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

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Abbreviated running title: Ultrasound-induced Maillard reaction under macromolecular crowding

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Abstract

High intensity ultrasound (HIUS) can promote Maillard reaction (MR). Macromolecular crowding conditions accelerate reactions and stabilize 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 (polyethylenglycole) 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 minimizing protein aggregation.

Key words: whey proteins, ultrasound, Maillard reaction, macromolecular crowding, protein oxidation

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

1. Introduction

The Maillard reaction (MR) is 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, that has been usually used to prepare Maillard-type of 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, Qi, Yin, Yang, Zhu & Huang, 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 showed 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, Montilla, Megias-Perez, Olano, Moreno & Villamiel, 2014), as well intermediate and final stages of Maillard reaction (Guan et al., 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 possesses improved antioxidative capacity, with a minor influence on protein's secondary and tertiary structure.

Macromolecular crowding (MC) nonspecifically enhances reactions due to the reduction of total excluded volume that results in 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 that drives reactions to completion and releasing short-lived, high-energy chemical species to solution. Strong sheer 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 polymerization should be minimized, even if harsher process conditions are used. So far there are no many reports about MC effect on

Maillard reaction. Zhuo et al. (Zhuo et al., 2013) prepared soy protein isolate–dextran conjugates and Zhang et al. (Zhang et al., 2012) glycated β-conglycinin with dextran via MR in macromolecular crowding conditions by heating in the solution. In both studies crowding conditions enabled shortened reaction time and prevented excessive protein denaturation and aggregation. Also, 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 any literature data on 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 attempt to increase 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), polyethylenglycole 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 dialyzed (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 A280, 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 Branson Sonifier 150 (Branson Ultrasonic Corp., Danbury, CT, USA) for 60 min. The ultrasound probe, with output power of 9.5 W (135 W/cm2), was immersed in a tube with 2.5 ml of sample, with 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 dialyzed 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 A420 (late MRPs) was measured in undiluted samples. For monitoring of contribution of caramelization 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 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 hydrophobic ligand binding experiment the fluorescence spectra of dialyzed 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 dialyzed 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 analyzed by the CONTIN program to determine the proportions 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 remained 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 remained free thiol groups was determined by derivatization with Ellman's reagent according to Morgan et al. (Morgan, Leonil, Molle & Bouhallab, 1999). The results are expressed as a percentage of the number of amino/sulfhydyl 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, 2000). Fresh egg yolks

(150 µl, 20 % in DW, v/v) were mixed with FeSO4 (60 µl, 10 mM) and WP sample (390 µl, 7.5 mg/ml). The mixture was incubated at 37ºC for 1 hour, followed by the addition of 300 µl of saturated TCA (TCA) and 200 µl of TBA (14.4 mg/ml in 100 mM NaOH). The reaction mixtures were incubated for 20 min at 80 °C. After centrifugation at 10,000 g for 10 min, the absorbance of the supernatant was monitored at 532 nm. The inhibition rate was calculated according to the following equation:

% inhibition = [(Abs control – Abs sample / Abs control)] x 100

The reducing power of WP samples (2.0 mg/ml) was determined according to (Stanic-Vucinic et al., 2013).

2.8. Determination of protein carbonyls and hydroperoxydes

Protein carbonyls were determined by 2, 4-dinitrophenylhydrazine assay. Dialyzed protein samples (1 ml, 1 mg/ml) were precipitated with 10 % TCA and precipitates were treated with 1.5 ml 2, 4-dinitrophenylhydrazine (10 mM in 2 M HCl) for 1 h with mixing. After deproteinization with 1.5 ml 20% TCA, pellet was washed three times with 300 µl methanol/ethyl acetate (1:1). The precipitates were solubilized in 1.25 ml 7M urea and carbonyl concentration was measured by spectrophotometry at 370 nm with ε_{370}= 22 000 M^{-1} cm^{-1}. Protein hydroperoxides and total peroxides were determined by ferrous oxidation-xylenol orange (FOX) assay. For determination of protein hydroperoxides WP (5 mg) from dialyzed samples were pelleted by 10 % TCA. The resulting pellet was washed 3 times with 1 ml acetone and dissolved in 450 µl 8 M guanidine hydrochloride. After addition of 50 µl of FOX reagent (2.5 mM xylenol orange, 2.5 mM ferrous ammonium sulphate and 1 M sorbitol in 1.1 M HClO4), the reaction mixture was incubated for 60 min at room temperature and the absorbance of supernatant was measured after centrifugation (12000g, 20 min) at 560 nm. For determination of total peroxide 50 µl of FOX reagent was

added 450 µl of WP samples (5.5 mg/ml). The peroxide concentration was determined using calibration curve for H2O2.

2.9. Thioflavin T and Congo red binding

Dialyzed protein solutions, diluted to 0.6 mg/mL, were incubated with 10 µM Congo red in 10 mM phosphate buffer (pH 7.4) for 30 min and absorption spectra were acquired. Dialyzed WP samples were diluted to 0.5 mg/mL protein in 20 µM ThT in 50 mM phosphate buffer (pH 7.4). The intensity of fluorescence emission at 485 nm was monitored using an excitation wavelength of 435 nm. The intensities of samples with no protein were subtracted from intensities for corresponding samples containing protein.

2.10. Solubility at different pH and thermal stability

Dialyzed WP samples were diluted in buffers of pH 2, 3, 4, 5, 6, 7, 8, 9 and 10 to 1 mg/ml. After 30 min of stirring at room temperature, the samples were centrifuged for 15 min and 10 000g. Solubility was determined by measuring the absorbance at 280 nm of the supernatants and expressed as the percentage of the WP concentration at starting pH (pH 8). For thermal stability dialyzed whey protein samples (1 mg/ml) at pH 7 were heated at different temperatures (60–100 °C) for 15 min. Samples were then cooled to room temperature and centrifuged for 15 min at RT and 10 000g, to precipitate aggregates. Finally, protein content of the supernatants was measured as indicated above, and was compared with that of the corresponding unheated samples.

