Maillard Reaction Product from Bovine Plasma Proteins as Antioxidant on Model Systems

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Abstract

A Maillard Reaction Product (MRP) was obtained by reacting bovine plasma proteins and aldehydes arising from oxidized soybean oil. SDS-PAGE electrophoresis and IR studies were developed. Antioxidant activity was analysed by Reducing Power (RP), Chelating Activity (CA) and Antiradical Activity (AA) determinations, using different concentrations of MRP (0 - 8 mg/ml) and BHA (0.01% w/v) as a reference. Oxidative stability of linoleic acid emulsions with 0; 1% and 3% w/v of MRP and 0.01% w/v of BHA was studied. The presence of carboxylic groups was evidenced from IR spectrum, while electrophoresis showed the appearance of new molecular weight constituents. MRP exhibited high RP and CA, but AA was much lower than BHA (35% vs 96%). Inhibition Percentages of Lipid Oxidation were 94% and 97% for MRP and BHA respectively. Consequently, MRP might be an alternative to the synthetic antioxidant, although further studies should be conducted for its application in food systems.

Keywords: Maillard reaction, lipid oxidation, antioxidant activity, oxidative stability, synthetic antioxidants

1. Introduction

Food industry resorts to the use of antioxidants as oxidation inhibitors, and these substances play an important role in improving food stability and people health. Nevertheless, the industry has decreased its use of artificial antioxidants such as Butylhydroxyanisole (BHA), Butilhidroxytoluene (BHT) and Terbutilhidroxyquinone (TBHQ) (Naveena et al, 2008), and it is specially focused on the development of natural substances for this purpose. Maillard reaction has been associated with the formation of compounds with strong antioxidant activity (Jayathilakan& Sharma, 2006) and even when natural antioxidants are primarily plant phenolics (Mathew & Abraham, 2006), Maillard reaction Products (MRPs) could be considered as natural since this reaction occurs during heating, storage and processing of food products (Fernández et al, 2012). The nonenzymatic MRPs formed during processing, packaging and storage via amino and carbonyl compounds interactions are common to many food systems, and several authors proved that these MRPs have antioxidant activity and they can act as radical scavengers and metal chelators, thus potentially providing more than one mechanism of action to prevent lipid oxidation (Vhangani et al, 2013).Mostof research papers reportthe antioxidant activity of MRPs obtained from mixtures of pure amino acids and sugars (Rufian-Henares & Morales, 2007; Sun et al, 2004) and other amino and carbonyl groups sources (Alamed et al, 2009; Drusch et al, 2009, Joubran, Mackie and Lesmes, 2013).

In this regard blood is one of the main waste product in slaughterhouses, and it has a great contaminant power due to the high amount of total solids (18%) and high Chemical Oxygen Demand (COD) of 500,000 mg $O_2.L^{-1}$ (Del Hoyo, 2007). However, any attempt to recover the protein content present in blood from the slaughterhouse would be very useful, especially in those regions where this material is highly available.

Despite all the information regarding the use of bovine plasma in the industry, there is no report of bovine plasma as a source of protein in Maillard reaction. Therefore, the aim of this work was to develop and characterize a MRP obtained from bovine plasma proteins, to evaluate the oxidative stability of model systems considering this MRP as an alternative to a synthetic antioxidant.

2. Materials and Methods

2.1. Preparation of Maillard Reaction Product

MRP was obtained from bovine plasma proteins and aldehydes arising from soybean oil oxidation. Bovine plasma was collected from local slaughterhouse, and mixed with sodium citrate (2.5% w/v) to prevent clotting. The mixture was centrifuged at 2500 g and 2 °C during 10 minutes (Hettich Zentrifugen, Rotina 380 R, Germany); the supernatant was refrigerated until use. Protein content was determined by the Biuret method as described by Robson, Goll and Temple (1968).

Soybean oil (3.5 g) was oxidized in rotary evaporator (Figmay SRL, Argentina) at 80 °C; aldehydes arising were removed by nitrogen flow and collected in Sorensen buffer (pH 7.6). Hexanal ethanolic solutions (1000 to 3000 mg/L) were used as standard and aldehydes content was expressed as mg Hexanal.mg^{-1} soybean oil, determined through spectrophotometric measures at 265 nm. After 6 hour of heating, absorbance measurements remained constant, being the final aldehydes content 0.006 mg Hexanal.mg^{-1} soybean oil.

