Interaction of whey protein with polyphenols from salal fruits (*Gaultheria shallon*) and the effects on protein structure and hydrolysis pattern by Flavourzyme®

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Summary: Milk whey can interact with polyphenols leading to the formation of complexes. In this research, whey protein was fortified with salal fruits (SB) extract and the effect on protein structure was investigated. Particle size and tertiary structure analysis indicates α-Lactalbumin-ligand interactions when whey is supplemented with SB extract. Circular dichroism spectroscopy suggests conformational changes of α-Lac to a partially unfolded state as indicated by the decrease in α-helix structures. Enzymatic treatment of whey protein mixed with SB revealed differences in the hydrolysis pattern. LC-MS/MS data analysis indicates that a higher number of peptides are released when whey is mixed with SB. Peptides of known bioactivity were identified in all hydrolysates. The supplementation of whey protein with SB extract can influence protein hydrolysis and the release of peptides following enzymatic treatment with commercial proteases which may affect the functional and health related properties of the hydrolysate.

Keywords: Whey protein, salal berry, Flavourzyme®, bioactive peptides, interaction

Introduction

Whey proteins are main components of mammalian milk accounting for about 20% of total milk proteins in bovine species. Whey proteins are secondary products of cheese manufacture and their disposal as waste raises environmental and food sustainability concerns (Das *et al.,* 2016). In recent years, considerable efforts and investments in separation technologies have been made for the recovery of whey

proteins from food waste to yield valuable products with desirable functional and nutritional properties (Ganju & Gogate, 2017). Enzymatic hydrolysis of recovered and purified whey to produce protein hydrolysates (WPH), has recently attracted attention as a method for adding value to whey. From a nutritional perspective WPH are beneficial since they are better digested and absorbed in the human gastrointestinal tract. In addition WPH formulations are potential sources of bioactive peptides with a range of biological activities such as anti-inflammatory, anti-glycemic, anti-hypertensive, antimicrobial and may play a role in the dietary prevention and management of chronic diseases (Sousa *et al.,* 2012; Madureira *et al.,* 2010; Pellegrini *et al.,* 2001). Microbial proteases offer many downstream processing advantages compared to animal or plant sources such as low-cost mass production and long shelf life and are therefore preferred by the food industry for a wide range of applications related to protein modification (Sharma *et al.*, 2017.) Flavourzyme® is an industrial peptidase from the food-grade fungus *Aspergillus oryzae*, which is widely used for protein hydrolysis in various industrial and research applications (Merz et al., 2015). Flavourzyme® is used to hydrolyze proteins from various sources including whey and the hydrolysates have shown promising functional properties (de Castro & Sato, 2014).

Reformulation of existing food and drink products is a burgeoning approach to enhance nutritional properties of processed foods. Fruits or fruits extracts are good sources of bioactive compounds with antioxidant activity and are thus commonly included into dairy product formulations to improve their health related properties

67 (Lila *et al.,* 2017). On the other hand, recently published research indicates that the
68 addition of polyphenols has a major impact on the functional properties of whey
69 proteins; chlorogenic acid is known to improve the solubility, foaming capability and
70 foam stability of whey proteins (Jiang *et al.,* 2018); the complex formation between
71 whey protein and cinnamon-derived polyphenols can suppress the production of
72 TNF-α (Lila *et al.,* 2017); the high content of polyphenols in the Argentinean green
73 tea can improve the antioxidant and antimicrobial properties of whey proteins
74 (Staszewski *et al.,* 2011).

75 *Gaultheria shallon*, formerly known as salal berry (SB), is currently a novel and
76 underutilized fruit species in Scotland which shows potential for food applications
77 due to its promising agronomic performance and phytochemical composition. Recent
78 research suggest that extracts of salal fruits have a high phenol and anthocyanin
79 content, which is comparable with the one detected in black currant varieties and
80 greater than strawberry and raspberry varieties (McDougall *et al.,* 2016). Previous
81 research suggested that yogurt reformulation with salal fruits extract may induce
82 changes in milk protein conformation and can affect the susceptibility to enzymatic
83 hydrolysis by lactic acid bacteria resulting in the release of casein-derived peptides
84 with antidiabetic properties (Ni *et al.,* 2018).

