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Influence of molecular weight on *in vitro* immunostimulatory properties of instant coffee

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ABSTRACT

Instant coffee was prepared and fractionated into higher (>100 kDa), medium (5–10, 10–30, 30–100 kDa) and lower (1–5, <1 kDa) molecular weight fractions. Sugars and linkage composition characteristics of arabinogalactans and galactomannans were recovered in all fractions. Also, amino acid analysis performed after hydrolysis showed similar compositions in all fractions. On the contrary, free chlorogenic acids and caffeine were only detected in the lowest molecular weight fraction (<1 kDa). A direct relationship between the melanoidins browning index and the molecular weight was observed. The fractions obtained were incubated *in vitro* with murine spleen lymphocytes in order to evaluate their possible immunostimulatory abilities. The surface expression of CD69 (early activation marker) on different lymphocyte sub-populations showed that the fraction with 1–5 kDa was able to induce activation of B-lymphocytes. This was the only fraction to induce B-lymphocyte activation, since all the other fractions failed, even when higher concentrations were used.

1. Introduction

Under the drastic industrial processing conditions in the preparation of instant coffee, when compared to espresso coffee, several modifications can be observed in its composition (Capek, Matulova, Navarini, & Liverani, 2009; Wolfrom & Anderson, 1967). One of the main observations is a decrease in the molecular weight of polysaccharides, although roasting promotes the formation of new glycosidic linkages, observed in model compounds in both galactomannan (Moreira, Coimbra, Nunes, Simões, & Domingues, 2011) and arabinogalactan (Moreira, Coimbra, Nunes, & Domingues, 2013), as well as in coffee polysaccharides (Simões, Maricato, Nunes, Domingues, & Coimbra, 2014).

The amount of chlorogenic acids (CGA) decreases by one third during roasting, depending on the roasting degree, mainly due to their high reactivity. These compounds react with Maillard-reaction compounds and may be included in melanoidins (Nunes & Coimbra, 2010), which are high molecular weight browning compounds. The molecular weight attributed to "melanoidins" can vary according to different authors: more than 100 kDa (Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002), more than 10 kDa (Delgado-Andrade & Morales, 2005) or higher than a few thousands of Daltons (Wang, Qian, & Yao, 2011). Premelanoidins have been defined as compounds formed in the initial stages of the Maillard reaction. These subsequently polymerise or cross-link with other Maillard reaction products to produce the high molecular weight melanoidins during the later stages of the Maillard reaction (Adrian & Frangne, 1973; Adrian & Susbielle, 1975; Morales, Somoza, & Fogliano, 2012; Wang et al., 2011).

The melanoidin content has been estimated from the difference between the known (carbohydrates and proteins) and unknown compositions, through the measurement of the specific extinction coefficient (K_{mix}) at 405 nm (Bekedam, Schols, Van Boekel, & Smit, 2006), or relating both unknown composition and the measure of the brown colour, expressed as the "melanoidin browning index" (MBI) (Nunes, Cruz, & Coimbra, 2012).

Several studies have shown the health implications of coffee and coffee derived products (George, Ramalakshmi, & Rao, 2008; Higdon & Frei, 2006; Moreira, Nunes, Domingues, & Coimbra,

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2012; Ramalakshmi, Rao, Takano-Ishikawa, & Goto, 2009). The immunostimulatory potential of coffee has been attributed to the pro-inflammatory potential of the arabinogalactan-proteins present in instant coffee (Nosal'ova et al., 2011), as well as galactomannans from coffee infusions (Simões et al., 2009). Melanoidins, caffeine and chlorogenic acids, on the other hand, have shown anti-inflammatory properties (Frost-Meyer & Logomarsino, 2012; Vitaglione et al., 2010). Altogether, due to the high complexity of the components present in the coffee bean, incremented by the formation of new compounds during roasting, it is difficult to untangle the molecular responsibilities for each biological effect. Fractionation methodologies such as those based on molecular weight and solubility can be used to decrease the mixtures complexity and help further the understanding of complex matrices, such as coffee (Bekedam, Roos, Schols, van Boekel, & Smit, 2008; Bekedam et al., 2006; Borrelli et al., 2002; Nunes & Coimbra, 2007).

In order to study the influence of compounds bearing different molecular weights on the immunostimulatory properties of instant coffee, instant coffee powder was solubilised in water and fractionated into six sequential molecular weight fractions. These fractions were structurally characterised, incubated *in vitro* with murine mononuclear spleen cells and analysed by flow cytometry in order to evaluate lymphocyte activation.

2. Material and methods

2.1. Sample preparation

The instant coffee powder was obtained from a local supermarket from a commercial batch (Auchan, Portugal). All reagents used were of analytical grade or higher available purity. A sample of 50 g of instant coffee was dissolved in 125 ml of water at 80 °C and stirred for 10 min at 80 °C. The solution was then cooled down at room temperature, and was then placed at 4 °C for a minimum of 48 h. The solution was decanted and centrifuged at 15,000 rpm for 10 min at 4 °C. About 86.3% of the material was recovered in the supernatant named as "WSn", which was frozen and freeze-dried.

