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Macromolecular crowding conditions enhance glycation and oxidation of whey proteins in ultrasound-induced Maillard reaction

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ABSTRACT

High intensity ultrasound (HIUS) can promote Maillard reaction (MR). Macromolecular crowding conditions accelerate reactions and stabilise protein structure. The aim of this study was to investigate if combined application of ultrasound and macromolecular crowding can improve efficiency of MR. The presence of crowding agent (polyethylene glycol) significantly increased ultrasound-induced whey protein (WP) glycation by arabinose. An increase in glycation efficiency results only in slight change of WP structure. Macromolecular crowding intensifies oxidative modifications of WP, as well as formation of amyloid-like structures by enhancement of MR. Solubility at different pH, thermal stability and antioxidative capacity of glycated WP were increased, especially in the presence of crowding agent, compared to sonicated nonglycated proteins. The application of HIUS under crowding conditions can be a new approach for enhancement of reactions in general, enabling short processing time and mild conditions, while preserving protein structure and minimising protein aggregation.

1. Introduction

The Maillard reaction (MR) is a spontaneous reaction between amino groups, usually amino acids or proteins, and reducing compounds, such as reducing saccharides. The usage of the MR was demonstrated to be a promising approach to improve protein functional properties, such as solubility, foaming, thermal stability, emulsifying (Oliver, Melton, & Stanley, 2006) and antioxidative properties (Amarowicz, 2009). Whey proteins (WP) are extensively used as food ingredients because of their valuable nutritional and techno-functional properties, and many methods have been developed to modify whey proteins *via* MR (Chevalier, Chobert, Popineau, Nicolas, & Haertle, 2001; Foegeding, Davis, Doucet, & McGuffey, 2002).

Dry heating, which has been usually used to prepare Maillard-type protein–saccharide conjugates, has several disadvantages: it can take up to several days/weeks; the reaction extent is uncontrollable, which may lead to excessive browning development; the reaction is limited by the uneven contact between reactants; and, for compact or rigid proteins, it can result in inefficient glycation (Zhuo et al. (2013)). Wet heating largely shortens the reaction time, which provides better control of browning, but high temperatures cause protein aggregation and lower degree of glycation (Zhu, Damodaran, & Lucey, 2008).

Low-frequency (20–100 kHz) high-intensity ultrasound (HIUS) is a new technology that has great potential for applications in food processing (Chandrapala, Oliyer, Kentish, & Ashokkumar, 2012). It was tested in several dairy applications and was shown to enhance physical and functional properties of whey (Jambrak, Mason, Lelas, Herceg, & Herceg, 2008; Zisu et al., 2011). High-intensity sonication can modify secondary structure of proteins and can lead to increase in surface hydrophobicity and propensity of WP to aggregate (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011). Several studies demonstrated that HIUS can promote Maillard reaction by accelerating early (Corzo-Martinez et al., 2014), as well as intermediate and final stages of Maillard reaction (Guan, Wang, Yu, Xu, & Zhu, 2010; Guan et al., 2011; Stanic-Vucinic, Prodic, Apostolovic, Nikolic, & Velickovic, 2013). In our previous study (Stanic-Vucinic et al., 2013) we have shown that HIUS can efficiently promote glycation of β-lactoglobulin (BLG) by MR, and that obtained glycoconjugates possesss improved antioxidative capacity, with a minor influence on protein's secondary and tertiary structure.

Macromolecular crowding (MC) non-specifically enhances reactions due to the reduction of total excluded volume that results in

Abbreviations: HIUS, high intensity ultrasound; WP, whey proteins; MR, Maillard reaction; MRPs, Maillard reaction products; BLG, β-lactoglobulin.

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increased activity coefficient of reactants (Zhou, Rivas, & Minton, 2008). MC is generally expected to increase the rate of slow, transition-state limited, association reactions and to decrease the rate of fast, diffusion-limited, association reactions. In crowded conditions there is increased viscosity, and mass transfer and diffusion rates are reduced, especially in the presence of crowding agent with high molecular mass (Kim & Yethiraj, 2009). However, if done in macromolecular crowding conditions, sonication should increase overall reaction rates. Ultrasound-accelerated chemical reactions arise from implosive collapse of cavitation bubbles, generating enough kinetic energy to drive reactions to completion and releasing short-lived, high-energy chemical species to solution. Strong shear forces and microstreaming, as well as rapid heating and cooling rates ($\sim 10^6$ K s^{-1}), generated during cavitation, enable effective mixing, highly improve mass transport and reduce viscosity, and therefore should overwhelm reduction of diffusion and eliminate cage effect due to MC. In addition, thermal stability of proteins in the crowded conditions is increased, due to a decrease in the entropy of protein unfolding and denaturation (Sasahara, McPhie, & Minton, 2003). In that way the extent of protein denaturation, aggregation and polymerisation should be minimised, even if harsher process conditions are used. So far there are not many reports about MC effect on Maillard reaction. Zhuo et al. (2013) prepared soy protein isolate–dextran conjugates and Zhang et al. (2012) glycated β-conglycinin with dextran *via* MR in macromolecular crowding conditions by heating in solution. In both studies crowding conditions enabled shortened reaction time and prevented excessive protein denaturation and aggregation.

