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Angiotensin-converting enzyme (ACE) inhibitory activity of hydrolysates generated from whey protein fortified with salal fruits (*Galtheria shallon*) by enzymatic treatment with Pronase from *Streptomyces griseus*
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Summary

Whey proteins mixed with salal fruits extract (0% - 20% w/w) were hydrolyzed with Pronase E from *Streptomyces griseus* for a period of 8 h. The ACE inhibitory activity of the hydrolysates was highest (IC_{50}: 0.087 mg/mL) for samples fortified with the highest extract concentration (20%). Peptides (>7 amino acids) with documented ACE inhibitory activity (DAQSAPLRVY, ALPMHIR, DKVGINY, LHLPLPL, YPFPGPI, YPFPGPIPN, VYPFPGPIPN) were identified bv LC-MS/MS data analysis using a database search approach. Fluorescence spectra of the whey proteins mixed with salal fruits extract indicates fluorescence quenching for α-lactalbumin. SDS-PAGE analysis suggests that α-lactalbumin is less susceptible to proteolysis when the extract is included in the formula. Data indicate that α-lactalbumin may be interacting with phenolic compounds naturally present in salal fruits. These interactions and the formation of complexes between a-Lac and phenolic compounds may affect the hydrolysis pattern of whey protein and the release of peptides with ACE-inhibitory activity.

Keywords: Whey protein, salal berry, protease, peptides, ACE-inhibition

Introduction

Whey is a secondary product of the dairy industry, which is typically generated during cheese manufacturing. Considering that millions of tons of whey are produced worldwide and that this product retains the soluble fraction of the milk proteins, valorization strategies such as membrane separation technologies have been exploited for the development of valuable protein ingredients from whey (Brans *et al*., 2004). Whey-derived protein products differ in protein content and can possess variable nutritional and functional properties, which is highly desirable by the food and drink industry for product formulation (Prazeres *et al*.,2012).

In recent years, there is an increasing demand for the development of new or reformulation of existing food and drink products with enhanced nutritional properties.

58 In addition, there is a growing interest for inclusion in foods of natural products with
59 bioactive ingredients which may beneficially affect one or multiple physiological
60 processes upon consumption. There are numerous examples of composite foods which
61 contain milk proteins and fruits in various forms, among other ingredients. A typical
62 example of such products in terms of composition are mixed beverages, which are
63 gaining popularity in the food market (Zulueta *et al*., 2007). Another example is the
64 addition of fruits or fruits extracts in dairy products such as yogurts (Trigueros *et al*.,
65 2011).

66 The incorporation of fruits in food formulations containing whey proteins is very
67 likely to affect the nutritional and health-related properties of the product. Whey
68 proteins have demonstrated ACE inhibitory activity upon hydrolysis by diverse
69 commercial enzymes (Brandelli et al., 2015). ACE inhibition is important in blood
70 pressure regulation and synthetic inhibitors are currently used for treatment of arterial
71 hypertension (Erdmann et al., 2008). Thus, peptides released from β-lactoglobulin and
72 α-lactalbumin in composite foods may exert their physiological activity against ACE if
73 they can withstand gastric digestion, reach the small intestine and be made bioavailable
74 in the systemic circulation. On the other hand, previous in vitro research has indicated
75 that whey proteins can bind to and interact with dietary polyphenols and binding
76 affinities are influenced by the structure of the later (Xiao *et al*., 2011). These
77 interactions can be of hydrophobic nature, hydrogen bonding or electrostatic and may
78 result in protein structural rearrangement and partial protein unfolding (Zhang *et al*.,
79 2014). Interactions between milk proteins and phenolic compounds have also been
80 confirmed in real food systems with a high degree of structural complexity and may
81 affect protein bioavailability (Oliveira *et al*., 2015).