2.11. Statistical analyses

The data are presented as mean ± S.E. for three independent experiments of ultrasound treatment, each done in minimum duplicate. Differences between the variables were tested for significance by one-way ANOVA accompanied with Tukey's post hoc test using Origin Pro

198 8.5.1 (OriginLab, Northampton, MA, USA). Differences at $p < 0.05$ were considered to be
199 significant.

200 **3. Results and discussion**

201 ***3.1. Monitoring of Maillard reaction***

202 In the present study whey protein solution with arabinose was used to investigate effect of
203 ultrasound processing on Maillard reaction in crowding conditions. The crowding conditions
204 were provided by addition of common crowding agent, the highly water-soluble polymer
205 polyethylene glycol (PEG 6000). A significant increase ($P<0.05$) of UV absorbance at 294 nm in
206 WP samples sonicated with arabinose (Figure 1A), with and without PEG, suggests formation of
207 UV-absorbing intermediate MRPs upon sonication. Also, a significant increase ($p < 0.05$) in
208 browning intensities (A420) of the WP/Ara system was observed after sonication, compared to
209 untreated protein/saccharide system (Fig 1A), and the presence of crowding agent further
210 dramatically increased browning, due to more intense formation of chromophores. Although
211 brown pigment (A420) was formed in parallel to the generated intermediate products (A294), in
212 the presence of PEG, lower A294/A420 ratio, compared to sample without its presence, suggests
213 that crowding conditions enhance Maillard reaction toward more advanced stages. The formation
214 of UV-absorbing compounds and browning upon sonication of sugar/BLG and sugar/amino acid
215 in solution has been recently reported (Guan, Wang, Yu, Xu & Zhu, 2010; Guan et al., 2011;
216 Stanic-Vucinic et al., 2013). Development of fluorescent compounds occurs in the Maillard
217 reaction prior to the generation of brown pigments (Jing & Kitts, 2002). In the presence of sugar
218 sonicated WP samples have shown increased fluorescence, with maximum at about 425 nm
219 when excited at 350 nm (Fig 1B), originating from MRPs. In the crowding conditions

fluorescence of Maillard products was further increased and it was in accordance with spectrophotometric properties of tested samples.

The pH change can be also used as an indication for the degree to which the Maillard reaction has occurred, as organic acids are formed along with the reaction, resulting in a pH drop. The presence of crowding agent caused significantly higher pH drop ($P < 0.05$) in comparison to its absence, due to intensified Maillard reaction (Figure 1 C).

The occurrence of the Maillard reaction was further monitored by the loss of available –NH2 groups after sonication (Figure 1C). Ultrasound treatment in the presence of arabinose induced a significant ($P < 0.05$) loss of WP amino groups, and in the crowding conditions amino groups loss was higher ($P<0.05$), suggesting enhancing effect of crowding on Maillard reaction. All these results demonstrate that the crowding conditions, e.g. presence of PEG, increase the rate of Maillard reaction.

During the development of brown color caused by the MR, caramelization reactions can contribute to overall non-enzymatic browning, especially in the alkaline pH ranges, and at high temperatures and pressures, yielding colored polymers (Ajandouz, Tchiakpe, Dalle Ore, Benajiba & Puigserver, 2001). When arabinose was sonicated with and without the presence of PEG, although ultrasound treatment induced slight caramelization and crowding enhances it, ultrasound-induced caramelization contributed less than 1% to A294 and less than 5% to A420 absorbtivity, without and with the presence of crowding agent (Figure 1D). Ultrasound treatment resulted in low extent of caramelization probably due to rapid heating and cooling rates.

In contrast to classical heating, in sonicated system local spots, with intense heating and high pressures (~5000 K and ~1000 atm inside the bubbles) (Suslick et al., 1999), enable increased reactivity of chemical species at gas-liquid interface and in the vicinitiy of collapsing bubbles,

243 which provide short reaction times. Macromolecular crowding conditions further increased
244 reaction rates by increasing chemical potential e.g. effective concentrations of reacting protein
245 molecules due to volume exclusion. Although excluded volume conditions reduce diffusion
246 coefficient of reactants, highly turbulent flowing conditions due to high velocity of liquid jet
247 streams (~400 km/h), and strong sheer forces enable high mass transfer velocities and
248 frequencies of collisions, even under crowding conditions.

249 ***3.2. Characterization of WP conjugates by electrophoresis***

250 The SDS-PAGE results are shown in Fig. 2A and 2C. After glycation due to sonication, bands
251 corresponding to α-lactalbumin (ALA), and especially β-lactoglobulin, smeared toward higher
252 molecular masses, due to conjugation of carbohydrate. In the presence of PEG smearing to
253 higher masses was more pronounced, indicating more efficient glycation. Under nonreducing
254 conditions, ultrasound treatment resulted in the appearance of high-molecular-weight
255 constituents in the stacking gel and near the top of the separating gel in sWP and sWP/Ara/PEG
256 samples (Fig 2A). However, SDS PAGE under reducing conditions (Figure 2B) clearly shows
257 that high molecular mass species are covalently bonded by disulphide linkages. In the samples
258 with Maillard derivatives formation nondisulphide BLG dimmers and smear of high molecular
259 mass nondisulphide polymers can be observed, with higher yield in the crowding conditions.
260 These results suggest that sonication itself induce generation of disulphide polymers and that
261 presence of arabinose reduce them, probably due to stabilizing effect of saccharide. However,
262 with more progressive Maillard reaction protecting effect of saccharide is partly nulled, with
263 more intensive formation of nondisulfide polymers due to Maillard reaction. In the native SDS
264 PAGE (Figure 2B) it is more obvious that all three main whey proteins, ALA, BLG and BSA,
265 were glycated by sonication in the presence of arabinose, and that glycation was more efficient in

266 the crowded conditions, especially for both forms of BLG, A and B. Isoelectrofocusing results
267 (Figure 2D) demonstrate that while native WP pattern showed bands near pH 5, after sonication
268 in the presence of arabinose, and in particular under crowding conditions, several bands ranging
269 pH 4 to 5 appeared, indicating heterogeneity of glycated derivatives.

