The purpose of the trails was to evidence, before the ex vivo assays, the existence of in vitro antioxidant activity of the samples (Tab. 2). In line with studies of WIJEWICKREME and KITTS [23], the GL60 and GL90 samples of the present assay exhibited antioxidant activity as assessed by the TBARS method, confirming that the presence of advanced and higher-molecular-weight MRP contribute to slowing down lipid oxidation [38]. The level of activity appeared to parallel the browning or roasting degree, as have been previously established in the presence of model melanoidins [38], melanoidins isolated from beer and sweet wine [39] and coffee brews [40]. Antioxidant properties of foods like ginger cakes have also been associated with the presence of intermediary and final MRP compounds, discarding the antioxidant power of early MRP [41]. It has been suggested that the effectiveness of MRP in reducing the propagation of lipid autoxidation reactions could signify an ability to scavenge free radicals [42]. However in our experimental conditions, the capacity of GL mixtures to reduce the stable radical DPPH increased drastically at the beginning of heating (15 min), with the lower-molecular-weight MRP formed, but dropped significantly at prolonged heating. Therefore, browning may not be a good marker to evaluate the free-radical-scavenging activity of MRP formed, in agreement

Tab. 2. Lipid peroxidation inhibition and DPPH-scavenging activity of glucose-lysine mixtures.

Sample	Lipid peroxidation inhibition [%]	DPPH-scavenging activity [%]
GL0	16.80 ± 3.12[a]	1.64 ± 0.37[a]
GL15	7.74 ± 0.38[a]	48.38 ± 9.01[b]
GL60	58.88 ± 4.14[b]	27.23 ± 0.22[c]
GL90	71.26 ± 6.77[b]	25.17 ± 0.66[c]

GL0 – unheated mixture, GL15, GL60, GL90 – mixtures heated at 100 °C for 15, 60 and 90 min, respectively.
Values are mean ± standard deviation (n = 4).
Different letters in the same column indicate significant differences (one way ANOVA followed by Duncan's test; $p < 0.05$).
Lipid peroxidation inhibition was calculated using eq. 1.
DPPH-scavenging activity was calculated using eq. 2.

with some authors [9] but in disagreement with others [30]. Our results agree with those of ANESE et al. [43], who suggest that the highest scavenging properties of glucose-glycine heated mixtures are located at the first stages of MR. They agree also with those of YILMAZ and TOLEDO [44], who stated that heating glucose-histidine mixtures at high temperatures over long periods degrades the antioxidant MRP formed in the early stages of the reaction. Thus, some authors have pointed out that compounds thermally induced by severe temperature and/or time conditions may contribute to colour development but not to antioxidant activity [45]. On the other hand, it seems that the amino acid could play an important role in the final antiradical activity of the compounds [46], and previous assays showed the ability of lysine to scavenge free radicals [10]. Therefore, the disappearance of free lysine with increased heating time (Tab. 1) could have contributed to the reduced antiradical properties of the samples This hypothesis is supported by the significant correlation between levels of free lysine and antiradical activity of the samples ($r = 0.6716, p = 0.0168$).

Cell culture oxidative markers

Cell damage

As a starting point, the cytotoxic effects of the isolate MRP were tested. Thus, it was pointed out that incubating Caco-2 cells with GL mixtures for 3 h at a concentration of 1 mg·ml^{-1} did not induce significant cell damage compared to basal conditions (control), as measured by the neutral red cell viability procedure (Fig. 2; experiment 1). The heating time of samples did not affect the percentage of cell viability. Cytotoxic effects of some MRP have been reported by some authors using different cell cultures, such as mouse C6 glioma cells [47], human lymphocytes [48] or intestinal embryonic cells [19]. However, studies carried out using Caco-2 cells show no effect of MRP on cell growth inhibition [10, 19], except after 24 h of incubation time and testing high-molecular-weight fractions [21]. Thus, different cell models respond differently to MRP and the Caco-2 cell line seems to be less sensitive than other cells to xenobiotics, according to authors who tested glucosinolate hydrolysis products in HT-29 and Caco-2 cells [49].

