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Protective effect of β-Lactoglobulin against heat induced loss of antioxidant activity of resveratrol

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ABSTRACT

Resveratrol exhibits many health benefits however, low water-solubility and instability to processing conditions such as heating can be some of the main challenges for its processing and formulation. Here the complexation of β-lactoglobulin (β-Lg) with resveratrol was investigated to improve its solubility and stability. The solubility of resveratrol in water was determined as 7mg/100ml. Resveratrol-β-Lg nanoparticles (181.8 nm) were produced at pH 6 and 75°C for 45 min. Heating resveratrol solutions at 75°C for 45 min resulted in isomerization of resveratrol and reduced antioxidant activity. However, resveratrol-β-Lg nanocomplexes which had undergone the same heat treatment exhibited improved antioxidant activity. Heating at pasteurisation conditions led to similar results and both native β-Lg and nanoparticles exhibited a protective effect against heat induced chemical changes in resveratrol resulting in enhanced antioxidant activity.
Fluorescence measurements revealed strong interactions of resveratrol with both, native protein and nanoparticles.

Keywords: Resveratrol; β-lactoglobulin; nanoparticles; antioxidant activity; heating

1. Introduction

Nowadays, consumers are attracted to dietary supplements instead of drugs to improve health. Oral administration is the most convenient and commonly applied method to develop new nutraceuticals. Based on many studies, the predominant problem of nutraceuticals is poor bioavailability due to their low aqueous solubility which, affects over 40% of the new chemical entities produced by high throughput screening processes (Lipinski, 2002). Several reasons contribute to the solubility problem: 1) high molecular weight; 2) high Log of the octanol-water partition coefficient (log P), which is a measure of a drug's lipophilicity. In order to improve the aqueous solubility, numerous researchers have developed many approaches such as particle size reduction, crystal engineering, salt formation, solid dispersion and surfactant complexation (Savjani, Gajjar., & Savjani, 2012). New techniques like nanoparticles, nanotubes, nanosuspensions and nanocomplexes are employed to improve the solubility of poorly soluble drugs and nutraceuticals (Rabinow, 2004; Tran, Tran, & Lee, 2013). Proteins like zein, whey proteins, soy proteins and other natural polymers like cyclodextrins have been applied not only in pharmaceutical but also in food applications (Duarte et al., 2015; Gorji et al., 2015; Pujara, Jambhrunkar, Wong, McGuckin, & Popat, 2017). Simple and safe carrier candidates, which meet the GRAS (Generally Recognised As Safe) qualification and possibly have essential health benefits, should be investigated to improve aqueous solubility and stability of bioactives.
Resveratrol (3,5,4'-Trihydroxystilbene), is a typical non-flavonoid phenol that belongs to the stilbene family and has various properties including antioxidant, anti-cancer, anti-inflammatory, and antineoplastic (Bhat & Pezzuto, 2002; Jang et al., 1997; Soleas, Diamandis, & Goldberg, 1997). Resveratrol is thought to be one of the main contributors to the famous “French paradox”: despite the French people’s high-fat intake, a low cardiovascular diseases incidence was found. It has been ascribed partly to the red wine consumption (Catalgol, Batirel, Taga, & Ozer, 2012). Since then, the studies of resveratrol have been carried out broadly in the food and pharmacy field.

There are 72 different natural resources which contain resveratrol, especially grapes, peanuts and blueberries (Burns, Yokota, Ashihara, Lean, & Crozier, 2002; Jimenez-Garcia et al., 2012; Lyons et al., 2003). Even though resveratrol is widely distributed in various plants and fruits, there are some pharmacokinetic limitations which lead to its low bioavailability: The low water solubility, labile properties, and rapid metabolism (Amri, Chaumeil, Sfar, & Charrueau, 2012; Pund, Joshi, & Patravale, 2016). The low solubility of resveratrol in oral administration is due to the chemical structure of resveratrol: two phenolic rings bonded together by a double styrene bond which leads to two isometric forms, cis- and trans-resveratrol (Gambini et al., 2015). It is believed that the trans-isomeric form is more abundant than cis-resveratrol, while the trans-isomer is able to transform into cis-isomer under light exposure and heating. Light exposure allows a rapid isomerization of trans-resveratrol, especially in the ultraviolet radiation at 366nm or at low concentration (Flieger, Tatarczak-Michalewska, & Blicharska, 2017; Gambini et al., 2015). Heating not only leads to increased solubility but also results in degradation, isomerization and reduction of antioxidant activity. The effect of heating on resveratrol can be complex depending on temperature (Flieger et al., 2017; Lee et al., 2014). In order to overcome its low solubility and low stability, encapsulation might be a solution. The encapsulation of resveratrol with a number
of biopolymers has been investigated including chitosan and γ-poly (Jeon, Lee, & Lee, 2016), soy protein isolate (Pujara et al., 2017), milk protein (Gorji et al., 2015), zein (Penalva et al., 2015) and β-lactoglobulin (Zhang, Liu, Subirade, Zhou, & Liang, 2014). The main whey protein, β-lactoglobulin, plays an interesting role in transporting retinol molecules and binds small hydrophobic molecules. Resveratrol has been found to bind to the surface of the hydrophobic pocket of β-lactoglobulin (Liang & Subirade, 2010; Liang, Tajmir-Riahi, & Subirade, 2008). Thus, β-lactoglobulin can be an attractive candidate as a carrier to offer a solution to both, poor solubility and low stability to processing /storage conditions such as heating and light exposure.