270 ***3.3. Secondary and tertiary structure changes***

271 The far CD spectra of sonicated samples, as well as samples of glycated WP, are only slightly
272 different from spectra of untreated proteins (Fig 3A). Differences in the percentages of secondary
273 structures are not significant, except of slightly increased random structures in sWP and α-helix
274 in sWP/Ara samples (Fig. 3B). These results suggest that sonication, as well as glycation by
275 arabinose does not disturb significantly secondary structure of WP under applied conditions,
276 even by more extensive glycation under macromolecular crowding conditions. This is in
277 accordance with previously obtained results showing that sonication-induced glycation of BLG
278 results only in slight secondary structure changes (Stanic-Vucinic et al., 2013). Near CD spectra
279 of untreated WP, with or without addition of arabinose, show peak at 295 nm, originating mainly
280 from BLG (Fig 3C). After sonication this peak is diminished, suggesting that tertiary structure is
281 partly distorted by sonication treatment. The presence of arabinose conserves protein structure,
282 while with increase of glycation extent, in crowding conditions, WP tertiary structure is also
283 partly disturbed due to protein glycation. However, in spite of the fact that crowding conditions
284 enhance Maillard reaction and extent of glycation, tertiary structure of WP was not dramatically
285 altered due to stabilizing effects of arabinose and PEG preventing excessive WP denaturation
286 and aggregation.

287 Upon non-covalent binding of polarity-sensitive hydrophobic fluorescent probe ANS to
288 hydrophobic patches on protein surfaces, its fluorescence intensity increases and the wavelength

of maximal emission shifts. WP sonicated without the presence of sugar have shown increased quantum yield after ANS addition, compared to native proteins (Fig. 3d). The presence of saccharide did not increase peak intensity, but shifted the maximum emission toward lower wavelengths, indicating that glycated WP possesses newly exposed hydrophobic patches on the protein surface. This effect was the most prominent in the presence of PEG, where the highest glycation extent occurred, with maximum shift from 468 nm to 461 nm. In our previous study we have shown that sonication induced BLG glycation resulted in increased surface hydrophobicity, due to newly exposed hydrophobic regions (Stanic-Vucinic, 2013).

In contrast to classical heating, during ultrasound treatment extremely high heating and cooling rates due to cavitation seems to enable proteins to mostly retain their conformation, even under density power of >100 W/cm^2. The localized hot spots generated by the rapid collapse of acoustic cavities are very short lived (<1 μs) (Suslick et al., 1999). This short time is probably long enough to expose protein reactive groups by transient partly unfolding of proteins, and enable reaction to occur, but it is too short to induce dramatic protein conformational change. MC conditions additionally stabilize protein structure, together providing mild reaction conditions.

3.4. Oxidative modifications

During sonication water sonolysis generates free radical species, including hydroxyl radical. Several studies demonstrated that nonenzymatic glycation of amino groups of model proteins increase generation of free radicals (Rizzi, 2003). Therefore, we intended to investigate if ultrasound-induced Maillard reaction results in oxidative modifications of WP, as well as the effects of macromolecular crowding on these processes.

311 Sonication of WP dramatically reduces content of free SH-groups (Fig 4A). However, after
312 sonication in the presence of arabinose, there is a very low extent of SH group oxidation, and
313 therefore low disulphide polymer yield (Fig 2A), due to protecting effect of saccharide. Excluded
314 water volume in the presence of crowding agent provided intensified Maillard reaction, as well
315 as more efficient oxidation of SH groups, resulting in disulfide-linked polymers (Fig 2A).
316 However, in spite of intensified Maillard and increased protein oxidation, protecting effect of
317 arabinose and PEG is evident.
318 It is well known that hydroxyl radicals can result in formation of protein carbonyl derivatives. In
319 addition, it was shown that during Maillard reaction reactive α-dicarbonyls are generated,
320 leading to oxidative desamination of basic amino acids (Villaverde & Estevez, 2013). We
321 proposed that forming of protein carbonyls could occur during sonication due to action of either
322 hydroxyl radicals generated by water sonolysis, and/or due to the effects of α-dicarbonyls
323 generated in Maillard reaction. Fig 4A shows that protein carbonyl content in sonicated WP
324 samples in the presence of arabinose is significantly increased ($P<0.05$), in comparison to control
325 samples. Dramatically higher protein carbonyl content in the samples with PEG suggests that the
326 formation of carbonyls is in direct relation to extent of Maillard reaction e.g. with generation of
327 α-dicarbonyls. Absence of a significant increase in carbonyl content in sonicated WP
328 demonstrate that protein carbonyls were formed entirely due to α- dicarbonyls generated in
329 Maillard reaction. Meltretter et al. have shown that when WP has heated with lactose, oxidative
330 modifications of proteins also occurred, including oxidation of lysine to aminoadipic
331 semialdehyde (Meltretter, Seeber, Humeny, Becker & Pischetsrieder, 2007).
332 Cavitation-generated extremely reactive OH• radicals can undergo various reactions resulting in
333 formation of other reactive oxygen species (ROS), which can react with different chemical

moieties on proteins producing protein radicals. Protein radicals react with O_2 to form peroxyl radicals, which may abstract hydrogen atom yielding protein hydroperoxides (Hawkins & Davies, 2001). In Maillard reaction, H_2O_2 can be generated in the pathway of Amadori product degradation via 1, 2- and 2, 3-enolization and the oxidation of the enolate anion, but also due to saccharide autooxidation (Elgawish, Glomb, Friedlander & Monnier, 1996). Generated H_2O_2 readily reacts with metal ions present in traces to form hydroxyl radical. Therefore we proposed that protein hydroperoxides could be formed as consequence of sonication itself and/or due to ultrasound-promoted Maillard reaction. In sonicated samples with arabinose small amount of protein hydroperoxides were detected, in contrast to WP, WP/Ara and sWP samples (Fig 4 B). Protein hydroperoxide content under detection limit in sonicated WP without sugar imply that protein hydroperoxides were generated only due to Maillard reaction and not due to sonocatalysis itself. In the presence of PEG there was a significantly higher content of protein hydropeoxide, suggesting that intensified Maillard reaction resulted in increased protein oxidation in addition to increased protein glycation. Total hydroperoxide was detected in all samples containing arabinose, but at significantly higher levels in sonicated samples (Fig 4B). In the presence of PEG total peroxide did not increase significantly and it seems that crowding conditions increase protein oxidation but not arabinose autooxidation.