The reports on the effects of ultrasound on the MR are still scarce, and there is no data about protein oxidative modifications due to ultrasound-induced MR. In this moment, there is no literature data on the effect of macromolecular crowding on HIUS-promoted/enhanced reactions in general. The objective of this study was to generate glycated whey proteins by ultrasound in macromolecular crowding conditions, in an attempt to increase the efficiency of glycation at mild temperatures in aqueous solutions with maintained protein structure/stability.

2. Materials and methods

2.1. Materials

D-Arabinose monohydrate, DPPH (1,1-diphenyl-2-picryl-hydrazyl), OPA (*o*-phthalaldehyde), ANS (8-anilino-1-naphtalensulfonic acid), polyethylene glycol of MW 6000 (PEG 6000), DTNB (5,5′-dithiobis(2-nitrobenzoic acid)), TBA (2-thiobarbituric acid), xylenol orange, thioflavin T and Congo red were obtained from Sigma–Aldrich (Traufunken, Germany). All other reagents were of analytical grade. Whey proteins were isolated from fresh raw (thermally untreated) bovine milk. Bovine milk was defatted by centrifugation (10 min at 12,000*g*, 4 °C), and then casein was precipitated by adding 0.1 M hydrochloric acid to pH 4.6. Precipitated casein was separated by centrifugation (10 min at 12,000*g*). Obtained whey was then additionally defatted by tetrachloroethylene extraction (3 times with 0.4 volumes of tetrachloroethylene), extensively dialysed (MW cut off 3 kDa) against 10 mM sodium phosphate buffer, pH 8.0, and concentrated by ultrafiltration (MW cut off 3 kDa), giving whey protein of 99% purity. Protein concentration was determined by the A_{280}, using $\varepsilon = 1.274$ mL mg^{-1} cm^{-1} as average extinction coefficient.

2.2. Preparation of ultrasound induced Maillard reaction products

Whey proteins (50 mg/mL) were mixed with or without arabinose (150 mg/mL), with and without PEG 6000 (120 mg/mL), in 10 mM sodium phosphate buffer (pH 8) and pH was adjusted to pH 8 using 1 M NaOH. Sonication (20 kHz frequency) was carried out with a Branson Sonifier 150 (Branson Ultrasonic Corp., Danbury, CT) for 60 min. The ultrasound probe, with output power of 9.5 W (135 W/cm^2), was immersed in a tube with 2.5 mL of sample, at a depth of 2 cm, and the tube was kept at 5–10 °C by using an ice bath.

After the treatment pH in undiluted samples was measured. An aliquot of every sample was dialysed against 10 mM sodium phosphate buffer (pH 6.5) at 4 °C (MW cut off 10 kDa). After the treatments samples were kept at −20 °C until use.

2.3. Spectrophotometric and spectrofluorimetry measurements

The absorbance at 294 nm (early MRPs) was measured in 50-fold diluted samples and A_{420} (late MRPs) was measured in undiluted samples. For monitoring the contribution of caramelisation to 294 nm/420 nm absorption, samples were prepared as mentioned above but without WP, and absorbance was measured in undiluted samples. Fluorescence measurements were performed using a Horiba Scientific Fluoromax-4 spectrofluorimeter (Horiba, Kyoto, Japan). The fluorescence of the Maillard reaction products was measured at an excitation wavelength of 350 nm in WP samples of 0.5 mg/mL in 10 mM potassium phosphate buffer (pH 8). For the hydrophobic ligand binding experiment the fluorescence spectra of dialysed WP solutions (0.4 mg/mL) saturated by ANS (80 μM) in 10 mM sodium phosphate buffer pH 8 were recorded at excitation of 350 nm.

2.4. Electrophoresis and isoelectrofocusing

Protein components were resolved by SDS PAGE on 14% polyacrylamide gels. SDS PAGE and isoelectrofocusing were done as described in Stanic-Vucinic et al. (2013). Native PAGE was done without SDS and β-mercaptoethanol in gels and sample buffer.

2.5. CD spectra measurements and CD spectra analysis

CD spectra were recorded on a JASCO J-815 spectropolarimeter (JASCO, Tokyo, Japan) with dialysed WP in 10 mM sodium phosphate buffer (pH 6.5). Each spectrum was acquired four times, and the results were averaged. The results were expressed as residue average molar ellipticity using average residue mass of 114 Da. Far-UV spectra were analysed by the CONTIN program to determine the proportion of secondary structures using the CDPro software package (http://lamar.colostate.edu/~sreeram/CDPro/main.html) and reference protein set SP29 (29 soluble proteins).

2.6. Determination of remaining free amino group and sulfhydryl content

The content of free amino groups was determined by the OPA method (Guan, Qiu, Liu, Hua, & Ma, 2006). The concentration of remaining free thiol groups was determined by derivatisation with Ellman's reagent according to Morgan, Leonil, Molle, and Bouhallab (1999). The results are expressed as a percentage of the number of amino/sulfhydryl groups determined for the native (untreated) whey proteins expressed as 100%.

2.7. Determination of DPPH radical-scavenging activity, reducing power and inhibition of lipid peroxidation

DPPH radical-scavenging activity of WP samples (0.3 mg/mL) was determined according to Stanic-Vucinic et al. (2013). Lipid peroxidation was monitored by TBA assay using egg-yolk homogenates as lipid-rich media (Ruberto, Baratta, Deans, & Dorman,