In previous work of our group, β-lactoglobulin nanoparticles were found to complex with caffeine (Guo, Harris, Kaur, Pastrana, & Jauregi, 2017). Higher binding affinity of β-lactoglobulin in nanoparticles form rather than in the native form was found. The aim of the present work was to apply this method to produce resveratrol-β-lactoglobulin nanoparticles and investigate the effect of these nanoparticles and native β-lactoglobulin on the solubility and stability of resveratrol under different thermal processing conditions. The main hypothesis tested here was that complexation of protein (native and/or nanoparticles) with resveratrol in aqueous solution can improve its solubility and/or stability. The stability was measured in terms of antioxidant activity. In addition, fluorescence measurements were carried out to gain an insight into the interactions between these two molecules.
2. Materials and methods

2.1. Material

Trans-resveratrol (98% w/w) was supplied by Evolva RES140332. The β-Lactoglobulin from bovine milk ≥85.0 % (PAGE), lyophilized powder L2506 was purchased from Sigma. Folin-Ciocalteu reagents: Folin-Ciocalteu (F9252), and Sodium carbonate BioXtra, ≥99.0% (S7795) were purchased from Sigma. Reagents of ABTS method: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) ≥98.0 % (HPLC) (A1888), Potassium persulfate (K2S2O8) ACS reagent, ≥99.0% (21622, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 97% (238813), and ethanol, ≥99.8% (GC) (32221) were purchased from Sigma-Aldrich.

2.2 Determination of resveratrol solubility in water solution

A series of resveratrol solutions/suspensions were prepared from 1mg/100ml, 3mg/100ml, 5mg/100ml, 7mg/100ml, 8mg/100ml, and 9ml/100ml to determine the solubility of resveratrol. All the solutions/suspensions were covered by tissue paper and were stirring with a magnetic stirrer at room temperature for 2 hours before any measurements. Two methods were applied to determine resveratrol solubility in water solution: the direct spectrophotometric method and total phenolic content by Folin-Ciocalteu method.

The direct spectrophotometric method: The λ_max value of resveratrol aqueous solution (2mg/100ml) was determined by using a Lambda 20 spectrophotometer (Perkin-Elmer®) and...
scanning UV absorption from wavelength 190nm to 600nm with 1 nm spectral bandwidth. The absorbance of resveratrol solutions/suspensions was measured at the maximum wavelength by the Spectrophotometers (Ultrospec ® 1100 pro) using a quartz cuvette with a path length of 1cm and deionized water was used as the blank. The solubility of resveratrol at room temperature was determined from the plot of the absorbance against concentration as the concentration after which no further significant increases were observed.

**Folin-Ciocalteu method:** The total phenolic content of all concentrations of resveratrol solutions/suspensions were measured by Folin-Ciocalteu method at 760nm. In brief, 0.2 ml of resveratrol solution was added to around 6.0ml of deionized water into a 10ml volumetric flask. Folin-Ciocalteu reagent (0.5ml) was added and mixed. After 1 minute and no longer than 8 minutes, 1.5 ml 20% sodium carbonate solution was added and the volume adjusted to 10ml. After 2 hours incubation in the dark, the absorbance was recorded at 760nm using a UV-Vis Spectrophotometer (Ultrospec ® 1100 pro). Deionised water was used as the blank. All the experiments were carried out in triplicates, and the results were expressed in gallic acid equivalents (GAE; mg/L) using a gallic acid (10-120mg/L) standard curve. The solubility of resveratrol was determined as the concentration at which the GAE value reached a peak and remained constant afterwards.

2.3 Production of resveratrol-β-lactoglobulin nanoparticles

Native β-lactoglobulin powder (0.01 g) was added into 5ml resveratrol standard solutions/suspensions of varying concentrations in a 7ml Sterile container. Then the pH of the sample was adjusted to 6.0 using a pH meter (Mettler Toledo, Switzerland) with 0.1M HCl and 0.1M NaOH. After this, the sample was introduced into a water bath (Grant Instrument Ltd., Cambridge, United Kingdom) that have been previously heated at 75 °C. The sample was kept for 45 minutes at this
temperature; it must be noted that the temperature of the sample reached the set temperature after about 13 minutes. On completion of the incubation time, samples were transferred to an ice bath for 10 minutes to terminate incubation. After 10 minutes in the ice bath, the pH of the sample was measured. The particle size was determined by dynamic light scattering (DLS).

2.4 The effect of heating and native β-lactoglobulin and β-lactoglobulin nanoparticle on the stability of resveratrol

After determination of solubility of resveratrol, all the following studies were carried out in a range of concentrations of resveratrol at the solubility or below. A set of resveratrol aqueous solutions in the range of solubility were used as a control and compared against samples of the same concentration of resveratrol that underwent different treatments. There were three different treatments: heating (see details below), the addition of native β-lactoglobulin (details below) and β-lactoglobulin nanoparticles (see section 2.3).