82 Previous research suggests that yogurt reformulation with salal fruits extract may
83 induce changes in protein conformation and can affect the susceptibility to enzymatic
84 hydrolysis by lactic acid bacteria resulting in the release of peptides with altered
85 functionality (He *et al*., 2018). To further investigate the effect on peptide functionality
86 due to possible interactions of whey proteins with phenolics from salal fruits, an

87 enzymatically-controlled hydrolysis process with a protease from *Streptomyces griseus*
88 was employed. The objective of this study was to determine the ACE-inhibitory activity
89 of hydrolysates released from the controlled hydrolysis of whey protein isolate
90 supplemented with salal fruits using a protease from *Streptomyces griseus* and interpret
91 the findings based on modifications of protein structure and interactions with phenolics.
92 Mass spectrometry data combined with a database search approach was adopted to
93 compare hydrolysis patterns between treatments and identify literature-cited bioactive
94 peptides. The aim of this work is to better understand the enzymatic hydrolysis of whey
95 proteins in mixtures with plant phenolic compounds and identify optimum conditions
96 for the release of ACE-inhibitory peptides from an edible source.
97
98 **Materials and methods**
99 Materials
100 Pure whey isolate™ 90 (food grade) was purchased from Bulk Powders (Colchester,
101 UK). Dried and powdered salal fruits were supplied by James Hutton Institute (Dundee,
102 Scotland). Protease from *Streptomyces griseus* (Pronase E), L-serine, O-
103 Phthaldialdehyde (OPA), Angiotensin converting enzyme (ACE) from rabbit lung,
104 Hippuric acid, N-Hippuryl-His-Leu hydrate (HHL) and trifluoroacetic acid were
105 purchased from Sigma-Aldrich (Dorset, UK). Amicon® Ultra-0.5 (3kDa) centrifugal
106 filter units were purchased from Sigma-Aldrich (Dorset, UK). Muslin squares (45 cm)
107 were purchased from Lakeland (Aberdeen, UK). Precast gels and all reagents used for
108 protein electrophoresis were purchased from Bio-Rad Laboratories Ltd (Hertfordshire,
109 UK). α-lactalbumin (α-Lac) and β-lactogobulin (β-Lg) were purchased from Shanghai
110 YuanYe Biotechnology Co. Ltd (Shanghai, China). All other reagents used were of
111 analytical grade.
112
113 Preparation of whey protein fortified with salal berry (SB) and enzymatic hydrolysis
114 14 gr of purified water was added to 1 g of dried fruit for preparing each aqueous extract.
115 The extracts were mixed for 1 h on a Stuart SRT6 tube roller (Cole-Palmer,

116 Staffordshire, UK) at room temperature and then centrifuged at 2290 x g for 10 min
117 using an EppendorfTM 5702R (Fisher Scientific, Loughborough, UK). The supernatant
118 was collected and filtered with muslin squares to remove any residues. The extraction
119 process was repeated 2 times and liquid extracts were combined and stored at -20 °C.
120 Pure whey isolate™ 90 (6% w/w) was hydrated overnight at 4 °C and aqueous berry
121 extracts were added (5%, 10% and 20%, w/w) and allowed to mix on a tube roller for
122 4 h at room temperature before samples were stored at -20 °C until further analyses.
123 The recipes were adjusted with water for samples with lower extract concentrations.
124 Whey protein-SB mixtures were diluted for 10 times using 0.1 mol/L phosphate buffer
125 (pH 7.4). 20 ml of diluted samples were mixed with 200 μl of 5 mg/mL Pronase E and
126 then incubated at 37 °C for 8 h. Aliquots of the hydrolysate were collected before
127 enzyme addition and every 1 h, and reactions were stopped by heating the samples in a
128 water bath at boiling temperature for 10 min. Hydrolysates were stored at -20 °C for
129 further analyses.
130
131 Determination of particle size
132 Particle size was determined using static multiple light scattering with a Turbiscan 2000
133 apparatus (Formulaction, Ramonville St. Agne, France). The light source scanned the
134 samples (whey protein-SB mixtures) at 5-min interval from top to bottom and measured
135 the percentage of light transmitted during a 1 h period at 25 °C. The transmission level
136 of the continuous phase (water) was set at 89%, volume fraction at 6% and the refractive
137 indices for particle size calculation were 1.54 for the dispersed phase and 1.33 for the
138 continuous phase (Purwanti *et al.*, 2012).
139
140 Degree of hydrolysis
141 The OPA method as described by Rao *et al.* (2018) was used to determine the degree
142 of hydrolysis (DH) of the samples incubated with Pronase E for different incubation
143 periods. The reaction was initiated by adding 400 μL of hydrolysate into 3 mL of OPA
144 reagent. After vortexing, the samples were incubated for 2 min at room temperature and

145 the absorbance was measured at 340 nm using a spectrophotometer (SpectraMax 190,
146 Molecular Devices Limited, Berkshire, UK). L-serine (0.9516 meq/L) was used as
147 positive control and distilled water as negative control.
148