3.5. Formation of amyloid-like structures

Stathopulos et al. (Stathopulos, Scholz, Hwang, Rumfeldt, Lepock & Meiering, 2004) reported that sonication of the range of structurally diverse proteins results in the formation of amyloid-like aggregates. Therefore we examined if amyloid-like structures (ALS) were formed after ultrasound induced glycation of WP and if they are more prominent in crowded conditions. We have monitored binding of Congo red (CR) (Klunk, Jacob & Mason, 1999) and thioflavin T

(ThT) (LeVine, 1993), standard dyes used to monitor formation of ALS (Fig 4 C and D), by WP. Upon CR binding to sonicated WP there was an increase in CR absorbance with concomitant spectral shift with maximum at 530 nm, characteristic maximum difference observed for ALS (difference spectra, Fig 4 C insert), suggesting ALS formation. In the presence of sugar, ALS formation was reduced by sonication due to protecting role of arabinose preventing WP structural changes, while in the presence of PEG formation of ALS, which is inhibited by arabinose, is overwhelmed. Similarly, sonicated WP caused a marked enhancement of ThT fluorescence (Fig 4D), due to binding of ALS to ThT, and although arabinose reduced ALS formation, extensive Maillard reaction in MC conditions induced ALS formation to the level of sonicated WP. ALS formation was in accordance to changes in tertiary structure changes (Fig 3 C), and in correlation with the yield of disulphide polymers observed on electrophoresis (Fig. 2). It was shown that crowding conditions lead to acceleration in the rate of the formation of amyloid fibrils, and that it increases polymer concentration (Munishkina, Cooper, Uversky & Fink, 2004). Interestingly, it was demonstrated that glycation with D-ribose induces BSA to misfold rapidly and to form globular amyloid-like aggregations (Wei, Chen, Chen, Ge & He, 2009). It seems that the presence of arabinose during sonication prevents formation of disulfide polymers and nonnative disulfide bonds particularly relevant for amyloid formation (Lee & Eisenberg, 2003), while intensive glycation and presence of crowding agent enhance them. The results of this study imply that protein glycation by Maillard reaction in crowded conditions is an important enhancer of amyloid like fibril formation.

3.6. Thermal stability and stability at different pH

Thermal stability of native and treated WP at concentration of 1 mg/ml is shown in Fig. 5A. Thermal stabilities of WP and WP/Ara are quite similar. Sonicated WP demonstrate significant

decrease in solubility at temperatures higher than 75 °C. This is most likely due to tertiary structure changes and increase in surface hydrophobicity induced by ultrasound treatment. However, glycated proteins show increased thermal stability, with higher one in samples obtained in macromolecular crowding conditions. This result can be explained by attached saccharide units which interfere with aggregation due to steric hindrance (Liu & Zhong, 2012). Several studies demonstrated reduced thermal stability of heat-treated WP and increased thermal stability of WP glycated in Maillard reaction induced by heat treatment (Chevalier et al., 2001; Li, Enomoto, Ohki, Ohtomo & Aoki, 2005; Liu et al., 2012). More intensive glycation, in samples under crowding conditions, provided better thermal stability, especially at higher temperatures.

CorzoMartinez at al. (Corzo-Martinez, Sanchez, Moreno, Patino & Villamiel, 2012) demonstrated reduced solubility at pH 5 of heat-treated WP, while it was increased in WP glycated in Maillard reaction induced by heat treatment. Chevalier et al. (Chevalier et al., 2001) observed decrease in solubility of BLG after heating in the pH range 4.5–5.5, while modification of BLG with different saccharides improved its thermal properties at acidic pH. Therefore we have investigated solubility of native and treated WP measured as a function of pH (Fig. 5B). Whey proteins showed good solubility at wide pH range (2-10). Sonication reduce WP solubility in pH range around pI of these proteins (3-5), but after sonication in the presence of arabinose (with and without PEG) good solubility of WP in wide pH range is retained, suggesting a protective effect of glycosylation towards the decrease of solubility due to sonication.

3.7. Antioxidative properties

Several studies have shown that ultrasound-induced Maillard reaction products show antioxidative activity (Shi, Sun, Yu & Zhao, 2010; Stanic-Vucinic et al., 2013). The native WP

have some capacity to inhibit lipid peroxidation (Fig 5C). However, sonication in the presence of arabinose, where Maillard reaction products are formed, increased capacity to inhibit lipid peroxidation was observed, and it was higher in macromolecular crowding conditions. The free radical scavenging activity, measured as DPPH scavenging activity, followed similar pattern (Fig 5C). Similar effects were observed in the reducing power (Fig 5D). Sonication in the presence of arabinose and particularly in the presence of macromolecular crowding agent, significantly increases reducing power originating from generated Maillard reaction products. Wang et al. (Wang, Bao & Chen, 2013) improved antioxidative activity of WPI by heat induced glycation under wet reaction conditions. During Maillard reaction free radical intermediers are generated. Although protein hydroperoxides are long-lived, they can be catalytically degraded (e.g. by iron or copper ions) to yield highly reactive and damaging free radicals. Also, saccharides can be autooxidized during Maillard reaction by traces metal ions generating H_2O_2 (Jiang, Woollard & Wolff, 1990). Therefore, high inherent antioxidative activity of MRPs can eventually neutralize effects of free radicals generated by oxidatively modified proteins and saccharides.