When studying the effect of heating, each of the resveratrol solutions/suspensions were heated at 75°C in a water-bath for 45 minutes and moved to an ice bath for 10 minutes afterwards. Then, samples were taken for analysis. This temperature was chosen as this was the temperature at which the nanoparticles were formed.

When exploring the effect of adding native β-lactoglobulin, 0.01g native β-lactoglobulin powder was added to 5ml resveratrol standard solutions/suspensions of varying concentrations in a 7ml Sterile container. Then samples were subjected to heat treatment.

The effect of heating, adding native β-lactoglobulin and β-lactoglobulin nanoparticles on the stability of resveratrol were determined by total phenolic content and the total antioxidant activity. For the total phenolic content, the Folin-Ciocalteu method mentioned above was applied, and when
measuring the samples with native β-Lactoglobulin and β-Lactoglobulin nanoparticles, the interference of protein was eliminated by substracting the GAE value of protein from the GAE value of samples. All the samples were prepared and determined in duplicate.

For the total antioxidant activity, the [2,2’-Azinobis(3-Ethylbenzothiazoline-6-Sulphonic Acid)] (ABTS) Free Radical Scavenging Activity Assay and Ferric ion Reducing Antioxidant Power assay (FRAP) were used which are described in detail below.

**ABTS method**: The total antioxidant activity of all samples was measured by ABTS assay at 734nm, which was modified from Re et al. (1999). In general, the ABTS•+ stock solution was prepared by mixing solution 5ml ABTS solution (7mM, 50ml volume, stored in an amber flask and kept under refrigeration at 0-4°C up to one month) and 88μl Potassium Persulfate (K2S2O8) solution (140mM, 10ml volume, stored in an amber flask and dark place at room temperature up to one month) together. Then the mixture was kept in the dark and room temperature for at least 16h prior to use. For the study of phenolic compounds, the working solution of the ABTS•+ was obtained by diluting the ABTS•+ stock solution with phosphate buffered saline (PBS pH 7.4) to an absorbance of 0.70±0.02 at 734nm. 20μl of samples was added into 2ml ABTS•+ working solution, and the mixture was homogenised by 1mins vortex. The mixture was then incubated in the dark for 6 minutes, and the absorbance (ABSsample) was recorded at 734nm using a UV-Vis Spectrophotometers (Ultrospec ® 1100 pro). The absorbance of ABTS•+ working solution was measured at the same wavelength and used as control (ABSControl). The PBS was to blank the spectrophotometer. The percentage of scavenging activity of each sample on ABTS•+ was calculated as the inhibition% (I%) using the following equation (Shah & Modi, 2015): Eq.(1)

\[
I% = \frac{(\text{ABS}_{\text{control}} - \text{ABS}_{\text{sample}})}{\text{ABS}_{\text{control}}} \times 100
\]  

**(1)**
When measuring the samples with native β-Lactoglobulin and β-Lactoglobulin nanoparticles, the interference of protein was eliminated by subtracting the I% of protein from the I% of samples. All the samples were prepared and determined in duplicate.

**FRAP method:** The total antioxidant activity of all samples was also assessed by FRAP method at 595nm, which was modified from Benzie and Strain (1996). In principle, The stock solution of FRAP method included: 300 mM Acetate buffer (pH 3.6, 2.699 g sodium acetate trihydrate and 16ml (16.8g) glacial acetic acid dissolved in 1L deionised water), 2,4,6-tripyridyl-s-triazine (TPTZ) (10mM) in 10ml HCl (40mM), and 20mM Ferric Chloride Hexahydrate aqueous solution. The FRAP reagent was mixed with the 25ml Acetate buffer, 2.5ml TPTZ and 2.5ml Ferric chloride solutions. Then, 10 μl of the sample/standard was added into 300μl FRAP reagent in a microcentrifuge tube and vortexed for 10 seconds. Then 100μl of this mixture, in triplicates, was transferred into the microwell plate (96well, NUNC, FB) and absorbance was measured at 595nm by a computer controlled Tecan Microplate reader. Results were express as the ascorbic acid equivalents (AAE) using an ascorbic acid (0.001761mg/ml-0.1761mg/ml) standard curve.

Increased absorbance of the reaction mixture indicated greater reduction capability. When measuring the samples with native β-Lactoglobulin and β-Lactoglobulin nanoparticles, the interference of protein was eliminated by subtracting the AAE value of protein from the AAE value of samples. All the samples were prepared and determined in triplicate.

### 2.5 The effect of native β-lactoglobulin and β-lactoglobulin nanoparticle on resveratrol under pasteurisation conditions

Different concentrations of resveratrol standard solutions/suspensions, resveratrol-native β-lactoglobulin solutions and resveratrol-β-lactoglobulin nanoparticles solutions prepared using the
The methods above were incubated at pasteurisation conditions, 63°C for 30 minutes. Then the total phenolic content was determined by Folin-Ciocalteu method. The total antioxidant capacity of samples was measured by the ABTS method. All the samples were determined in duplicate.

