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Effect of Maillard reaction conditions on browning and antiradical activity of sugar–tuna stomach hydrolysate model system

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Abstract

The antiradical activity of Maillard reaction products (MRPs) made from sugar–tuna stomach hydrolysate model system was tested. The antiradical activity of the MRPs derived from ribose was 11-fold higher than that of MRPs derived from glucose due to the acyclic form of the ribose. The activity reached the plateau at a 30 mg/mL ribose concentration. The ribose caramelization contributed to the antiradical activity and browning reactions at 95 °C and 115 °C. The increase in DPPH$^{\bullet}$ radical scavenging of MRPs is attributed not only to the temperature but also to the buffer type and buffer concentration. Phosphate buffer showed the most efficient compared to citrate or Tris–HCl buffers. A positive correlation ($R^2 = 0.98$) was observed between the antiradical activity, the browning and the phosphate concentration. The MRPs obtained under these mild experimental conditions exhibited no toxicity towards Vero cells and 3T3 cells, despite their high antiradical activity.

Keywords: Maillard reaction; Protein hydrolysate; Ribose; Antiradical activity; DPPH$^{\bullet}$; Browning; Phosphate buffer; Temperature

1. Introduction

The Maillard reaction (MR), which links the carbonyl group of reducing carbohydrates and the amino group of free amino acids as well as of lysyl residues in proteins, may have either beneficial or detrimental effects (Ajandouz, Tchiakpe, Ore, Benajiba, & Puigserver, 2001). This reaction is classified as non-enzymatic browning reactions and has been associated with the formation of compounds with strong radical scavenging activity. The Maillard reaction takes place in three major stages:

- At an early stage of the reaction, the free amino groups of proteins such as the ε-NH_2 groups of lysine, react with carbonyl groups of sugar to form a reversible Schiff base, which rearranges to stable, covalently bonded Amadori products (Jing & Kitts, 2002; Rizzi, 1994). The radical scavenging activity derived from the uncolored reaction products is smaller than the brightly colored pigments (Murakami et al., 2002).
- At intermediate stages, highly-UV-absorbing and colorless compounds are continually formed. In the advanced phase of the reaction, Amadori products undergo further transformation to fluorescent, colored substances and cross-linked polymers (Ames, 1990; Morales, Romero, & Jimenez-Perez, 1996; Van Boekel, 1998; Van Boekel, 2001).
- Formation of melanoidins and heterocycles compounds in the advanced stage of the Maillard reaction could explain the ability of glycated hydrolysate to react with radical compounds (Friedman, 1996).

Thus, the Maillard reaction is a complex reaction, since it is influenced by many factors such as temperature, pH, time, water activity, type and concentration of buffer, reactant source and sugar involved (Ames, 1990; Wijewickreme, Krejpcio, & Kitts, 1999). Changing

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any of these factors will alter reaction rate, reaction pathways and reaction end-products. Little information is available on the chemical structure of the hundreds of unknown products which are formed by a series of consecutive and parallel reactions including oxidations, reductions, and aldol condensations among others (Eriksson, 1981). In addition, since the majority of studies was carried out on model systems, little is known about sugar–protein hydrolysate systems. Chevalier, Chobert, Genot, and Haertle (2001) reported that the radical scavenging activity of glycated β-lactoglobulin and its tryptic and peptic hydrolysates depended on the sugar used for the modification. Guerard and Sumaya-Martinez (2003) showed that the antiradical scavenging effect was improved by 75% when protein hydrolysates from casein or fish were incubated in the presence of glucose. Although certain protein hydrolysates had been reported to be antioxidative per se, the effect was considerably improved when reacting the hydrolysates with glucose (Lingnert & Eriksson, 1981).

Finally, the MR is a cascade of consecutive and parallel reaction steps and has been shown to produce some desirable components such as antioxidative molecules, other undesirable compounds such as mutagenic, DNA-damaging, and cytotoxic substances. Brands, Alink, Boekel, and Jongen (2000) demonstrated that ketose sugars (fructose and tagatose) exhibited a remarkably higher mutagenicity compared with their aldose isomers (glucose and galactose), which was due to a difference in reaction mechanism. The cytotoxic effects of MRP have been studied in different model systems and are associated with the use of high temperatures (Jing & Kitts, 2002).The degree of browning is often used analytically to assess the extent to which the MR has taken place. Simple positive or complex correlation between color and antioxidant properties have been found depending on the composition and technological history of the product (Manzocco, Calligaris, Mastrocola, Nicoli, & Lerici, 2001).