Plasma proteins were incubated with aldehydes in a ratio 3.5:1. The mixture (pH 7.6) was kept in rotatory evaporator at 80 ºC for 4 h under moderated agitation (56 rpm). The resultant mixture was lyophilized to obtain the dry product named MRP.

2.2. Colour Determination

Colour measurements were performed using an Evolution 600 UV-Vis (Thermo Scentific equipped with an integrated sphere using a VisionliteColourCalc Software, Germany) to obtain CIE L*, a* and b*values.

2.3. FT-IR Measurements

FT-IR spectra were recorded in a Nicolet 8700 FT-IR spectrometer, using Turbo mode of the EverGlo infrared source. Series of 20 scans were made with a selected resolution of 2 cm^{-1}.

2.4. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polypeptide composition of bovine plasma proteins and MRP were analyzed by SDS-PAGE using a separating gel (12% w/v in polyacrylamide) with a stacking gel (4% w/v in polyacrylamide) in a minislabs system (Bio-Rad Mini-Protean II Model) (Laemmli, 1970). Protein molecular weights were estimated using standard molecular weights markers (Sigma Aldrich) that included albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20,1 kDa), and a-lactalbumin (14,400kDa).

2.5. Reducing Power Determination

Reducing power of MRP was determined according to Romero et al (2010); 0.5 to 8 mg of MRP were dissolved in 1 ml of 0.2 M of sodium phosphate buffer (pH 7.6) and mixed with 1 ml of 1% potassium ferricyanide ($K_3Fe(CN_6)$). The reaction mixture was incubated in a temperature–controlled water bath at 50ºC for 20 minutes, followed by the addition of 1 ml of 10% trichloroacetic acid. The mixture was then centrifuged at 650 rpm for 10 minutes. The supernatant obtained (1 ml) was treated with 1 ml of distilled water and 200 µl of 0.1% $FeCl_3$. The absorbance of the reaction mixture was measured at 700 nm in a UV–visible Beckman DU 640B, Fullerton, CA, USA spectrophotometer. An increase in the absorbance was used as the measure for reducing power. A higher absorbance indicated a higher reducing power.

2.6. Metal Chelating Activity

The ability of samples to chelate ferrous ions was assessed using the method of Decker &Welch (1990). One milliliter of solution of MRP (2.5 $mg.mL^{-1}$) was first mixed with 3.7 mL of distilled water. It was then reacted with a solution containing 0.1 mL of $FeCl_2$ 2 mM and 0.2 mL of Ferrozine 5mM.

After 10 minutes, the absorbance of the reaction mixture was measured at 562 nm. Chelating activity was calculated as a percentage using {1−(absorbance of the sample at 562 nm−absorbance of the blank sample at 562 nm)/ (absorbance of control at 562 nm)}×100.

2.7. Scavenging Effect on DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ Radicals

1,1-diphenyl-2-picrylhydrazyl radical scavenging activity of dry extract (0.5 to 8 mg.mL^{-1} ethanol) was determined in a0.12 m Methanolic solution of DPPH$^{\bullet}$(Sigma-Aldrich). An aliquot of 100 µl of the antioxidant solution was added to 3 ml ethanolic solution of DPPH$^{\bullet}$. The decrease of the absorbance at 517 nm was read for ten minutes, extrapolating the result to infinite time, using ethanol as a blank and solution of DPPH$^{\bullet}$as a control.
The radical scavenging activity by the 2, 2´-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) radical cation discoloration assay was measured as described by Re et al. (1999). The bleaching rate of ABTS$^{\bullet+}$ in the presence of the sample was spectrophotometrically monitored at 734 nm. The reaction was started by adding 100 µl of an antioxidant solution (containing 0.5 to 8 mg MRP.mL^{-1} ethanol). ABTS$^{\bullet+}$ bleaching was monitored at 25 ºC for at least 30 min and the discoloration after 10 min was used as the measure of antioxidant activity.
The inhibition of free radicals DPPH$^{\bullet}$ and ABTS$^{\bullet+}$was expressed in terms of Antiradical Activity, calculated using %AA={1− (Absorbance of the sample − absorbance of the blank)/(absorbance of the blank)}×100.