### 2.6 Fluorescence measurement of resveratrol-β-lactoglobulin solutions

The concentrations of resveratrol solutions were 0.23mg/100ml, 0.46mg/100ml, 0.92mg/100ml, 1.84mg/100ml, and 2.48mg/100ml. The protein concentration of resveratrol-native β-Lg solutions and resveratrol-β-Lg nanoparticles solutions was kept constant at 0.2% w/v. According to Liang, L., & Subirade, M. (2012), the protein intrinsic fluorescence due to tryptophan (Trp) and tyrosine (Tyr) residues is sensitive to the fluorophore environment and thus potentially an indicator of changes in protein conformation. Trp in the protein emits fluorescence when excited at a wavelength (λex) of 295 nm. The protein conformational changes as a result of interactions with the quencher can be studied by the changes of the intensity of emission. Therefore, the degree of β-Lg conformational changes was determined based on fluorescence emission of tryptophan (Trp), which was measured by fluorescence spectrophotometer with the temperature controller (Varian Cary Eclipse, United Kingdom). Fluorescence spectra were obtained after excitation at 295 nm, scanning an emission wavelength range between 300 nm to 510 nm, using 5 nm excitation and emission slits wavelength. The data was collected by Cary Eclipse software version 2 (Varian Cary Eclipse, United Kingdom) and samples were analysed at a constant temperature of 20°C. According to Liang and Subirade (2012), the fractional residual fluorescence or relative fluorescence intensity (RFI = Fmax/F0×100) was the fraction of the total protein fluorescence that was not quenched, and thus the fraction of β-Lg not bound to the ligand; where, F0 is fluorescence intensity of pure β-Lg; Fmax is the intensity at the emission maximum (λmax). A low ratio of RFI demonstrated a strong binding of the ligand, while a ratio of 100% indicated no binding.
For the calculation of binding constant and binding number, the fluorescence quenching data were analysed by fitting to the Stern-Volmer equation (Liang et al., 2008) in dynamic quenching.

The fluorescence quenching data were analysed by fitting to the Stern-Volmer equation (Liang et al., 2008) in dynamic quenching.

\[
\frac{F_0}{F_{\text{max}}} = 1 + K_q \times \tau_0 \times [\text{Resveratrol}] = 1 + K \times \text{[resveratrol]}
\]  

(2)

\(F_0\) and \(F_{\text{max}}\) are the fluorescence emission intensities without and with resveratrol; [resveratrol] is resveratrol concentration; \(K_q\) (M\(^{-1}\)s\(^{-1}\)) is the fluorescence quenching rate constant; \(\tau_0\) (s) is the fluorophore fluorescence lifetime without quencher, and \(K (K_q \times \tau_0)\) is defined as the Stern-Volmer quenching constant. By plotting \(F_0/F_{\text{max}}\) as a function of [resveratrol] and according to Eq. (2) \(K\) was obtained from the slope of the straight line.

For the static quenching, the binding constant \(K_s\) and binding number \(n\) can be calculated according to the double logarithmic equation (He et al., 2016) Eq. (3):

\[
\log\left(\frac{(F_0 - F_{\text{max}})/F_{\text{max}})}{F_0}\right) = \log K_s + n \log [\text{resveratrol}]
\]  

(3)

From a plot of log \((F_0 - F_{\max})/F_0\) as a function of log [resveratrol] and according to Eq. 3 the \(K_s\) value was obtained from the intercept of the resulting straight line and \(n\) from the slope.

2.7 The stability of resveratrol to light

Resveratrol solutions/suspensions were exposed to overhead light (Philips, TL-D 840, 70w, and distant was 1 meter) in the laboratory during 0, 1, 3, 6 and 12 hours. The total phenolic content was measured at 303nm by a spectrophotometer (Ultrospec\textsuperscript{®} 1100 pro) and the Folin-Ciocalteu method as described above. The total antioxidant capacity of samples was measured by ABTS as described above. All the samples were determined in duplicate.

2.8. Statistical analysis
Figures were plotted using MS Excel, MS Office 2013 (Microsoft Corporation, Redmond, WA, USA). The results were statistically analysed by analysis of variance using IBM® SPSS® Statistics version 20.0. Means and standard deviations from at least two measurements carried out on two samples repeated. The significance level was set at 0.05. Data fitting of experimental data to models was performed with Excel MS Office 2013 (Microsoft Corporation, Redmond, WA, USA)

3. Results and discussion

3.1. The solubility of resveratrol in aqueous solutions

The solubility of resveratrol in water was determined by measuring total phenolic content using the direct spectrophotometric method and Folin-Ciocalteu method (Fig S1). In the direct spectrophotometric method, the $\lambda_{\text{max}}$ of 2mg/100ml resveratrol aqueous solution was determined as 303 nm. Therefore, solubility was measured by measuring absorbance of a range of concentrations at this wavelength. The absorbance of resveratrol reached a peak and levelled off at resveratrol concentration 7mg/100ml after an approximately linear increase. Results from Folin-Ciocalteu method showed the same trend, and at 7mg/100ml the GAE also remained constant at about 35.5 mg/L. Both these results indicated that 7mg/100ml corresponded to the limit of solubility of resveratrol in water. Surprisingly, it was in disagreement with the reported solubility value (3mg/100ml), but this was determined by high-performance liquid chromatography (HPLC) (Jeon et al., 2016; Pujara et al., 2017). Moreover, the solubility value reported by Camont et al. (2009) using the UV spectrophotometric method was 6.85 mg/100ml, but they claimed that this increased solubility was due to sonication which helped to dissolve resveratrol. Another possible explanation of this different result was that, in our method, samples were not filtered prior to absorbance measurements, so any insoluble particles of resveratrol in suspension could have led
to an overestimation of the solubility. However, in the Folin assay, 0.5ml samples were taken and 20 times dilution was applied so any interferences by suspended particles would be minimal. In any case filtration was not an option in this study as filtration of aqueous solutions of resveratrol with a 0.45 μm Polyethersulfone (PES) membrane proved to be very difficult and all resveratrol was retained (results obtained by HPLC but not shown here). Only the 0.22 μm Polypropylene (PVDF) filters showed high permeability for resveratrol but still, a reduction in absorbance (7.69%) was observed. Moreover, with this filter high retention of β-Lg nanoparticles (about 80%) was obtained and that also resulted in retention of resveratrol. Therefore, as filtration is a key step before injecting the samples into the column, HPLC was not used in this study to determine the concentration of resveratrol.