The present investigation was undertaken to study the effect of experimental conditions (sugar type and concentration, buffer type and temperature incubation) on the development of Maillard reaction, the degree of browning and the formation of the antiradical compounds. The objective was to maximize the antiradical activity without the production of the cytotoxic effect related to MRPs. The contribution of the caramelization of the reducing sugar in the development of antiradical activity was also evaluated.

2. Materials and methods

2.1. Materials

D-Glucose, D-ribose and 1,1-diphenyl-2-picryl-hydrazyl (DPPH·), citrate buffer salts, TRIS buffer salts and phosphate buffer salts were purchased from Sigma/Aldrich (St Louis, MO, USA). All reagents were of analytical grade.

2.2. Preparation of the tuna stomach hydrolysate

The tuna (*Thunnus Albacares*) stomachs were taken from frozen fish and heat inactivation of endogenous stomach enzymes (100 °C, 20 min) was carried out prior pH adjustment and addition of Alcalase®2,4L (Novo Nordisk). Hydrolysis experiments were performed in a batch reactor using the pH-stat method in controlled conditions (pH, temperature and stirring speed) as follows: The grinded stomachs (25 kg) were mixed with an equal amount of water and temperature of the mixture was adjusted to 50 °C. The enzyme concentration was fixed to 4% (w/w protein) and pH was kept constant (pH 8) by addition of a 5 N NaOH solution. The reaction was stopped after 15 min by heating treatment at 85 °C. The hydrolysis degree was calculated from the amount of base consumed according to Alder-Nissen (1982) and was stated to be 18% after 4 h hydrolysis. The DPPH scavenging activity of native hydrolysate was 72 ± 5 μmol equiv TROLOX/L.

2.3. Preparation of MRPs from sugar–tuna stomach hydrolysate model system

Glucose, fructose and tuna stomach hydrolysate (5 mg/mL of protein according to Lowry assay) were used to prepare different model aqueous solutions: A mixture of sugar and tuna stomach hydrolysate was heated for 17 h in pyrex tubes (15-mL) at different temperatures ranging from 35 to 115 °C. The assay conditions were: sugar concentration (ranging from 5 to 90 mg/mL), buffer type (Tris–HCl, citrate and phosphate), buffer concentration (ranging from 0 to 0.5 M) and pH (ranging from 4 to 12).

The DPPH· scavenging activity was evaluated by triplicate after appropriate dilution of heated solutions.

2.4. Measurement of browning

Browning of the samples was evaluated by reading the absorbance at 420 nm on a Hitachi U-2000 UV–vis spectrophotometer using a 1 cm path length cell after appropriate dilution.

2.5. Measurement of free radical scavenging activity

The antiradical activity of MRPs was evaluated according to the procedure reported by Morales and Jimenez-Perez (2001), which was sightly modified. An aliquot of sample (200 μL) was added to 1 mL of a daily-prepared solution of 1,1-diphenyl-2-picryl-hydrazyl (DPPH·) in ethanol (74 mg/L). The mixture was shaken

vigorously for 1 h at 25 °C. The sample was centrifuged at 10 000 × *g* for 5 min, then the absorption of the supernatant was measured at 520 nm. The DPPH· concentration in the reaction medium was calculated from the following calibration curve, determined by linear regression: $[DPPH^{\cdot}]_t = 0.0241\ (A_{520nm}) + 0.022\ (r^2 = 0.9995)$. For all experiments, osmosed water instead of sample solution was used as blank. The antiradical activity (AA) of the sample was expressed as percentage disappearance of DPPH·, $AA(\%) = (100 - ([DPPH^{\bullet}]_t/[DPPH^{\bullet}]_{H_2O}) * 100)$ where $[DPPH^{\bullet}]_{H_2O}$ is the concentration of DPPH· in the presence of water instead of MRPs. Trolox was used as the control.

2.6. Cytotoxicity assays

Cells: African green monkey (*Cercopithecus aethiops*) kidney cells (Vero cell line no ATCC CCL-81) and Swiss albino mouse (*Mus musculus*) fibroblast cells (3T3-Swiss albino cell line no ATCC CCL-92) were respectively grown in RPMI 1640 and in DMEM (BioWest, Nuaille France) containing 100 UI/m, Penicillin, 100 μg/mL Streptomycin and 2 mM L-Glutamine (BioWest, Nouaille France), supplemented with 10% fetal calf serum (FCS). Cells were grown at 37 °C under 5% CO_2 and routinely passed before they reached confluence every 3 days. Cytotoxicity of MRPs was assessed using determination of cell viability performed by neutral red dye method as previously described by McLaren, Ellis, and Hunter (1983) and Langlois, Allard, Nugier, and Aymard (1986). The IC50 of the tested MRPs was defined as the concentration that reduced the absorbance to 50% of that of controls.