2.2. Fractionation by ultrafiltration

The WSn solution was subjected to sequential ultrafiltration at room temperature using a stirred ultrafiltration unit (Stirred Cell Model 8200, 200 ml) with regenerated cellulose membranes with molecular weight cut-offs of 100, 30, 10, 5 and 1 kDa (Millipore, Darmstadt, Germany). The WSn sample was redissolved in 200 ml of water. When the volume of retentate reached 40 ml after passing through the membrane, refilling with water to a volume of 200 ml was performed and this procedure was repeated several times. To control the efficiency of the separation, the conductivity in the filtrates recovered was measured until it presented a value below 20 $\mu S.cm^{-1}$. On average, this procedure was repeated 10 times for each membrane pore size: the fraction remaining as retentate was recovered as high molecular weight material (>100 kDa) and all the filtrate volume recovered was concentrated to 200 ml and submitted to a new ultrafiltration using a 30 kDa cut-off membrane; the filtrate of 30 kDa was submitted to a new ultrafiltration using a 10 kDa, followed by 5 kDa and 1 kDa cut-off membranes. At the end of the procedure, six fractions corresponding to the 100, 30, 10, 5 and 1 kDa retentates and one corresponding to the 1 kDa cut-off filtrate were recovered. All fractions obtained were freeze-dried and stored under an anhydrous atmosphere.

2.3. Size exclusion chromatography (SEC)

About 4–5 mg of each soluble fraction was dissolved in 500 µl of 0.1 M $NaNO_3$ aqueous solution at 20 °C for 60 min and further filtered through a 0.4 µm filter reaching a sample concentration of ca. 1%. Glucose (Glc) was dissolved in the sample and used as control. The SEC analysis was carried out using two PL aquagel-OH MIXED 8 µm 300 × 7.5 mm columns protected by a PL aquagel-OH Guard 8 µm pre-column on a PL-GPC 110 system (Polymer Laboratories, UK). The columns, injector system and the detector (RI) were maintained at 36 °C during the analysis. The eluent (0.1 M $NaNO_3$) was pumped at a flow rate of 0.9 ml/min. The columns were calibrated with pullulans (Polymer Laboratories, UK) in the range 0.7–100.0 kDa. The injected volume was 100 µl (Mendes, Xavier, Evtuguin, & Lopes, 2013).

2.4. Chlorogenic acids (CGA) and caffeine identification and quantification

The methodology used for detection and quantification of CGA and caffeine was adopted from Nunes et al. (2012). Aliquots of 10 g/l of each fraction were prepared and characterised for their free chlorogenic acids (CGA) and caffeine content through RP-HPLC, using an Ultimate 3000 solvent delivery system equipped with a PD-100 UV–Vis diode array detector. Separation was performed by gradient elution on an ACE 5 C18 column (5 µm particle size; 250 mm × 4.6 mm, Advanced Chromatography Technologies, Scotland). Analysis conditions were as follows: solvent A was a mixture of 95:5 water/formic acid (v/v), and solvent B was methanol. A linear gradient analysis for a total run time of 80 min was used as follows: starting from 5% solvent B during 2 min, increase to 80% solvent B over 68 min and then isocratic for 8 min, decreasing to 5% solvent B over 2 min and finally isocratic for 5 min. The sample volume injected was 50 µl, the flow rate was 0.8 ml/min and the column temperature was maintained at 30 °C during the run. The eluent was continuously monitored from 240 to 600 nm with a photodiode array detector. Quantification of all chlorogenic acids were performed by using a standard curve made with 5-caffeoylquinic acid and expressed as 5-cafeoylquinic acid equivalents. Caffeine was quantified by using a calibration curve made with pure caffeine.

2.5. Specific extinction coefficients (K_{mix} at 280, 325 and 405 nm) determination

The specific extinction coefficients at 280, 325 and 405 nm, respectively $K_{mix,280\ nm}$, $K_{mix,325\ nm}$ and $K_{mix,405\ nm}$, were determined using the methodology described by Bekedam et al. (2006). A 1.0 g/l sample of each lyophilised fraction was prepared in water. Subsequently, several dilutions were prepared from this sample (between 1 and 500 mg/l). The absorption at 280, 325 and 405 nm was measured in a double beam ultraviolet–visible (UV/Vis) spectrophotometer (Lambda 35, Perkin-Elmer, USA). Each curve was prepared from at least 5 measurements.

2.6. Protein total content and individual amino acids composition

The individual amino acids were determined after acid hydrolysis, derivatisation to the isobutyl and heptafluorobutyrate derivatives and analysis by GC-FID (Coimbra, Nunes, Cunha, & Guiné, 2011). The total protein content was determined by the sum of the amount of the individual amino acids. Each result represents the average of two replicates.

2.7. Sugar and glycosidic-linkage analysis

The individual neutral sugars were determined after acid hydrolysis, derivatisation to alditol acetates and analysis by GC-FID as described by Passos and Coimbra (2013). The total sugar content was determined by the sum of the amount of the individual sugars,