To sum up, in this study, the solubility of resveratrol in deionized water at room temperature was 7mg/100 ml. As our solubility result was higher than some of the reported values, it was assumed that at 7mg/100 ml, a nearly saturated solution of resveratrol was obtained. Therefore, the rest of experiments were carried out at the range of 1mg/100ml to 7mg/100ml.

3.2 The effect of light on resveratrol solubility and stability

For the stability to light, the resveratrol suspensions were exposed to the lab light for 12 hours. Based on the measurement of the direct spectrophotometric method, the concentration of resveratrol had an increasing trend with one exception at 1mg/100ml (Fig 1). Also, according to Figure 1, there was a reduction of GAE value at 1mg/100ml, but no significant difference at 3mg/100ml-7mg/100ml during 12 hours light exposure with a wide variance. Corresponding to the total phenolic content, inhibition% had the same increasing trend at 3mg/100ml-7mg/100ml. A significant positive correlation between total phenolic content (the direct spectrophotometric
method) and total antioxidant capacity was observed as Pearson value was 0.844. Therefore, it was concluded that the resveratrol solution was not stable when exposed to lab light. Simiarly, Pinelo, Rubilar, Sineiro, and Núñez (2005) found that resveratrol solution achieved maximum antioxidant activity at 22°C in water bath after three days. Also, according to Orgován, Gonda, and Noszál (2017), the trans-resveratrol is more lipophilic than its isomer. Moreover, they also found that the cis-resveratrol has over ten times more solubility than trans-resveratrol. Another potential explanation could be that at high concentration, the trans-resveratrol converted to cis-resveratrol under the light condition and more cis-resveratrol dissolved in the solution leading to an increase in antioxidant activity.
Figure 1: the stability of resveratrol to light by total phenolic content at 303nm (A), total phenolic content by Folin-Ciocalteu method (B) and total antioxidant capacity by ABTS (C).

3.3. The characterization of resveratrol-β-lactoglobulin nanoparticles

After heating the mixture of resveratrol and β-lactoglobulin (0.2% w/v) to form nanoparticles, the size of nanoparticles was measured by dynamic light scattering. The average size of resveratrol-β-lactoglobulin nanoparticle was 181.80 nm ± 1.48 nm with polydispersion index 0.048. The nanoparticle size was close to that reported by Fonseca, Khalil, and Mainardes (2017) who encapsulated resveratrol with bovine serum albumin nanoparticles (175nm) by desolvation method with ethanol. There was no significant difference in size among different concentrations of resveratrol nanoparticles and nanoparticles alone. So the nanoparticles (200nm) produced were not affected by the concentrations of resveratrol. On the other hand, the size of nanoparticles was smaller than the nanoparticles we produced in our previous work (Guo et al. 2017) and this is possibly because no hydration step (at 4 °C overnight) was applied prior to the production of
nanoparticles. Here no hydration step was applied in order to reduce any risk of resveratrol degradation during storage.

3.4. Effect of heating, native β-lactoglobulin, and resveratrol-β-lactoglobulin nanoparticles on the stability of resveratrol

The effect of heating was investigated under the same conditions as those used in the β-lactoglobulin nanoparticles preparation: heating at 75°C for 45 mins. After heating, 10 minutes in the ice bath allowed rapid reduction of samples temperature to room temperature. The concentration of native β-lactoglobulin added to the resveratrol solution was the same as that used in the preparation of nanoparticles. The stability of resveratrol solutions was assessed after: (i) heating, (ii) protein addition and (iii) nanoparticles production; this was assessed by comparing the total phenolic content (Folin-Ciocalteu method), and total antioxidant activity (ABTS method and the FRAP method) of treated samples with those of the control (resveratrol aqueous solution 1mg/100ml-7mg/100ml).