3. Results and discussion

3.1. Effect of sugar concentration on DPPH· scavenging activity and browning

In this study, glucose, an aldose sugar and ribose, an aldopentose sugar, were selected as sugar reactants due to differences in the reaction rates associated with their chemical structure. Generally, pentoses are more reactive than hexoses in the sugar–amino-acid model. The relationship between radical scavenging activity, browning intensity and sugar concentrations is shown in Fig. 1. The antiradical activity of the MRPs derived from ribose was 11-fold higher than that of MRPs derived from glucose. The activity increased with increasing concentration and reached the plateau at a 30 mg/mL ribose concentration. The high ribose reactivity could be related to the acyclic form of the ribose, thus making this sugar more reactive than glucose. The rate of the reaction depends on the rate at which the sugar ring opens to the reducible, open-chained form (Davies, Kaanane, Labuza, Moskowitz, & Guillaume, 1998). The progress of the browning was followed by monitoring absorbance at 420 nm. The brown color development is largely due to the formation of chromophores which have been widely studied in different model systems, and studies on melanoidin formation have been summarized in different review articles (Friedman, 1996; Rizzi, 1997). However, Murakami et al. (2002) showed that DPPH· radical scavenging activity was found in the early stages of the Maillard reaction, and the scavenging activity appeared before the formation of the pigments.

The development of MRP color was maximum at a 50 mg/mL ribose concentration. A correlation between antiradical activity and browning for both ribose and glucose hydrolysates was observed ($R^2 = 0.86$ and 0.92, respectively). These results confirmed that the pentose sugar, ribose, is more reactive than hexoses, such as glucose, and are in agreement with the data reported on a casein–sugar system (Jing & Kitts, 2002). Due to its high reactivity and to its positive effect on the enhancing of antiradical activity, the ribose has been used for the further experiments at a 30 mg/mL concentration.

3.2. Effect of buffer type and concentration on DPPH· scavenging activity and browning

The DPPH· scavenging activity and the browning were five and three times higher, respectively, when phosphate buffer was used, compared to Tris–HCl buffer and about two times higher compared to that of citrate buffer (Fig. 2). The catalytic effect of phosphate in Maillard reaction was in agreement with previous studies in model systems (Burton & McWeeny, 1963; Potman & Van Wijk, 1989). It was suggested that phosphate may act as a bifunctional catalyst for the nucleophilic reaction of the amine with the carbonyl due to the ability of the small phosphate anion to simultaneously donate and accept the proton necessary for the conversion (Bell, 1997; Watkins et al., 1987). Phosphate catalysis of hydrolysate glycation was concentration-dependent as shown in Fig. 3. At 0.5 M phosphate buffer concentration, antiradical activity was 10 times higher and the browning was 16 times higher than those of the unbuffered system. A positive correlation ($R^2 = 0.98$) between the antiradical activity, the browning and the phosphate concentration, was observed. Rizzi (2004) suggested that phosphates may be useful for enhancing browning during food processing.

In addition to the buffer effect, it is well-known that Maillard reaction is greatly influenced by the pH of the system. When the pH is not controlled, it falls throughout the reaction, giving a different profile of reaction products compared to the analogous system reacted at constant pH (Monti et al., 1999). Low pH values (⩽7) favour the formation of furfurals (when pentoses are involved) or hydroxymethylfurfural