4 Conclusions

In this work, for the first time, we have demonstrated that combined application of HIUS with macromolecular crowding conditions increased reaction rate of model system Maillard reaction. We have shown that degree of WP glycosylation by arabinose under macromolecular crowding conditions, using PEG 6000 as crowding agent, was significantly higher than without crowding. In this study we have shown that HIUS treatment can lead to Maillard reaction, protein oxidation, as well as caramelization, especially under crowded conditions e.g. under water volume excluded conditions. These effects of HIUS can influence protein/saccharide based food

quality, and in iron-enriched food formulas it is expected that protein oxidation will be even more favored.

Our results indicate that Maillard reaction promoted by ultrasound in the presence of the macromolecular crowding agent is an effective and promising method for attaching saccharides to proteins, resulting in proteins with improved functional properties, such as antioxidative capacity and thermal and pH stability. The usage of ultrasound in crowded conditions could be a new approach for acceleration of reactions in general, especially with proteins involved. Many food proteins are heat sensitive, and if elevated temperatures and/or advanced stages of reaction are used, protein denaturation and aggregation are likely to occur. The sonication under crowding conditions can be a valuable method for modification of labile proteins, as it enhances reactions under lower temperatures and can shorten reaction time, while stabilizing protein structure at the same time.

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560

561 **Figure captions**

562 **Figure 1.** The effects of ultrasound on A294 and browning intensity (A420) (A), fluorescence
563 (B), remained amino group content and pH change (C) of WP solution, with and without the
564 presence of arabinose and crowding agent. (D) The effects of ultrasound on A294 and A420 of
565 arbinose solution with and without the presence of crowding agent. WP- untreated whey
566 proteins, WP/Ara – untreated whey proteins in the presence of arabinose, sWP – sonicated whey
567 proteins, sWP/Ara - sonicated whey proteins in the presence of arabinose, sWP/Ara/PEG -
568 sonicated whey proteins in the presence of arabinose and PEG. Different small superscripts (a -
569 d) denote the significant difference ($P < 0.05$).

570 **Figure 2.** Molecular weight estimation of WP, sonicated with and without the presence of
571 arabinose and crowding agent, by SDS PAGE: under nonreducing (A) and reducing conditions
572 (C). Native electrophoresis (B) and isoelectrofocusing (D) of WP sonicated with and without the
573 presence of arabinose and crowding agent. Figure legend for the samples is according to Figure
574 1. MM – molecular weight markers, BLG-β-lactoglobulin, ALA-α-lactalbumin, BSA-bovine
575 serum albumin, IgG L- immunoglobulin light chain, IgG H-immunoglobulin heavy chain, LF/LP
576 – lactoferrin and lactoperoxidase.

577 **Figure 3.** Circular dichroism spectra of WP sonicated with and without the presence of arabinose
578 and crowding agent in far UV (A) and near UV (C) spectral range, and determination of the
579 secondary structure fractions (B). ANS binding to WP sonicated with and without the presence of
580 arabinose and crowding agent (D). Figure legend is according to Figure 1.

581 **Figure 4.** Protein carbonyls remained free sulfhydryls (A) and protein hydroperoxides (B)
582 generated by ultrasound treatment of WP with and without the presence of arabinose and
583 crowding agent. Spectral shifts induced in Congo red (C) and thioflavin T fluorescence

584 enhancement properties (D) by WP sonicated with and without the presence of arabinose as
585 crowding agent; inset shows difference spectra obtained by subtracting the spectra of native
586 protein solutions from those of sonicated protein solutions. Figure legend is according to Figure
587 1.

588 **Figure 5.** The heat stability at pH 7 (A) and the solubility at room temperature as function of pH
589 (B) of WP sonicated with and without the presence of arabinose and crowding agent. The results
590 are means of two independent experiments ± SD. Antioxidative activity of WP, sonicated with
591 and without the presence of arabinose and crowding agent, measured as DPPH-scavenging**,**
592 inhibition of lipid peroxidation (C), and reducing power (D). Figure legend is according to
593 Figure 1.

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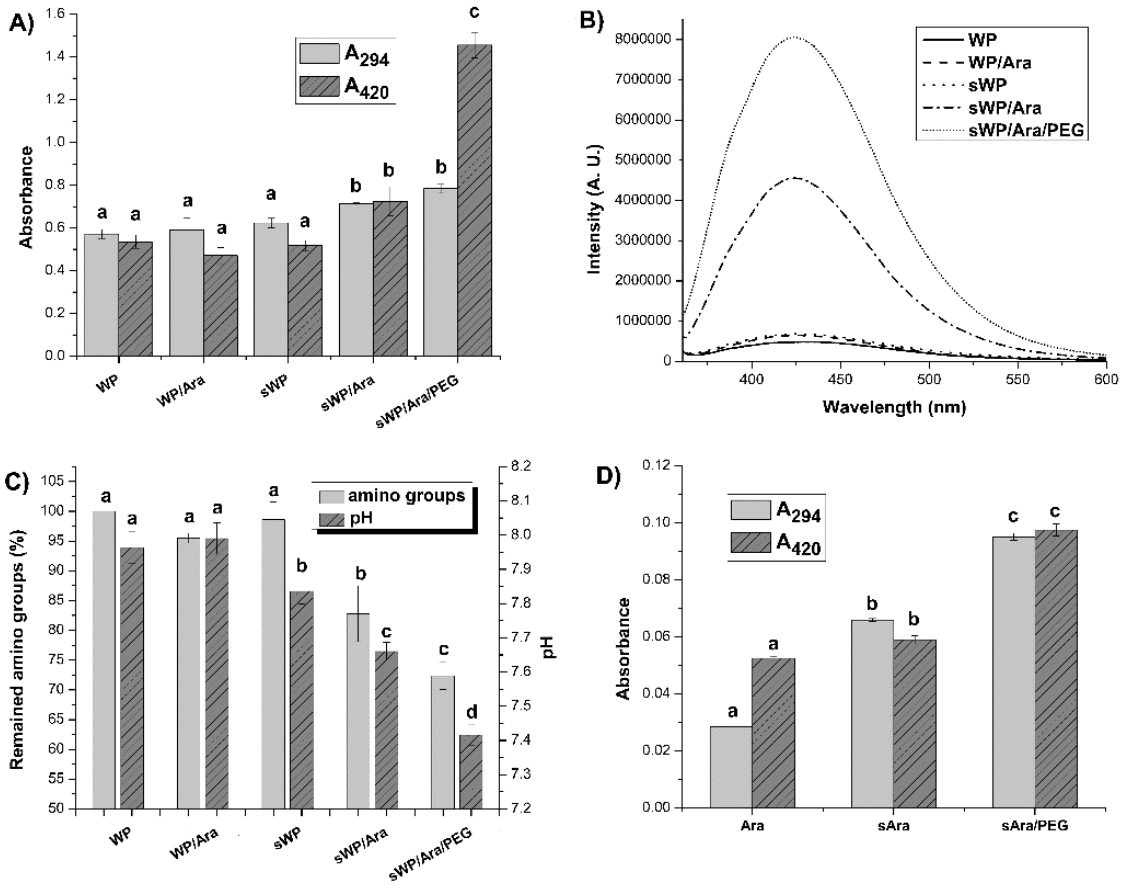
A)
Absorbance
A294
A420
WP
WP/Ara
sWP
sWP/Ara
sWP/Ara/PEG
B)
Intensity (A. U.)
Wavelength (nm)
WP
WP/Ara
sWP
sWP/Ara
sWP/Ara/PEG
C)
Remained amino groups (%)
pH
amino groups
pH
D)
Absorbance
A294
A420
Ara
sAra
sAra/PEG