3.4.1. Total phenolic content by Folin-Ciocalteu method

A clear increment of GAE value was observed after heating at all concentrations (Fig 2), indicating that the heating has a significant influence on resveratrol. Even though heating could improve the solubility of resveratrol in water dramatically (Filipa et al., 2003) since in this study all samples were subjected to an ice bath after heating, the increase of solubility due to heating would have been reduced or eliminated. So the increase in GAE observed at all concentrations, particularly those below the solubility (< 7 mg/100 ml) could be due to chemical changes in the molecule which resulted in changes in its oxidative status and/or oxidation power and led to enhanced blue colour production. However, at 7 mg/100 ml, the big increase in GAE from 30.01
to 81.46 mg/L may also be partly due to an increase in solubility since near saturation might have been reached at this concentration of resveratrol. Flieger et al. (2017) pointed out that when the temperature reached 75°C or above, heating could induce the isomerization of resveratrol from trans-resveratrol to cis-resveratrol even without light. Also, Orgován et al. (2017) found that the solubility of cis-resveratrol was over ten times more than that of trans-resveratrol in water as trans-resveratrol was more lipophilic than its isomer. Thus, a possible explanation for the increase in GAE observed here could be that some of the trans-resveratrol isomerized to cis-resveratrol and that led to an increase in solubility.

When adding native β-lactoglobulin, an increase in total phenolic content happened although this increase was not significant (P>0.05) compared with the control sample at all concentrations. The interference of protein on total phenolic content measurement has been eliminated by subtracting the GAE value corresponding to the protein to the total GAE value (see Methods in 2.3). So it can be assumed that any increases in GAE as a result of protein addition were due to interactions between native β-lactoglobulin and resveratrol.

Moreover, the samples of resveratrol with β-lactoglobulin nanoparticles had the largest increment in phenolic content among these three treatments, and it was around three times the phenolic content of the control sample at all concentrations (Fig 2). Interestingly the nanoparticles (combined effect of heat and protein addition) led to a much higher increase than the heating alone for those concentrations of RSV below solubility whereas for the highest concentration it had almost the same effect than heating alone.

Overall these results suggest that at concentrations below saturation heating enhanced resveratrol and β-lactoglobulin interactions as well as inducing isomerisation thus, these combined
Effects led to an increase in total phenol content. For the highest resveratrol concentration which was about saturation point heating had the strongest effect and led to a further increase in solubility.

Figure 2: The total phenolic content results by Folin-Ciocalteu method of: ■ resveratrol aqueous solution (control); ■ heated resveratrol; ■ resveratrol with native β-Lg; ■ resveratrol with nanoparticles of β-Lg. Results were expressed as Gallic Acid Equivalents GAE mg/L.

3.4.2. Total antioxidant activity by ABTS and FRAP methods

Besides total phenolic content, the total antioxidant capacity of resveratrol was measured to assess changes as a result of the different treatments and in this way assess the stability of resveratrol. In this work, two methods were applied to measure antioxidant capacity: ABTS method and FRAP method. Results in Fig 3. demonstrated a reduction in antioxidant activity (inhibition %) after heating at all concentrations (P<0.05). According to Mikulski, Gorniak, and Molski (2010), trans-resveratrol was a stronger antioxidant than cis-isomer due to the planar conformation; this conformation would ensure the main parameters contributing to antioxidant
activity: favourable spin and unpaired electron distribution. Therefore, this reduction in antioxidant activity supports further the hypothesis that trans-resveratrol changed to cis-isomer during heating with the subsequent reduction in antioxidant activity and increase in total phenols (as shown in Fig 2).

The addition of native β-lactoglobulin led to no significant changes in antioxidant activity when compared against the control samples, indicating that the antioxidant activity would not be masked by β-lactoglobulin.

On the other hand, the resveratrol-β-lactoglobulin nanoparticles had higher activity than heated samples (P<0.05) at 1-5 mg/100ml. So the reduction in activity due to heating was counteracted by the interactions of resveratrol with the nanoparticles which led to similar or higher activity than the control. This suggests a protective effect of β-lactoglobulin nanoparticles against heat-induced loss of antioxidant activity; possibly the interactions of β-Lg nanoparticles with resveratrol hinder its isomerisation. Interestingly, at 7 mg/100ml, the difference in antioxidant activity between the heated sample (14.52%) and resveratrol-β-lactoglobulin nanoparticle samples (21.31%) was not statistically significant (p=0.072). At 7 mg/100 ml, the heating led to two counteracting effects: (1) improving solubility which would increase activity and (2) isomerization which would result in reduced activity, this is why both the heating effect and the nanoparticles did not have a significant impact on antioxidant activity at this concentration.
Figure 3: The total antioxidant activity results by ABTS method of: ■ resveratrol aqueous solution (control); ▲ heated resveratrol; □ resveratrol with nativeβ-Lg ▪ resveratrol with nanoparticles of β-Lg. Results were expressed as inhibition% (I%)

Another antioxidant activity measuring method, FRAP, was also applied to study the effects of different treatments on resveratrol. The antioxidant activity results by FRAP method expressed as AAE (mg/ml) are shown in Fig 4. It was noted that there was no significant difference (P<0.05) in activity between 5mg/100ml and 7mg/100ml of control samples, which might suggest that solubility of resveratrol in water was 5mg/100ml instead of 7 mg/100ml.

A reduction in activity due to heating was observed for samples up to 5mg/100ml in agreement with ABTS measurements (Fig 3). Surprisingly, a sharp increase was observed at 7mg/100ml. This observation agrees with the sharp increase measured in total phenolic content at this concentration (Fig 2). Therefore, the increase in antioxidant activity at 7mg/100 ml could be explained by an increase in solubilized resveratrol in the form of cis-resveratrol as a result of heating.
Figure 4: The total antioxidant activity results by FRAP method of: ■ resveratrol aqueous solution (control); ■ heated resveratrol; ■ resveratrol with native β-Lg; □ resveratrol with nanoparticles of β-Lg. Results were expressed as Ascorbic Acid Equivalents (AAE mg/ml).