2.8. Model Systems

Linoleic acid emulsions were prepared by adding 0.285 g of linoleic acid (ICN biomedicals Inc, Ohio, USA), 0.289 g of Tween 20 as emulsifier and 50 ml of phosphate buffer (pH 7.17), The mixture was homogenized using a hand mixer (400 W, ATMA, Argentina), processing 30 seconds and resting 15 seconds each time, until 5 minutes of treatment. To determine the optimum antioxidant concentration, MRP was added to this emulsion at final concentrations of 0%, 1% and 3% (w/v). Butylhydroxyanisole (BHA) was used as a reference (0.1%).

2.9. Lipid Oxidation

Oxidation was monitored by measuring Peroxide Value (PV) and Thiobarbituric Acid Reactive Substances (TBARS).

The antioxidant activity at the end of the assay time was expressed for each indicator as inhibition percent of peroxidation (IP %), with a control without antioxidant:

IP %= [(Peroxidation indicator value $_{blank}$ - Peroxidation indicator value $_{sample}$)/ (Peroxidation indicator value$_{blank}$)] * 100

A higher IP indicated a higher antioxidant activity.

2.9.1. Peroxide Values

The FTC (ferric thiocyanate) method was adapted from the FIL-IDF method 74A:1991. Samples (0.02 g) dissolved in 9.8 ml methanol: chloroform (70:30) solution and 0.1 ml of 30 % (w/v) ammonium thiocyanate were added and mixed. Precisely 5 min after the addition of 0.1 ml of ferrous chloride in 3.5 % v/v hydrochloride acid to the reaction mixture, the absorbance of the resulting red colour was measured at 501 nm against solvent solution as blank. Peroxide values were expressed in terms of mmoles of oxygen per kg of sample.

2.9.2. Thiobarbituric Acid Reactive Substances (TBARS)

Lipid oxidation was evaluated by TBARs according to Sinnhuber and Yu (1997) with slight modifications. Sample (100 µg) was placed in 10 ml test tubes; then 10 µl of antioxidant solution (BHA, 0.01%) and 2 ml of TBA solution (20mM TBA in 15 % TCA) were subsequently added. The mixture was vortexed and heated in a water bath (90ºC) for 15 minutes. After cooling at room temperature, 2 ml of chloroform were added, followed by centrifugation at 2000 rpm for 15 minutes. The absorbance of supernatant was read at 532 nm against a blank containing 0.1 ml of distilled water and 2 ml of TBA/TCA solution. Malondialdehyde standard curve was prepared from different 1, 1, 3, 3-tetramethoxypropane solutions, and TBARS were expressed as mg of MDA /kg dry matter.

2.10. Statistical Analysis

All analyses were carried out in triplicate. Data were recorded as means±standard deviations and analysed using the software package Statgraphics Plus.

Analysis of variance (ANOVA) was carried out to test for any significant differences at ($p<0.05$).

3. Results and Discussion

3.1. L*, a* and b* Determination

It is very useful to evaluate the colour of a substance that will eventually be added to a food product since this is an important parameter which determines the food acceptance. L*, a* and b* measurement gives an idea of the appearance of the developed MRP. The colour of the product here studied was light beige, suggesting that the reaction has not progressed to a larger extent given the fact that red and yellow colour development is an indicative of the progress of Maillard reaction, being expected greater a* and b* values as the reaction is more advanced, while L* value will be lower. In this case, L* value (91.36±0.30) was higher than MRPs coming from different sources, such as proteins and dextran (Spotti et al, 2013) although a*(2.38±0.04) and b* (11.76±0.24) values were lower.

The MRP under study turned out to be a product of high luminosity, showing a little development of redness and yellowness probably due to the reaction temperature, which was not high enough to generate the characteristic polymers responsible for browning. Nevertheless, colour parameters were higher than that reported for similar substances (Fernandez et al, 2010), whose a* and b* values were lower than those obtained in this study.