85 To further investigate the interaction of whey proteins with phenolics from salal
86 fruits, α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg) were incubated with SB
87 extract and the effects on secondary and tertiary structure were determined by
88 fluorescence and circular dichroism (CD) spectroscopy. An enzymatically-controlled

hydrolysis process with Flavourzyme® was also employed to identify differences in hydrolysis patterns due to whey protein-ligand interactions and a database-search peptidomic approach was adopted to identify bioactive peptides from whey protein hydrolysates with and without the addition of SB extract. The main objective of the present study was to fortify whey proteins with extracts from salal fruits and to investigate the effect of protein-phenol interactions on protein structure and hydrolysis.

Materials and methods

Materials

Pure whey isolate™ 90 (90% purity) was purchased from Bulk Powders (Colchester, UK). SB powder was supplied by James Hutton Institute (Dundee, Scotland). Flavourzyme®, L-serine, and O-Phthaldialdehyde (OPA reagent) were purchased from Sigma-Aldrich (Dorset, UK). α-Lac and β-Lg (Emily add purity) were purchased from Shanghai Yuanye Biotechnology Co. Ltd (Shanghai, China). All other reagents used were of analytical grade.

Proximate analysis and total phenol content of SB

Moisture, ash and fat content of the dried SB powder were determined in accordance with standard methods of AOAC International (2000). Crude protein (*N* X 6.25) was determined by the generic combustion method of AOAC International (2005). The Englyst method (Englyst *et al.* 1992) was used to determine the fiber

content (nonstarch polysaccharides) and total carbohydrates were determined calorimetrically by a glucose oxidase procedure (Rosevear *et al*., 1969). Total phenol content of ethanolic extracts from the berry powder was measured using a modified Folin-Ciocalteau method (Deighton *et al*., 2000).

Fortification of whey protein with SB

1 g of SB powder was added to 14 g of purified water for preparing SB extract. The extract was mixed for 1 h on a Stuart SRT6 tube roller (Cole-Palmer, Staffordshire, UK) at room temperature and then centrifuged at 4000 rpm for 10 min. The extraction process was repeated 2 times and the supernatant were combined and stored at -20 °C. 6% (w/w) of whey was hydrated with distilled water overnight at 4 °C and then different content (0%, 5%, 10% and 20%, w/w) of SB extracts were added before samples were stored at -20 °C for further analyses. The recipes were adjusted with water for samples with lower extract concentration. The estimated phenol to milk protein ratio for the samples containing SB was 0.01, 0.02 and 0.05 (mole GAE equivalent/g protein) respectively. The pH of the samples decreased from neutral (6.98) to slightly acidic (6.38) with the addition of SB extract (0%-20% respectively).

Structural analysis of whey protein fortified with SB extract

Spectrofluorometric and circular dichroism (CD) analysis were applied to estimate the interaction of whey proteins (α-Lac and β-Lg) and SB extract. Fluorescence measurements were recorded with Hitachi F-4500 spectrofluorometer (Hitachi

High-Technologies Corp., Tokyo, Japan) according to the method by Arroyo-Maya *et al*. (2016). Protein fluorescence was measured at α-Lac or β-Lg of 0.5 mg/mL and SB extract was added based on the whey protein-SB mixtures. Samples were illuminated using an excitation wavelength of 280 nm and the resulting emission spectra were measured in the wavelength range from 300 to 500 nm.