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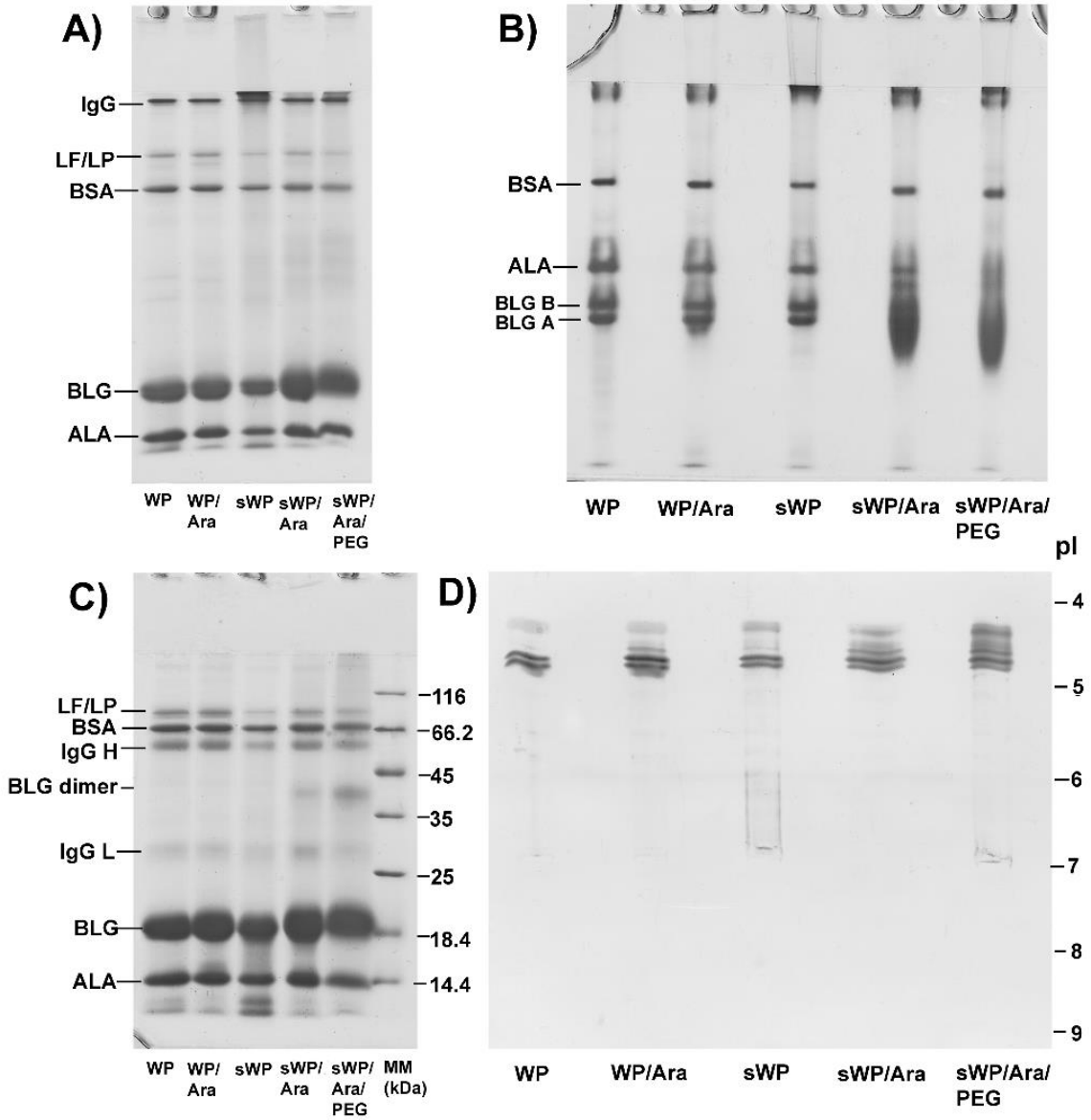