3.2. FT-IR

To learn more about structural changes arising from Maillard-type conjugation, FT-IR was recorded for MRP. In general, FT-IR spectra of MRPs are commonly accompanied by secondary structure changes that are expressed in the amide I (1600–1690 cm^{-1}) and amide II (1480–1575 cm^{-1}) bands of the spectra (Kong & Yu, 2007; Xavier et al, 2010).

Figure 1 shows the obtained IR spectrum and two highlighted absorption bands corresponding the first one (2000-3500 cm^{-1}) to the presence of -OH groups, showing a saw–tooth like shape at the bottom, being this a characteristic of the OH^- carboxylic acid constituents. Second remarkable absorption bands ranged from 1750 to 1500 cm^{-1}, denoting the presence of C=N, C–NH, C=O and C=C, which could correspond to Schifff bases, Amadori compounds and reductones, respectively (Wen-qiong et al, 2013).The results agreed with those reported in studies of structure of MRPs which showed that the progress of the Maillard reaction involves changes in chemical structure as a result of the disappearance of some functional groups, especially amino groups, and the appearance of other groups associated with the reaction products (S´miechowski et al, 2008; Srivastava et al , 2011).

The identified functional groups could be related to early stages of the Maillard reaction, suggesting the safety of the developed product.

3.3. Gel electrophoresis (SDS-PAGE)

Gel electrophoresis has been applied in numerous studies as a tool to confirm glycation reactions between reducing sugars and food proteins (Liu et al, 2012). It has always been observed that the more advanced the reaction, the greater the number of diffuse bands at the top of the separation gel, which is a direct indicator of the formation of new molecular weight compounds (Zhang et al, 2012; Qv et al, 2009).

SDS–PAGE analysis was made for both native protein and MRP, as it can be seen in Figure 2. Lines A and B represent the electrophoretic profile of the native protein, while lines C and D represent the MRP. A clear change in the protein profile was observed, since bands corresponding to 67kDawere more intense for the proteins, and their intensity decreased for the MRP. Moreover, in MRP the band corresponding to 45kDawas not present, while new species with molecular weight higher than 67kDa were formed, as it can be seen at the top of the figure. This might indicate that a covalently linked protein dimer was produced due to some cross-linking reaction as previously reported by Ajandouz et al, 2008 and Oliver et al, 2006.

Identified plasma proteins (lines A and B) were albumin (66 kDa), globulins (41 kDa) and low molecular weight polypeptides (<40 kDa), including trypsin (23 kDa). In lines C and D a single identified band (1) between 23 and 45 kDa representative of trypsin can be seen; between 45 and 67 kDa three bands were observed (2, 3 and 4) as well as three well-defined bands (5, 6 and 7) above 67 kDa, representing higher molecular weights compounds. It should be emphasized that these high molecular weights could not be related to final stages of the Maillard reaction, as high molecular weight honey melanoidins (> 235 kDa) (Brudzynski & Miotto, 2011), although a high molecular weight marker could show cross linked proteins bigger than 235 KDa which do not appear in the gel under these conditions.

Therefore, these results would indicate that the MRP under study is a mixture of low molecular weight compounds, which ensures the safety of the product since not molecular weights related to potentially toxic substances were detected.

3.4. Changes in Reducing Power (RP)

This assay measures the antioxidant activity of MRP and indirectly reflects the amount of OH^- groups, which play an important role in the generation of a reducing environment, due to redox potential of transferring electrons (Guet al, 2010; Hwang et al, 2011).Figure 3 shows the resulting RP, expressed as absorbance units (AU). RP increased with MRP concentration. The highest RP was achieved with the highest concentration of product, while the other tested concentrations showed a higher effect than the antioxidant used as a reference (BHA), with no significant differences between the lowest concentration of MRP and BHA. These results were similar to MRPs from sarcoplasmic proteins-malondialdehyde mixtures (Fernandez et al , 2012) (0.68 UA and 0.72 , respectively) and higher than those obtained when using different MRPs, such as those reported by Gu et al (2010) for MRPs obtained from casein-glucose.