In agreement with ABTS results the resveratrol-β-lactoglobulin nanoparticles had the highest AAE value at all concentrations among all treatments (except heated sample at 7mg/100ml), whilst resveratrol with native β-lactoglobulin samples had the same antioxidant activity as the control samples (P>0.05) (Fig 4). So these results again confirm a protective effect of β-lactoglobulin nanoparticles against heat-induced loss of antioxidant activity. Moreover, according to the FRAP method, there was a significant effect of β-Lg nanoparticles as compared to native β-Lg (Fig 4).

To conclude, the results of both antioxidant capacity methods indicated that β-lactoglobulin nanoparticles had the ability to prevent chemical changes in resveratrol during heating and in this way protect its antioxidant capacity.
3.5. The effect of pasteurization on stability of resveratrol with and without protein

In order to study further the protective effect of β-lactoglobulin against thermal degradation of resveratrol an industrial thermal process, pasteurisation, was applied to resveratrol with and without protein. Pasteurisation is a common commercial method to minimise health hazards from food pathogens and to extend shelf life for liquid food and drinks. Thus, it is important to examine the effect of pasteurisation on resveratrol stability and how would adding protein affect this.

Firstly, as shown in Fig 5A, the total phenolic content of resveratrol after pasteurisation showed the same results as the heating at 75°C: dramatic increase of GAE value after pasteurisation. Secondly, it demonstrated that pasteurising resveratrol with native β-lactoglobulin had no significant effect in total phenolic content at all concentrations as compared to control. However, pasteurization of resveratrol with nanoparticles showed a significant decrease in total phenolic content. A possible reason could be that the interaction with β-lactoglobulin suppressed the isomerization undergone by resveratrol at 63°C. This would agree with the observations above.

On the other hand, the antioxidant activity (%inhibition) of standard resveratrol slightly reduced after pasteurisation at 1mg/100ml and 3mg/100ml but increased at 5mg/100ml and 7mg/100ml (Fig 5B). These results were different to results of heating at 75°C, which indicated that heating at 75 °C led to more degradation than at 63°C, and here samples were not put in an ice bath after pasteurisation so heating might have led to increased solubility.

Moreover by comparing the pasteurised samples with and without protein it is clear that the presence of protein led to higher antioxidant activity. The increment of total antioxidant activity after pasteurisation in the samples containing protein could be explained as above, ie: the
interactions between resveratrol and protein hindered the isomerisation of resveratrol that led to reduced activity.

Figure 5: The total phenolic content (A) and the total antioxidant activity (B) of:
- resveratrol aqueous solution (control);
- resveratrol with native β-Lg;
- resveratrol with β-Lg nanoparticles. The plane colour bars were samples before pasteurisation and the patterned bars the same sample after pasteurisation.

To conclude, adding protein particularly in the nano form would suppress the heating effect on resveratrol. Pasteurisation led to a small decrease in AOC at all concentrations (except at the
highest) whilst resveratrol samples with protein (both in native and nanoparticle form) showed an increase in AOC.

3.6. Determination of β-lactoglobulin-resveratrol interactions by fluorescence

Fluorescence measurements were carried out in order to obtain a further insight into the resveratrol-protein interactions. The fluorescence measurements of resveratrol-β-lactoglobulin solutions were conducted at constant protein concentration (for both native and nanoparticles) with varying concentration of resveratrol from 0.23mg/100ml-2.5mg/100ml.
Figure 6: The fluorescence emission spectra of (A) resveratrol-native β-lg at 0mg/100ml-2.48mg/100ml resveratrol (1-6); (B) resveratrol-β-lg nanoparticle at 0mg/100ml-2.48mg/100ml resveratrol (1-6).