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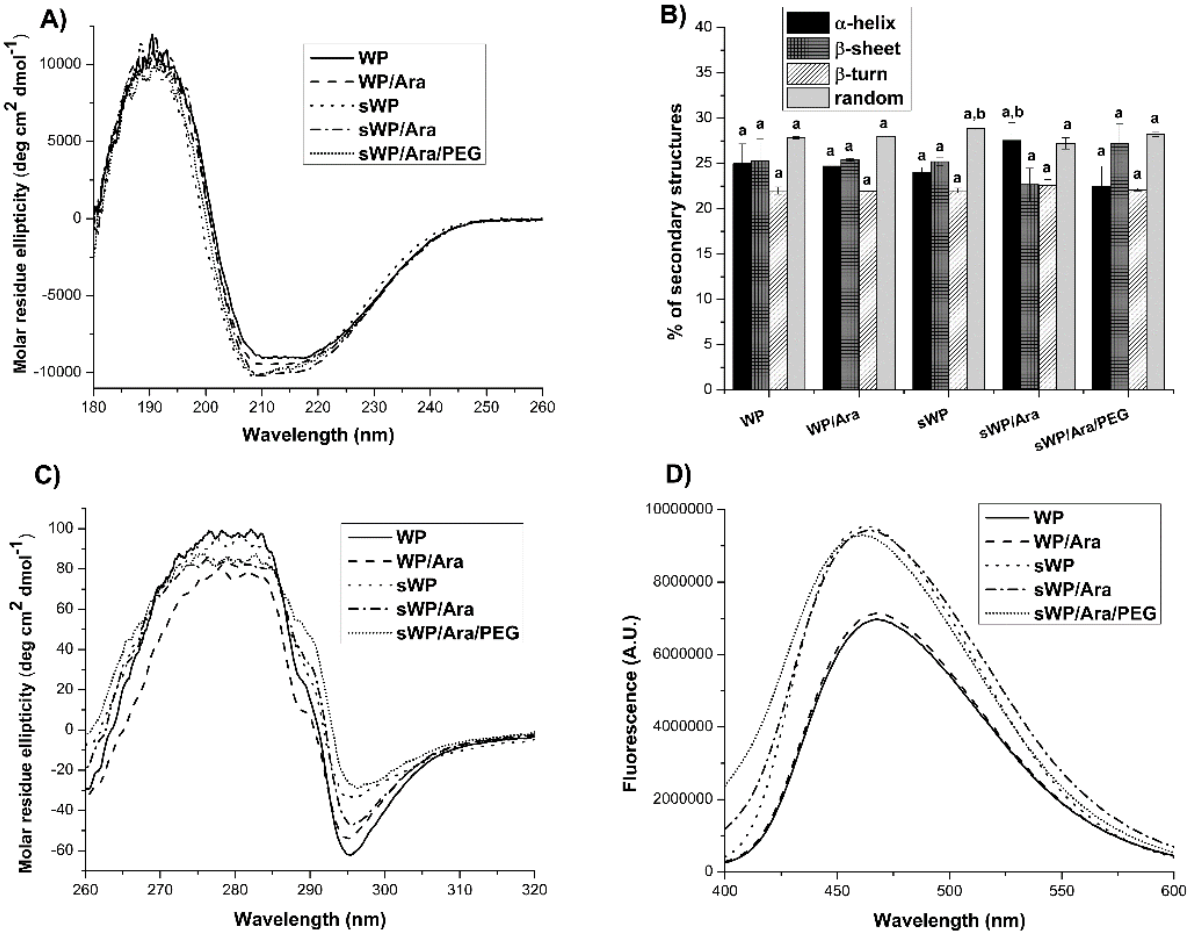
A)
Molar residue ellipticity (deg cm^2 dmol^{-1})
Wavelength (nm)
WP
WP/Ara
sWP
sWP/Ara
sWP/Ara/PEG
B)
% of secondary structures
α-helix
β-sheet
β-turn
random
WP
WP/Ara
sWP
sWP/Ara
sWP/Ara/PEG
C)
Molar residue ellipticity (deg cm^2 dmol^{-1})
Wavelength (nm)
WP
WP/Ara
sWP
sWP/Ara
sWP/Ara/PEG
D)
Fluorescence (A.U.)
Wavelength (nm)
WP
WP/Ara
sWP
sWP/Ara
sWP/Ara/PEG