The high reducing power exhibited by the MRP can be attributed to the presence of reductones, intermediate compounds in the Maillard reaction which have a great ability to donate hydrogen atoms (Amarowicz, 2009).

3.5. Chelating Activity (%ChA)

Figure 4 shows that 4 $mg.mL^{-1}$ of MRP had a chelating activity (% ChA) greater than EDTA (5 $mg.mL^{-1}$) used as a control. The %ChA was dependent on the concentration of MRP, being 23% and 71% for MRP at concentrations of 2 $mg.mL^{-1}$ and 4 $mg.mL^{-1}$ respectively.

Different studies have reported %ChA greater than 80% for MRPs obtained by heating proteins – glucose mixtures (Gu et al, 2010), but the high %ChA values are characteristic of high weight molecular compounds (HWMC) formed in the final stages of the Maillard reaction. However, MRPs formed in early stages have acceptable %ChA values, although lower. Nevertheless in both cases chelating activity is attributed to the presence of hydroxyl and pyrroles groups (Gu et al, 2010; Hofman, 1998).

3.6. $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ Radical Scavenging Activity

Concentrations of 0.5, 1, 2, 4 and 8 $mg.mL^{-1}$ of MRP were used for both assays.

$DPPH^{\bullet}$ assay is a measure of the electron transfer reactions between $DPPH^{\bullet}$ and the antioxidant compound (Prior et al, 2005); $DPPH^{\bullet}$ scavenging was monitored by the decrease in absorbance at 517 nm and the results showed that MRP antiradical activity was slightly dependent on the MRP concentration, as shown in Figure 5, probably due to the fact that this assay was conducted in an alcoholic medium where protein nature substances may precipitate, causing reaction interferences (Fernández et al, 2010).

The observed effect was smaller than the BHA, since the maximum %AA achieved was 12% for 8 mg $MRP.mL^{-1}$ and 96% for BHA respectively. These results were also lower than MRPs from whey protein (40%) and MRP derived from casein-glucose mixtures (80%) (Chawla et al, 2009; Guet al, 2010).

MRP antiradical activity measured as $ABTS^{\bullet+}$ scavenging activity was concentration dependent, since %AA ranged from 9.04% to 23.53% (for 0.5 mg $MRP.mL^{-1}$ and 8 mg $MRP.mL^{-1}$ respectively), these values were lower than BHA too (%AA of 96%).

This result together with the $DPPH^{\bullet}$ scavenging activity suggested that MRPs obtained from bovine plasma proteins had a poor free radical scavenging activity, in contrast with other MRPs with higher %AA (45%) (Limsuwanmanee et al, 2013). MRPs coming from early stages of the Maillard reaction with low %AA were expectable, while radical scavenging activity increased with the accumulation of brown-coloured MRP (Sacchetti et al, 2009).

3.7. Oxidative Stability of Model Systems

Global antioxidant effect of MRP was tested on linoleic acid/water model system. Lipid oxidation was evaluated by measuring PV and TBARS in model system added with 0%, 1% and 3% PRM (%w/v), being the reference the system with 0.01% of BHA.

The MRP exerted a remarkable inhibitory effect especially in the initial stages of the oxidation process, as seen in Figure 6.

The IP achieved with 1% and 3% of MRP were 94% and 92% respectively, with no significant differences with the effect of BHA on the hydroperoxides formation. Regarding TBARS, the inhibition effect of the MRP was independent of the concentration (76%), although BHA had an inhibitory effect significantly higher (98%). It is clear from the figure that 1% of MRP was enough to achieve an inhibitory effect comparable to that obtained with de maximum allowed

4. Conclusions

MRP turned into a pallid brown product with a high reducing power and a high effectiveness as metal chelator, although it showed a poor antiradical activity against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$.

According to SDS-PAGE and IR spectrum studies, the developed MRP was a mixture of low molecular compounds with functional groups that could be related to earlier stages of the Maillard reaction.

Since no significant differences between MRP and BHA inhibition effects were observed, MRP could be an alternative to replace this synthetic antioxidant in food model systems.

Further studies of MRP added to food systems must be developed to ensure the safety of this substance.

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