The treatment of cells with 0.5 mmol·l^{-1} H_2O_2 reduced cell viability to 30.51% ± 1.81%, clearly showing the harmful effect of peroxide (Fig. 2; experiment 2). H_2O_2 has been widely used as an in vitro oxidative stress-inductor, both to examine the cellular response [50] and to study the antioxidant properties of different compounds [51, 52]. Bibliographic data on the cytotoxic effect of H_2O_2 are disparate and depend greatly on the cell line and the experimental conditions used. Some authors did not find changes in Caco-2 cell viability after treatment with 0.25 mmol·l^{-1} H_2O_2 for 30 min [53] or with 0.5 mmol·l^{-1} H_2O_2 for 1 h [52], but lactate dehydrogenase (LDH) release, DNA damage, reactive oxygen species (ROS) intracellular accumulation or mitochondrial disturbance [52, 54] were observed. However, longer incubation periods (3 h) evoked a significant cytotoxic effect with cell viability loss, both at 0.25 mmol·l^{-1} H_2O_2 [20] and at 10 mmol·l^{-1} [17], which is in accordance with the results of the present assay. RAO et al. showed that the administration of 0.1–5.0 mmol·l^{-1} H_2O_2 to Caco-2 cells increased the cellular permeability in a time- and concentration-dependent manner during up to 150 min of incubation, by a tyrosine kinase-related mechanism [51].

The presence of GL samples in test buffer together with the pro-oxidant partially counteracted the cytotoxic damage of H_2O_2, with cell viability recovering significantly for the GL0 and GL15 samples (Fig. 2; experiment 2). Cell viability was not totally restored in any case in our experimental conditions; to do this, higher doses or longer incubation periods were probably required. The protective activity of MRP against stressed intestinal cells has been scarcely studied. JING and KITTS [20] found that glucose, fructose and ribose-lysine

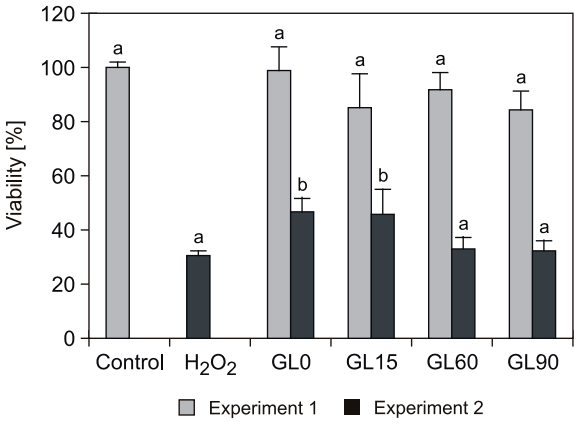

Fig. 2. Cell viability in Caco-2 cell cultures treated with GL mixtures.

Experiment 1 – treatment of cultures with GL mixtures in basal conditions. Experiment 2 – treatment of cultures with GL mixtures and 0.5 mmol·l^{-1} H_2O_2.
Values are means ± standard deviation, $n = 8$.
Different letters indicate significant differences between samples within each experiment (one way ANOVA followed by Duncan's test; $p < 0.05$).

model MRP exerted a marked protection against H_2O_2-induced toxicity in Caco-2 cells, in particular when high-molecular-weight fraction were assayed. In the same line, GOYA et al. attributed to the high-molecular-weight compounds in coffee, melanoidins, the preventive effect on cell damage evoked by *tert*-butylhydroperoxide in a human hepatoma cell model [54]. However, the present assay revealed a slight but significant recovery of cell viability in the presence of the less browned samples (15 min of heating), in line with other authors who reported higher antioxidant activities for MRP formed at the first stages of the reaction [43, 45]. The positive effect of the unheated sample (GL0) in restoring oxidative cell damage could be attributed to free lysine, which has been proven to exhibit antioxidant activity [10].

Lipid peroxidation

The presence of GL mixtures in the culture medium markedly reduced lipid peroxide Caco-2 levels, both in isolate form and in the combined presence with H_2O_2 (Fig. 3; experiments 1 and 2). In the case of the isolate presence of GL mixtures, the effect was greater in GL15 samples, pointing to the lower-molecular-weight compounds as possibly responsible for the effects, since in this sample high-molecular-weight MRP were barely present, based on the absorption spectra (Fig. 1). To the best of our knowledge, this is the first study reporting on the ability of MRP to counteract the effects of H_2O_2 on lipid peroxidation in a cell line

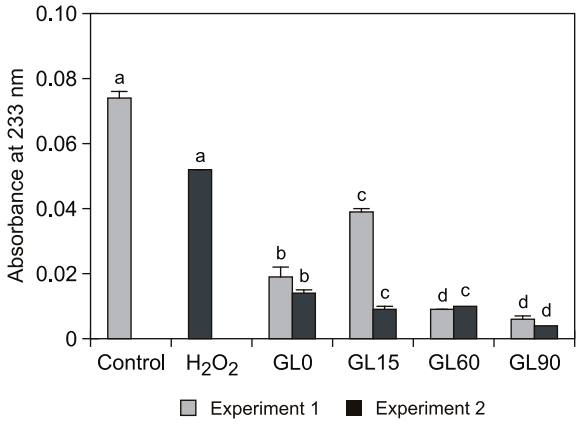

Fig. 3. Concentration of conjugated dienes in Caco-2 cell cultures, expressed as absorbance at 233 nm.