149 ACE-inhibitory activity of whey protein hydrolysate
150 The ACE-inhibitory activity of the hydrolysates (0-20% SB) was determined using a
151 Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) method as
152 described by He *et al.* (2012) with slight modifications. HHL was dissolved in 0.1 mol/L
153 Na-borate buffer (pH 8.3) containing 1.55 mmol/L NaCl. ACE was dissolved in the
154 same buffer at a concentration of 0.2 U/mL. 10 µL of hydrolysate were mixed with 5
155 µL of ACE solution, and then incubated at 37°C for 5 min. 50 mL of HHL solution were
156 added and the solution was incubated for 1 h. The reaction was stopped by adding 250
157 mL of 0.1% TFA. The amount of hippuric acid (HA) yielded by ACE catalysis was
158 measured by RP-HPLC with a Phenomenex Kinetex C18 column (250mm × 4.6mm, 5
159 µm). The activity of ACE was measured by the production of hippuric acid. The mobile
160 phase was 50% methanol (V/V) at a flow rate of 0.8 mL/min. The effluent was
161 monitored at 228 nm. ACE-inhibitory activity was calculated as follows:
162

163 $$\text{ACE inhibitory activity (\%)} = \frac{B - A}{B - C} \times 100\%$$

164

165 where A is the content of HA generated in the presence of hydrolysate, B is the content
166 of HA generated without hydrolysate, and C is the content of HA generated without
167 ACE. The IC_{50} was defined as the concentration of inhibitor required for 50% inhibition
168 of ACE.
169

170 Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE)
171 SDS-PAGE was carried out according to the method described by He *et al*. (2018) using
172 a Mini-Protean® 3 electrophoresis cell unit (Bio-Rad, Hertfordshire, UK). Hydrolysates
173 (0% and 20% SB) incubated with Pronase E for different incubation periods (0 – 8 h)

174 were analyzed on a 4-20% Mini-Protean® TGX™ precast gel under reducing
175 conditions (2-mercaptoethanol). Electrophoretic migration was performed at 200 V
176 (constant) for 30 min. The resulting gels were stained, de-stained and scanned
177 according to the detailed method referenced above. Stained bands were compared
178 against standards of known molecular weight.

179

180 Fluorescence spectroscopy

181 The interactions between whey proteins (α-Lac and β-Lg) and SB were analyzed with
182 a Hitachi F-4500 spectrofluorometer (Hitachi High-Technologies Corp., Tokyo, Japan)
183 according to the method by Zhang et al. (2014). Protein fluorescence was measured at
184 constant protein concentration (α-Lac or β-Lg) of 0.5 mg/mL and SB extract (2.2) was
185 added at different concentrations (0-20%, w/w). The emission spectra were obtained by
186 excitation at 280 nm and 295 nm and collecting the spectra in the range 300 and 500
187 nm. Slit widths were set at 1 nm for excitation and emission. The resulted spectra were
188 expressed as arbitrary units.

189

190 Peptide identification using Proteomics-Q Exactive LC-MS

191 Hydrolysates were prepared for peptide identification as described by Kunda et al.
192 (2012) with the addition of a size separation step to select peptides smaller than 3 kDa .
193 Briefly, 0.2 mL of samples (0% and 20% SB) hydrolyzed with Pronase E for 4 h and 8
194 h were centrifuged at 12000 ×g for 30 min in Amicon® Ultra-0.5 centrifugal filter
195 device (3 kDa), and the filtrate (<3 kDa) was collected. Solid-phase extraction (SPE)
196 with Bond Elut Plexa (Agilent, UK) polymeric SPE cartridges was used to elute
197 peptides before LC-MS experiments. Peptide samples were analyzed using the method
198 described in detail by He et al. (2018) by an UltiMate 3000 RSLCnano liquid
199 chromatography system (Thermo Scientific Dionex, MA, USA) configured for pre-
200 concentration onto a nano column fitted to an EASY-Spray ion source (Thermo
201 Scientific) and connected to a Q Exactive Plus quadrupole-Orbitrap hybrid mass
202 spectrometer (Thermo Scientific). Peptides were identified from database searches

against the *Bos taurus* sequences, using the Mascot search engine and no enzyme specificity, as described by He et al. (2018). Theoretical peptide sequences having Mascot ion scores of at least 13 were accepted as valid identifications of bioactive peptides. The bovine database (https://www.uniprot.org/) was used for protein identification. The whey peptides thus identified were screened for bioactivity using the Milk Bioactive Peptide Database (MBPDB, http://mbpdb.nws.oregonstate.edu/) (Nielsen et al., 2017).