CD measurement was conducted according to the methods by Zhang *et al.,* (2014) and Zhou *et al.* (2017) with minor modifications. JASCO-810 (JASCO Corporation, Tokyo, Japan) spectrophotometer was used to record CD spectra with path length 1 mm at room temperature. CD spectra were scanned in the far UV range (190-260 nm) at 50 nm/min. The protein concentration was kept constant at 0.2 mg/mL and SB extract was added based on the whey protein-SB mixtures (Section 2.2). The Spectra were analyzed with CDNN software.

Turbiscan

Particle size (mean spherical equivalent diameter) of control (0%) and 20% SB was determined using static multiple light scattering with a Turbiscan 2000 apparatus (Formulaction, Ramonville St. Agne, France) according to the method by Raikos *et al.* (2019) with minor modifications. The light source scanned the samples at 5-min interval from top to bottom and measured the percentage of light transmitted during a 1 h period at 25 °C. The transmission level of the continuous phase was set at 89%, volume fraction at 6% and the refractive indices for particle size calculation were 1.54 for the dispersed phase and 1.33 for the continuous phase.

155

156 Hydrolysis of whey proteins using Flavourzyme®

157 Whey protein-SB mixtures were diluted for 10 times using 0.1 mol/L phosphate
158 buffer (pH 7.4). 10 mL of diluted samples were mixed with 100 µL of Flavourzyme®
159 and then incubated at 50 °C for 8 h. The hydrolysate were collected before enzyme
160 addition and every 1 h, and reactions were stopped by heating the samples in a boiling
161 water bath for 10 min (Ahtesh *et al.,* 2016; de Castro & Sato, 2015; Samaranayaka &
162 Li-Chan, 2008).

163

164 Degree of hydrolysis

165 The OPA method was used to determine the degree of hydrolysis (DH) of the
166 samples as described by Spellman *et al.* (2003) with minor modifications. The OPA
167 assay was carried out by the addition of 400 µL of sample into 3 mL of OPA reagent.
168 After vortexing, the samples were incubated for 2 min at room temperature and the
169 absorbance was measured at 340 nm using a spectrophotometer (SpectraMax190,
170 Molecular Devices Limited, Berkshire, UK). L-serine (0.9516 meq/L) was used as
171 positive control and distilled water as negative control.

172

173 Identification of bioactive peptides from whey protein

174 Bioactive peptides smaller than 3 kDa from different hydrolysates was identified as
175 described by Raikos *et al.* (2019). 0.2 mL of samples (0% and 20% SB) hydrolyzed
176 with Flavourzyme® for 4 h and 8 h were centrifuged at 12000 r/min for 30 min in

177 Amicon® Ultra-0.5 centrifugal filter device (3 kDa) (Sigma-Aldrich, Dorset, UK), and
178 the filtrate (<3 kDa) was collected. Solid-phase extraction (SPE) with Bond Elut
179 Plexa (Agilent, UK) polymeric SPE cartridges was used to elute peptides before
180 LC-MS experiments. Peptide samples were analyzed by an UltiMate 3000 RSLCnano
181 liquid chromatography system (Thermo Scientific Dionex, MA, USA) configured for
182 pre-concentration onto a nano-column fitted to an EASY-Spray ion source
183 (ThermoScientific) and connected to a Q Exactive Plus quadrupole-Orbitrap hybrid
184 mass spectrometer (Thermo Scientific). Searches were carried out against the Bos
185 Taurus sequences. The whey peptides identified were screened for bioactivity using
186 the Milk Bioactive Peptide Database (MBPDB, http://mbpdb.nws.oregonstate.edu/)
187 (Nielsen *et al.,* 2017).
188
189 Statistical analysis
190 Results are expressed as mean±standard deviation (SD). Statistical analysis of the
191 data was performed using the statistical software SPSS Statistics 22 (IBM Corp,
192 Armonk, NY, USA). Data were analyzed by analysis of variance (ANOVA) followed
193 by the Scheffè's post hoc test ($P < 0.05$).
194
195 **Results and discussion**
196 Interaction of whey protein with polyphenols from SB extract and effects on protein
197 structure
198 The proximate composition of the SB powder including the crude protein content