Experiment 1 – treatment of cultures with GL mixtures in basal conditions. Experiment 2 – treatment of cultures with GL mixtures and 0.5 mmol·l^{-1} H_2O_2.
Values are means ± standard deviation, $n = 3$.
Different letters indicate significant differences between samples within each experiment (one way ANOVA followed by Duncan's test; $p < 0.05$).

of intestinal origin, although many studies demonstrated that they can reduce lipid peroxide levels in vitro [10, 39]. Assays performed in cell cultures of hepatic origin have also shown the capacity of certain MRP to lower induced lipid peroxidation [54] but in this case, the experimental design considered a pre-treatment of cells with MRP to later expose them to an oxidative insult. In this sense, the results found in the present assay were obtained in a more physiologic situation, when MRP and the pro-oxidant were supplied simultaneously to the cells.

The relationship between browning and antioxidant activity has been studied in vitro by several authors. Most of them agree that the ability to reduce lipid peroxidation increases with colour when MR is the prevalent event during processing [39]. Our study also reveals a greater capacity to reduce cellular peroxidation in the most browned samples. The antioxidant activity of melanoidins seems to depend on the ability to donate hydrogen atoms of the hydroxyl group and to neutralize the free radical formed during lipid oxidation; termination would take place by a coupling reaction of the melanoidin radical with another lipid radical [39]. This mechanism has also been proposed for other antioxidants such as phenolic diterpenes of rosemary leaves [18] and soya isoflavones [55]. The above mentioned compounds have shown an ability to reduce lipid peroxidation in Caco-2 cells but, unlike the samples of the present study, only in stressed conditions.

Antioxidant enzymes activity

In addition to radical-scavenging properties, lipid hydroperoxide decomposition may also be influenced by alterations in antioxidant enzyme activities. CAT, SOD and GPx activities were tested in Caco-2 cells in culture after treatment with the different GL samples and/or H_2O_2 (Fig. 4). Incubating Caco-2 cell cultures with the isolate GL samples for 3 h induced a general increase in CAT, SOD and GPx activities (experiment 1), with a more intense effect with the most heated samples (GL60 and GL90). For this reason, it seems logical to attribute to the higher molecular weight MRP the higher responsibility in this action.

The effects of MRP on antioxidant enzymes at the intestinal level have been scarcely investigated. The few studies performed in Caco-2 cells have shown that melanoidins isolated from roasted malt increase the cytochrome c reductase and gluthatione S-transferase activities [56], and that glucose, fructose or ribose-casein mixtures have no impact on CAT, SOD and GPx enzyme activity [19]. SOMOZA et al. [7] reported an increase in the

activity of chemopreventive enzymes in the liver and kidney of rats fed diets containing malt, bread crust or pronylated albumin. Our results show that GL mixtures supplementation of Caco-2 cells led to increased CAT, SOD and GPx activities compared to control cultures, to a greater extent as the heating time of samples increased. Moreover, negative correlations between conjugated diene levels and the three antioxidant enzymes were found (r = –0.76, p = 0.0102 for CAT; r = –0.89, p = 0.0004 for SOD; r = –0.86, p = 0.0012 for GPx). Therefore according to our results, the ability of the tested samples to reduce cellular lipid peroxidation in unstressed cells was directly related to the improvement in the activity of antioxidant enzymes. In other words, one of the mechanisms underlying lipid hydroperoxide reduction by GL mixtures seems to be by increasing the antioxidant enzyme activity levels. As mentioned above, the cellular antioxidant enzyme system plays an essential role in the defense against oxidative stress and in maintaining the cellular redox status required for normal cell functions. SOD is responsible for catalysing dismutation of superoxide radical in living tissues, whereas CAT removes H_2O_2 and GPx eliminates H_2O_2 as well as other organic peroxides [1]. Under physiological conditions, the most important enzyme for the removal of lipid hydroperoxides seems to be GPx [18].

Treatment of Caco-2 cells with peroxide led to increased levels of SOD and GPx activities, whereas the CAT level was unaffected with respect to control cultures (Fig. 4; experiment 2). Similar results were observed by other authors using H_2O_2 as an insult in the Caco-2 cell line [52,53] and *tert*-buthyl hydroperoxide in hepatoma HepG2 cells [50]. The increase in antioxidant enzyme activities after treatment with H_2O_2 clearly indicated a positive response of cell defenses to an oxidative insult, manifested by the increased activity of enzymes implicated in oxygen detoxification [57].