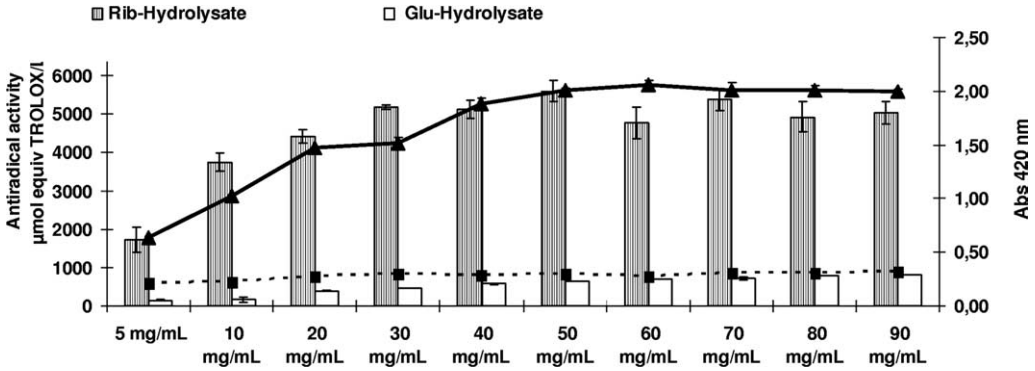

Fig. 1. Effect of sugar concentration ranging from 5 to 90 mg/mL on DPPH· scavenging activity and browning (as measured by absorbance at 420 nm). Reaction conditions: 55 °C, 17 h, pH 7, 0.05 M phosphate buffer. "Rib–Hydrolysate": ribose–hydrolysate mixture; vertical bar: antiradical activity in µmol equivalent TROLOX/L; full line: measurement of browning. "Glu–Hydrolysate": glucose–hydrolysate mixture; vertical bar: antiradical activity in µmol equivalent TROLOX/L; dotted line: measurement of browning.

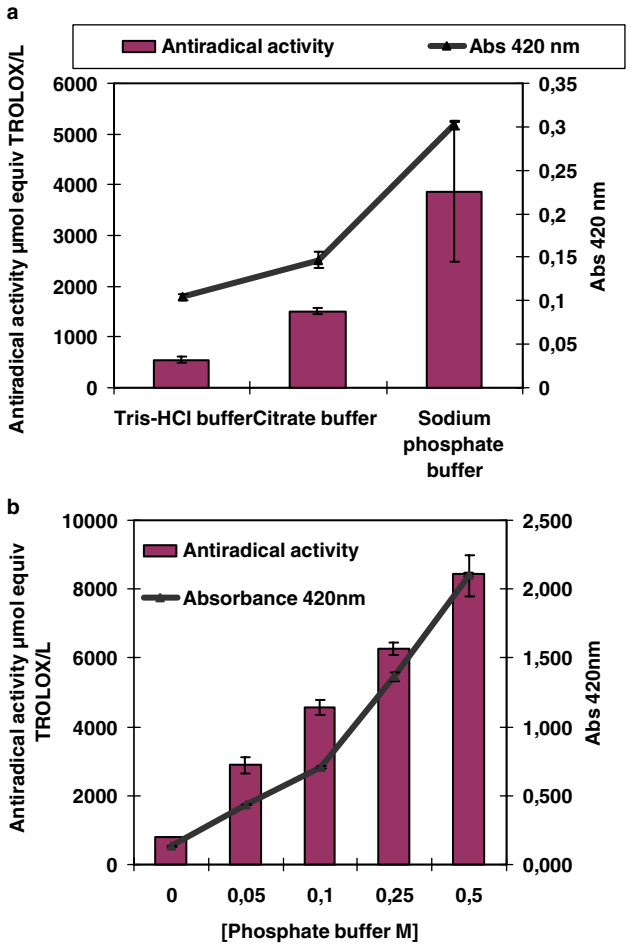

Fig. 2. Effect of buffer type and concentration on DPPH· scavenging activity and browning (as measured by absorbance at 420 nm). (a) Buffer concentration: 0.05 M, pH 7. Sample dilution for evaluation of browning:1/3. (b) Phosphate buffer concentration ranging from 0 to 0.5 M. Heating conditions for both experiments: 55 °C, 17 h, 30 mg/mL ribose, pH 7.

(HMF) (when hexoses are involved) from Amadori rearrangement products, while the formation of reductones (such as 4-hydroxy-5-methyl-2,3-dihydroxyfuran-3-one or HMFone) and a variety of fission products are preferred at a high pH (>7) (Hodge, 1953; Martins, Jongen, & Boekel, 2001).

3.3. Effect of temperature on DPPH· scavenging activity and browning

The influence of temperature on the Maillard reaction of the hydrolysate incubated in the presence of ribose ("rib–hydrolysate") is presented in Fig. 3. As expected, the incubation temperature showed a high correlation ($R^2 = 0.96$) with the increase of antiradical activity and browning. The DPPH· scavenging activity was increased by 400% when temperature rose from 55 to 95 °C. Manzocco et al. (2001) reported that color changes due to Maillard reaction are always associated with the formation of heat-induced antioxidants and a positive correlation between browning and antioxidant activity was identified in all the model systems analyzed. The linear correlation (R^2) varied from 0.81 to 0.99. Apart from Maillard reaction, caramelization also takes place simultaneously. According to Ajandouz et al. (2001), caramelization of fructose in a fructose–lysine model system accounted for more than 40% of total UV-absorbance and 10–36% of brown color development. From a practical point of view, this may therefore lead to overestimating the Maillard reaction in foods. In Fig. 3, the contribution of ribose caramelization to the antiradical activity and browning reactions is assessed at 95 °C and 115 °C. Based on the absorbance at 420 nm of the heated solutions, the caramelization reactions of ribose were found to account for 31% and 35% of the brown products of ribose–hydrolysate mixture. When ribose was heated alone at initial pH values ranging from 4.0 at 8.0, no antiradical activity, browning development and UV-absorbance were noticed (data not shown).