and the total phenol content of the extracts is presented in Table 1. Previous research has shown that anthocyanins (delphinidin and cyanidin derivates) and flavonols (myricetin and quercetin derivates) are the main phenolic components in salal fruit extracts (McDougall *et al*., 2016). Fluorescence quenching was employed to investigate potential SB polyphenol-whey protein (α-Lac and β-Lg) interactions. Protein intrinsic fluorescence properties are sensitive to the polar microenvironment and have been widely used to detect changes in the tertiary structure of proteins (Li *et al.,* 2008). Most proteins including α-Lac and β-Lg from whey contain Trp, Tyr, and Phe residues, which emit intrinsic fluorescence upon absorption of ultraviolet light. A decrease in fluorescence intensity of proteins indicates molecular interactions with ligands owed to collisional effect, ground-state complex and non-radical energy transfer (Zhang *et al.,* 2014). As shown in Figure 1, the fluorescence signal emitted from the SB extract was rather weak and therefore its interference to the protein fluorescence signal can be ignored. With the addition of SB extract, the fluorescence intensity of α-Lac was quenched significantly, and the maximum emission wavelength shifted from 348 nm to 330 nm with increasing SB extract concentration (Fig. 1A). The decrease of fluorescence intensity is caused by the interaction of α-Lac with polyphenols present in SB extract, and a blue shift of maximum emission wavelength indicates an increase of hydrophobic amino acid residues or phenol-deriving hydrophobic groups in the microenvironment of the fluorophores (Chen *et al.,* 2016). Interactions between whey protein and phenolic compounds from fruit extracts are predominantly noncovalent and include hydrophobic, van der Waals,

hydrogen bridge-binding and ionic interactions (Czubinski & Dwiecki, 2017). Phenolic structures contain hydrophobic benzenoid rings and hydrogen-bonding hydroxyl groups capable to strongly interact with proteins (Pereira *et al*., 2009). The main determinants of protein affinity to phenolic compounds are the amino acid composition and structure (particularly the presence of proline residues), as well as differences in hydrophobicity and the isoelectric point (Charlton *et al.,* 2002; Prigent *et al*., 2003). Unlike α-Lac, the fluorescence intensity of β-Lg was unaffected by the addition of SB extract, which suggested that there was no interaction of the most abundant whey protein with polyphenols from SB extract (Fig. 1B). Previous studies reported that phenolic compounds from tea, grapes, and berries could induce fluorescence quenching by molecular interaction with β-Lg (Oliveira *et al.,* 2015; Hao *et al.,* 2018, Hao *et al,*. 2014; Stănciuc *et al,*. 2017). The contradiction of this finding may be attributed to differences in the type, concentration and structure of phenolic compounds between the studies. Furthermore, the protein-polyphenol interaction is strongly dependent on the molar ratio for β-Lg (Nucara *et al*., 2013), which is different between α-Lac and β-Lg due to differences in molecular weight.

The whey protein-polyphenol interactions indicated by fluorescence spectroscopy may induce conformational changes in α-Lac and β-Lg and thus affect the secondary structure of the proteins. CD is a sensitive technique to detect quantitative changes in the proportion of α-helices and β-sheets in proteins induced by interactions with a ligand (Zhang *et al.,* 2014). The CD spectra of the whey proteins in increasing concentration of SB extract is presented in Figure 2. Data from native α-Lac featured