However, supplementing cells with the combined mixture of GL samples and H_2O_2 did not modify the enzyme activity observed in the presence of H_2O_2 (except in the GL15 sample for CAT and SOD compared to H_2O_2 values), the values remaining in similar ranges. The lower CAT values observed in the presence of H_2O_2 may be explained by the very low level of CAT activity in Caco-2 cells, significantly lower than in colonic epithelial cells [58]. Thus, CAT is probably the first enzyme to be "consumed" in stressed conditions. No previous studies have shown the effect of MRP on the antioxidant defense response to an oxidative insult in intestinal cells, but it has been observed that coffee melanoidins prevent the increased activity of GPx and glutation reductase induced by *tert*-buthyl hydroperoxide in hepatoma cells [54]. Our study shows that SOD and GPx activities in Caco-2 cells co-treated with GL mixtures

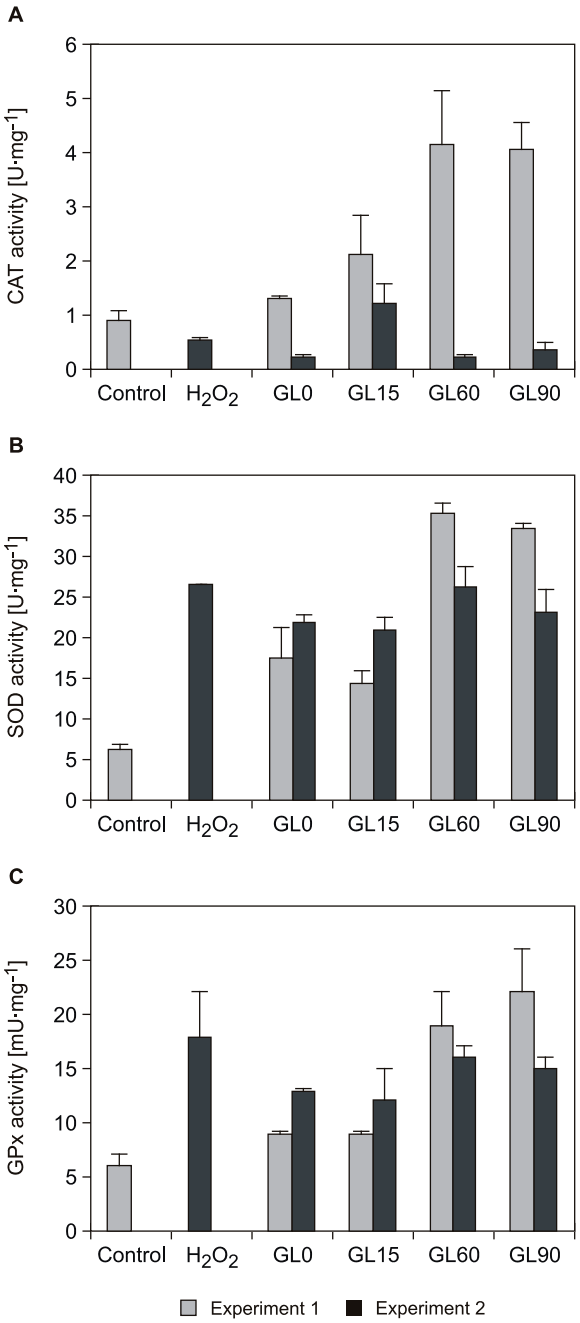

Fig. 4. Activities of CAT, SOD and GPx in Caco-2 cell cultures.

Experiment 1 – treatment of cultures with GL mixtures in basal conditions. Experiment 2 – treatment of cultures with GL mixtures and 0.5 mmol·l^{-1} H_2O_2.
Values are means ± standard deviation, n = 3.
Different letters indicate significant differences between samples within each experiment (one way ANOVA followed by Duncan's test; $p < 0.05$).

and H_2O_2, in the same manner as it occurs in the intestinal lumen, remain at values similar to those reached with H_2O_2. A parallel decrease in cell lipid peroxides was observed with the combined presence of MRP and the pro-oxidant, although no significant correlations between enzymatic activities and conjugated dienes were found.

CONCLUSION

Incubation of Caco-2 cells with GL samples together with H_2O_2 lowered lipid peroxides and maintained antioxidant enzyme activity levels, suggesting that MRP could be able to counteract, to some extent, the detrimental effect of the stressor agent on enterocytes. The compounds responsible for this effect were mainly the initial and advanced MRP, rather than the final MRP. However, the cell damage evoked by H_2O_2 was only partially restored with the combined presence of GL samples and the peroxide. On the other hand, the ability of these products to reduce lipid peroxidation and the positive correlation with the increase of the antioxidant enzymes activities was reported for the first time.

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