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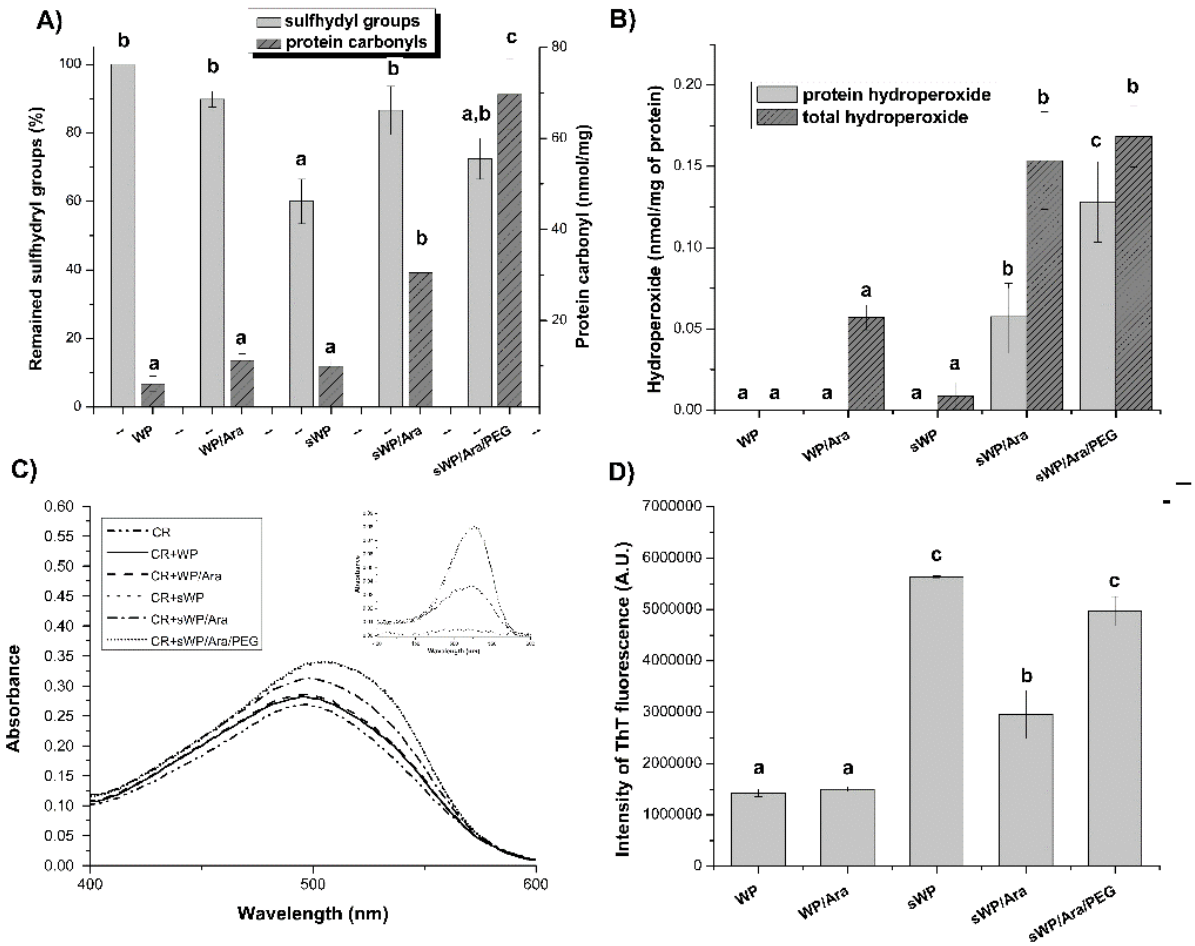

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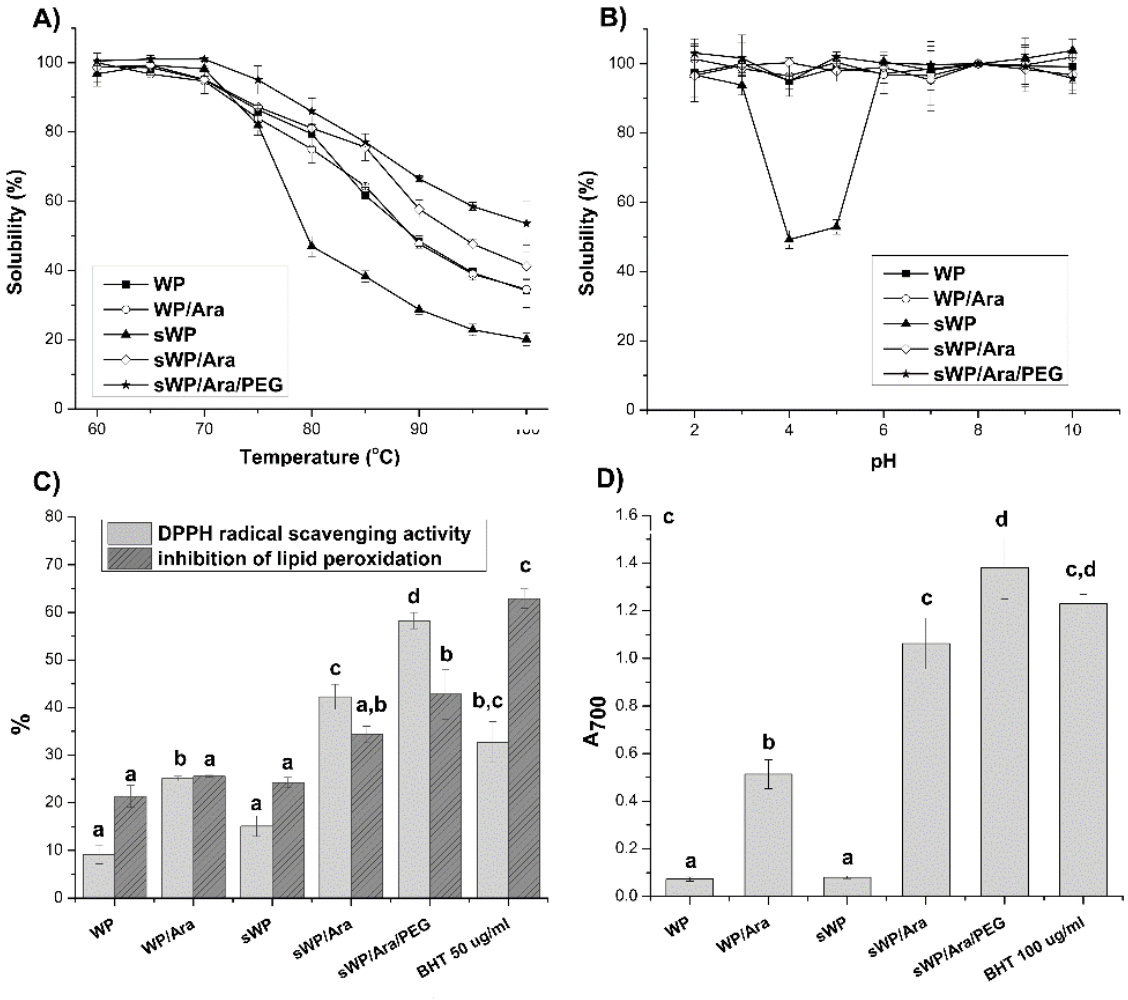
A)
Solubility (%)
Temperature (°C)
WP
WP/Ara
sWP
sWP/Ara
sWP/Ara/PEG
B)
Solubility (%)
pH
C)
DPPH radical scavenging activity
inhibition of lipid peroxidation
%
WP
WP/Ara
sWP
sWP/Ara
sWP/Ara/PEG
BHT 50 ug/ml
D)
A700
BHT 100 ug/ml

610