two negative bands at 208 and 222 nm, which are characteristic of the typical α-helix structure of proteins (Fig. 3A) (Zhang *et al.,* 2014; Hao *et al,.* 2018). The α-helix content of α-Lac decreased from about 26% to 21%, while the random coil increased from about 33% to 38% with increasing SB extract content (Table 2). According to previous studies, noncovalent interactions of polyphenolic compounds can destabilize protein structure (Velickovic *et al.*, 2018). In this study, a decrease in the amount of α-helix structures indicates partial unfolding of α-Lac (Hasni *et al*., 2011; Zhang *et al,.* 2014). Thus, our data suggests that interactions between α-Lac and SB phenols disrupt the secondary structure of the protein in a concentration-dependent manner and result in protein dissociation which are manifested as alterations in β-sheet structure and an increase in random coil conformation. In accordance with previously published literature the CD spectrum of β-Lg showed a typical β-sheet structure with a broad negative minimum around 216 nm (Fig. 2B). The supplementation of β-Lg with SB extract had no effects on the CD spectra, suggesting that the protein secondary structure remained unaffected (Table 2) (Li *et al.,* 2008; Hao *et al.,* 2018). CD data agrees with fluorescence results which suggest that there is no evidence to support interaction between β-Lg and SB phenols.

CD spectra of the whey protein samples fortified with SB supports the particle size data obtained using dynamic light scattering. Results indicate that particle size (hydrodymanic diameter) increases significantly for whey protein samples supplemented with SB extract at 20% (Fig. 3). This effect is also SB concentration-dependent. A similar trend with increasing total polyphenol

concentration was observed by Schneider *et al.* (2016) when whey protein was mixed with cranberry, blackcurrant and muscadine grape juice and they concluded that the observed increase in particle size was driven by the presence and interactions with polyphenols rather than any other juice component present. Similarly, the presence of green tea polyphenols promoted the formation of large whey protein aggregates, which are formed through weak intermolecular bridging interactions carried out by the polyphenols (von Staszewski et al., 2011). Changes in particle size can be driven by interactions of SB polyphenols with α-Lac, which result in protein dissociation and destabilization (protein partial unfolding). This conformational transition may subsequently lead to a more opened structure, followed by aggregate formation at the highest molar concentrations, as demonstrated by the changes in protein particle (aggregates) size (Nucara *et al*., 2013).

Enzymatic hydrolysis of whey proteins and identification of bioactive peptides

The effect of enzymatic treatment with Flavourzyme® on the hydrolysis of whey protein is presented in Figure 4. The degree of hydrolysis (DH) gradually increased with incubation time for all samples during the first 4 h and remained relatively stable during the last 4 h. The addition of SB extract affected the DH of whey proteins, and the effect was incremental with increasing SB concentration. The DH of whey protein mixed with 20% SB was significantly (P<0.05) higher compared to the control for the second half of the incubation period (4 h onwards). The DH of the sample with the highest SB concentration (20%) increased by 41% at the end of the treatment period,

287 which was the highest increase observed for all samples (Fig. 4). The improvement of
288 DH may be attributed to polyphenol-induced exposure of catalytic sites initially
289 buried in the interior of the molecular backbone of whey proteins (Tang *et al.,* 2009).
290 CD spectra analysis indicates partial unfolding of α-Lac due to protein-ligand
291 interaction. Partial protein unfolding may have facilitated the access of the protease to
292 catalytic sites in α-Lac and thus may have enhanced the hydrolysis process. Similarly,
293 phenolic compounds can bind to proteases and influence their catalytic activity by
294 altering their conformation particularly in the vicinity of the active site (Velickovic *et*
295 *al.,* 2017). Depending on the binding affinity of the phenolic compound and the effect
296 on the enzyme's molecular configuration, this may result in either enzyme inhibition
297 or activation (Bandyopadhyay *et al*., 2012). Although most of *in vitro* studies suggest
298 that polyphenols are more likely to have an inhibitory effect on protease activity, there
299 is evidence to show that an increase in enzymatic activity can also be mediated and
300 this effect is concentration-dependent (Tagliazucchi *et al*., 2005). However, the
301 mechanistic understanding of enzyme activation as a result of polyphenol binding
302 requires further investigation.