Fig 6 (A) showed the intrinsic fluorescence emission spectra of native β-Lg in presence of different concentrations of resveratrol. As resveratrol concentration increased, a reduction in intensity of fluorescence emission was observed. This was an indication of partial disruption of the protein structure which led to a change in the polarity of the fluorophore (Trp groups in the protein) and hence the reduced intensity. There was also a slight shift in $\lambda_{\text{max}}$ to longer wavelengths (from 337.0 nm to 357.14 nm) which is in agreement with the protein conformational changes induced by the resveratrol as reported by Liang et al. (2008). Interestingly the changes in fluorescence intensity at increased resveratrol concentrations were not as big for the β-Lg nanoparticles (Fig 6B) as for the native protein. The RFI value of native β-Lg (22.82%) was higher than the RFI value of β-Lg nanoparticles (46.65%) indicating stronger interactions between these two molecules when β-Lg is in native than in nanoparticle form. The nanoparticles were formed by heat denaturation of protein; as the protein conformation had been already altered the addition of resveratrol did not lead to important changes in fluorescence intensity. However, the $\lambda_{\text{max}}$ shift was much bigger for the nanoparticles than for the native protein (from 335.08nm to 377.89nm) suggesting a big change in the fluorophore environment towards a more hydrophilic environment. This could be explained by hydrophobic interactions between the resveratrol and the unfolded protein molecule which would lead to further exposure of the fluorophore to a more hydrophilic media. Therefore, there was a large effect on the protein tertiary structure. Overall this seems to agree with the stronger effect observed on the stability of resveratrol by the nanoparticles than the native protein.
Furthermore, from the linear plot of $F_{\text{max}}/F_0$ as a function of resveratrol concentration and according to Eq (2) the Stern-Volmer equation constant $K$ of Resveratrol with native $\beta$-Lg was $3.18 \times 10^4 \text{ M}^{-1}$ ($R^2 = 0.986$). Assuming that $\tau_0$ was 1.28 ns for Trp of $\beta$-Lg (EricDufour, Genot, & Haertlé, 1994; Stojadinovic et al., 2013) the $K_q$ was determined as $2.5 \times 10^{13} \text{ M}^{-1}\text{s}^{-1}$, which was higher than the maximal dynamic quenching constant ($1.20 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$). This suggests that binding of resveratrol with $\beta$-Lg followed a static quenching mechanism where the fluorophore formed a stable complex with another molecule that was non-fluorescent. This result was in agreement with that reported by Liang and Subirade (2012) and Liang et al. (2008), who confirmed that $\beta$-Lg fluorescence quenching induced by resveratrol is a static quenching. Thus, from the linear equation of $\log (F_0-F_{\text{max}})/F_{\text{max}}$ as a function of $\log [\text{resveratrol}]$ ($R^2=0.985$) and according to Eq. 3 the binding constant $K_s$ was determined from the intercept of the slope as $1.67 \times 10^5 \text{ M}^{-1}$ and from the slope the binding number, $n$ was obtained as 1.33. This means 1.33 resveratrol molecules bind one $\beta$-Lg molecule and also $K_s$ was in the range of $10^5$ and $10^6 \text{ M}^{-1}$, which was in agreement with binding constants reported for native $\beta$-Lg and a range of substrates such as folic acid with $K_s= 4.3 \times 10^5 \text{ M}^{-1}$ (Liang & Subirade, 2010) and EGCG with $K_s=1.05 \times 10^5 \text{ M}^{-1}$ (Shpigelman, Israeli, & Livney, 2010). However, the Stern-Volmer quenching equation might not be suitable to apply to the resveratrol-$\beta$-Lg nanoparticles binding since the $\beta$-Lg fluorescence emission maximum changed with resveratrol concentration.

Overall from our work, it was demonstrated that more quenching by resveratrol occurred when binding $\beta$-lactoglobulin in the native than in nanoparticle form. Resveratrol bound native $\beta$-Lg following the Stern-Volmer static quenching model at ratio 1: 1 in agreement with previously reported by Liang et al. (2008); most probably resveratrol bound at the surface of the protein. On the other hand, the heat denaturation undergone by the protein during the formation of the
nanoparticles exposed new binding sites to which resveratrol bound. The quenching was not as
strong as with native β-Lg but these type of interactions (most probably of hydrophobic nature)
led to further structural changes in the protein as shown by the shift to longer wavelengths. This
may also explain the protective effect of nanoparticles against chemical changes in resveratrol
upon heating and the increased antioxidant activity.

Denaturation of the protein affects the binding of different ligands in different ways. For
example, Liang and Subirade (2012) found that thermal denaturation of β-Lg reduced the binding
affinity for tocopherol whilst it increased for resveratrol. Moreover, Shpigelman et al. (2010)
reported a higher association of heated β-Lg with Epigallocatechin-3-gallate (EGCG) than native
β-Lg whilst Perez, Andermatten, Rubiolo, and Santiago (2014) demonstrated no significant
differences between native and heat-induced β-Lg aggregates binding to linoleic acid. Protein
denaturation leads to structural changes which then can change access to binding sites in the
protein; polarity and size of the ligands influences which binding sites their bind to.

4. Conclusions

In this work, the solubility of resveratrol in water was determined as 7mg/100ml by the direct
spectrophotometric method and Folin-Ciocalteu method. Resveratrol was not stable to light, and
light exposure resulted in higher phenolic content and total antioxidant capacity. Nanoparticles of
β-lactoglobulin were produced in the presence of resveratrol with average size around 181.8nm
and the concentration of resveratrol had no significant difference in the size of nanoparticles. The
heat induced isomerisation of resveratrol at 75°C led to increased solubility but reduced
antioxidant capacity. β-lactoglobulin nanoparticles showed a protective effect against heat induced
loss of antioxidant activity. Moreover, when resveratrol with and without protein was treated at
pasteurisation conditions both, native and β-lactoglobulin nanoparticles showed a protective effect against heat induced loss of activity and an increase in antioxidant activity. Fluorescence measurements of resveratrol binding to native β-Lg showed strong fluorescence quenching of β-Lg by resveratrol suggesting strong interactions between these two molecules. Interactions with β-Lg nanoparticles led to smaller quenching but a significant shift of λ_max to longer wavelengths which suggested also strong interactions with resveratrol. Overall it was found that the interactions of protein and resveratrol in combination with heating led to improved antioxidant capacity. Thus, complexation of protein with resveratrol proved to have a stabilization effect. These findings are very relevant for the optimum processing and formulation of resveratrol.

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Conflict of interest statement

The author declares that there are no conflicts of interest.

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