The DPPH-scavenging activity of MRPs from the ribose–tuna stomach hydrolysate model systems heated at

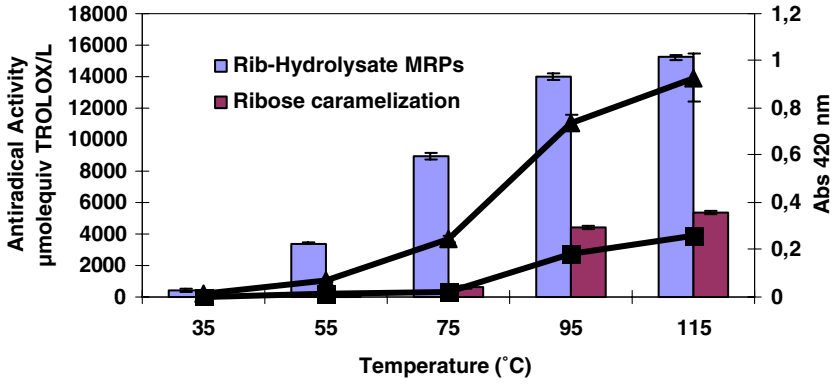

Fig. 3. Effect of temperature on DPPH· scavenging activity and browning development (as measured by absorbance at 420 nm) of a ribose–tuna stomach hydrolysate solution. Reaction conditions: temperature ranging from 35 to 115 °C, 17 h, 30 mg/mL ribose. Sodium phosphate buffer 0.05 M, pH 7. An aqueous ribose solution was separately heated alone under the same experimental conditions. Sample dilution for evaluation of browning: 1/25. Full line: measurement of browning.

Table 1
DPPH· scavenging activity of MRPs in rib–tuna stomach hydrolysate model system as affected by heating temperature and results of cytotoxic evaluation

Conditions of Maillard reaction	Antiradical activity (µmol equiv TROLOX/L)	IC50		
		3T3 cells	DIET cells	VERO cells
Not heated	436	>0.750 mg/mL	ND*	ND*
55 °C	8430	>1 mg/mL	ND*	>1 mg/mL
75 °C	14846	>1 mg/mL	>1 mg/mL	>0.750 mg/mL

*ND**: Not determined.
System consisted of ribose (30 mg/mL) and tuna stomach hydrolysate (5 mg/mL) in 0.5 M phosphate buffer, pH 6.5.

55 °C and 75 °C (17 h, pH 6.5, 0.5 M buffer phosphate) was dramatically enhanced in comparison with the unheated sample (Table 1). The radical scavenging activity of the rib–hydrolysate system heated at 55 and 75 °C was 19 and 34 times higher, respectively, than that of the unheated rib–hydrolysate system.

3.4. Cytotoxicity of MRPs

Cytotoxic evaluation showed that MRPs in rib–hydrolysate system (experimental conditions detailed in Table 1) did not show any cytotoxic effect at concentration up to 750 µg/mL when the cell type (Vero cell line no. ATCC CCL-81 and 3T3-Swiss albino cell line no. ATCC CCL-92) were used. Jing and Kitts (2000) demonstrated that the high molecular weight polymer of MRP derived from glucose– (or fructose–) lysine model heated at 121 °C possessed greater potential toxicity towards Caco-2 cells in comparison with low molecular MRP, despite apparent antioxidant properties.

4. Conclusions

The non-enzymatic browning reaction of the sugar–tuna stomach hydrolysate model system confirmed that the pentose sugar, ribose, is much more reactive than the hexose sugar, glucose both in browning intensity and in DPPH· radical scavenging activity. The contribution of ribose caramelization to the antiradical activity and browning reactions was found at 95 °C and 115 °C.

DPPH· radical scavenging effect of MRPs obtained from ribose–tuna hydrolysate systems is attributed not only to the temperature effect but also to the buffer type and buffer concentration. The phosphate buffer is the most efficient for the increase in radical scavenging activity compared to citrate or Tris–HCl buffers. The MRPs obtained under these mild experimental conditions exhibited no toxicity to Vero and 3T3 cells at high concentrations. Thus, by combining several variables of Maillard reaction such as buffer, temperature, reacting sugar, it is possible to maximize the DPPH· radical scavenging efficiency without using severe conditions of heating.

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