303 Peptidomics as a sub-field of proteomics is becoming an increasingly valuable tool
304 for the identification of peptides deriving from proteins subjected to food processing
305 (Giacometti & Buretić-Tomljanović, 2017). In this research, peptides released from
306 whey hydrolysates (0% and 20% SB extract) at 4 h and 8 h intervals of incubation
307 with Flavourzyme® were identified using a database-search approach to analyze the
308 data generated by LC-MS/MS. Most peptides identified belong to α-Lac and β-Lg

309 (Table 3). All peptides were screened for bioactivity using the Milk Bioactive Peptide
310 Database (Nielsen *et al.,* 2017). According to the literature, peptides with documented
311 ACE-inhibitory, DPP-IV inhibitory, cytotoxic, antimicrobial or cell proliferation
312 activity were identified to be present in WPH. Most bioactive peptides identified were
313 detected in both WPH (control and 20% SB) irrespective of the addition of SB extract.
314 Nevertheless, data indicates that a higher number of peptides are released when whey
315 is incubated with SB extract (Fig. 5). This finding suggests that protease activity is
316 enhanced at high polyphenol concentration. Interestingly, this effect is not confined to
317 α-Lac only but is also supported by β-Lg data. This may indicate polyphenol-enzyme
318 interactions resulting to a more favorable enzyme configuration for proteolytic
319 activity.
320
321 **Conclusions**
322 The present study investigated the effect of SB extract supplementation on the
323 structure and hydrolysis pattern of whey proteins by enzymatic treatment with
324 Flavourzyme®. The results demonstrated that SB extract increases the DH of whey
325 proteins by 41% and facilitates the release of peptides. The secondary and tertiary
326 structure analysis of whey proteins indicated that phenolic compounds present in SB
327 extract may interact with α-Lac and alter its conformation to a partially unfolded state.
328 This may have affected the susceptibility of α-Lac to proteolytic cleavage by
329 Flavourzyme®. Peptidomic data also suggested that β-Lg is also more readily
330 hydrolyzed in the presence of SB extract but there was no evidence to support

interaction with phenolics. SB polyphenols may also interact with the protease from *Aspergillus oryzae* and enhance its catalytic activity. The addition of SB extract in dairy formulations can affect the structure and hydrolysis of whey proteins and thus can impact on health-related properties of such foods beyond the well documented antioxidant effects.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. Fluorescence emission spectra of α-Lac (A) and β-Lg (B) lactoglobulin recorded from 300 to 500 nm with an excitation wavelength of 280 nm. The evolution of fluorescence intensity as a function of increasing SB extract concentration (0-20%) is recorded.

Figure 2. Circular dichroism spectra of α-Lac (A) and β-Lg (B) supplemented with different content of SB extract (0-20%).

Figure 3. Particle size (nm) of whey proteins (control) and supplemented with SB extract concentration (20%) using dynamic light scattering. Different small letters denote significant differences ($P < 0.05$) between samples.

Figure 4. Degree of hydrolysis of whey protein supplemented with SB extract (0-20%) obtained by treatment with Flavourzyme® at different incubation times (0-8 h). Asterisks (**) denote significant differences ($P < 0.05$) between samples at each time point. Bars represent the standard deviation of the mean.

Figure 5. Venn diagrams of identified peptides in whey proteins (control and 20% SB) treated with Flavourzyme® for 8 h.

507 **Table legends**

508 Table 1. Proximate composition and total phenol content (TPC) of SB powder.

509 Table 2. Secondary structure analysis (based on CD spectra) of α-Lac and β-Lg
510 supplemented with SB (0% and 20%).

511 Table 3. Bioactive peptides identified from whey hydrolysates (control) and
512 supplemented with SB (20% w/w) after 4 and 8 h incubation periods with
513 Flavourzyme®.

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528 Table 1

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% Dry basis	Dry matter	Ash	Crude protein	Fat	Carbohydrate	Fiber	TPC (mole GAE/L)
SB powder	92.63±0.0	2.50±0.0	6.02±0.1	5.45±0.0	13.93±0.0	12.27±0.2	13.62±1.3

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