Results and discussion

Effects of SB fortification on whey protein structure

Light scattering techniques have been previously used to study the complexation of proteins with polyphenols (Lin *et al.,* 2004; Poncet-Legrand *et al.,* 2006). The size of the particles detected in whey protein-SB extract mixtures are presented in Figure 1. Data show an incremental effect in particle size with increasing SB extract concentration. This observed effect becomes significant ($P<0.05$) for 10% and 20% SB compared to control (0% SB) and 5% SB respectively. At 20% SB extract concentration the average size of the particles is 1.7 times larger compared to the control sample (47.5 nm vs 25.5 nm respectively). Similar results by previously published studies indicate an increase in the size of complexes formed between whey proteins and plant polyphenols, with increasing content of the later (von Staszewski *et al*., 2011). The reactivity of whey proteins with plant phenols has been documented in both model (single phenolic compounds) and mixed (plant extracts) systems (Rawell *et al*., 2001).

To further investigate the reactivity of whey proteins with phenolic compounds present in SB extract, fluorescence spectra were obtained for α-lactalbumin and β-lactoglobulin. Whey proteins contain residues (Trp, Tyr and Phe) which can emit intrinsic fluorescence after absorbing ultraviolet light and thus this methodology is appropriate for investigating the structural transition and binding properties of whey proteins in solution (Yuan *et al*., 2007). Figure 2 presents the fluorescence spectra of α-lactalbumin (A and C) and β-lactoglobulin (B and D). The fluorescence intensity of α-

232 lactalbumin is quenched with increasing SB extract concentration and a significant shift
233 in λmax is also observed. This effect, known as fluorescence quenching, is attributed to
234 interactions between α-lactalbumin and phenolic compounds (Zhang *et al*., 2014).
235 Similar findings have been reported for whey proteins and natural polyphenols from
236 grapes (Liang *et al*., 2008; Stănciuc *et al*., 2017). According to the literature, protein-
237 polyphenol interactions are mediated primarily through hydrophobic bonds between
238 amino acid side chains and polyphenol aromatic rings and less commonly with
239 hydrogen bonds and is therefore regarded as a surface phenomenon (Charlton *et al*.,
240 2002). Interestingly, the fluorescence spectra of β-lactoglobulin were unaffected by the
241 addition of SB extract and thus there is no indication of molecular interaction of the
242 most abundant whey protein with phenolic compounds. This finding contradicts
243 previous research which suggests that complex formation between whey protein and
244 strawberry-derived phenolic compounds in yogurt are mainly attributed to β-
245 lactoglobulin (Oliveira *et al*., 2015). This suggests that the formation of complexes is
246 determined by the nature of the polyphenol as well as the presence of other components
247 in the food system (Prigent *et al*., 2003). Salal fruits are a good source of anthocyanins
248 and may also contain considerable amounts of flavonols, hydroxycinnamic acid and
249 proanthocyanidin components (McDougall *et al*., 2016).

250 The analysis of the electrophoretic mobility of whey protein hydrolysates revealed
251 that α-lactalbumin is not fully digested after the 8 h incubation period (Fig. 3). On the
252 contrary, β-lactoglobulin is not visually detectable after 1 h of incubation with the
253 protease. Furthermore, the intensity of the bands corresponding to α-lactalbumin in the
254 presence of SB (20%) extract suggests that the protein is even less digested in the later
255 case. Thus, the formation of complexes between α-lactalbumin and phenolic
256 compounds from SB extract may induce protein conformational changes which affect
257 the susceptibility of the protein to proteolytic cleavage. Interestingly, for both non-
258 hydrolyzed samples (A and B), fade but distinctive bands are visible in the range of 25-
259 35 kDa, which are likely to be attributed to caseins. This suggests that caseins are not
260 fully removed from the isolate during the purification process.

261

262 **Peptide identification and ACE-inhibitory activity of hydrolysates fortified with**

263 **SB**

264 The use of proteolytic enzymes for the release of peptides with ACE inhibitory activity

265 from whey proteins has been previously documented (Otte *et al*., 2007; Tavares *et al*.,

266 2011; Morais *et al*., 2013; Jeewanthi *et al*., 2017). The whey-derived peptides typically

267 contain 2-12 amino acids residues and the C-terminal position is occupied by

268 hydrophobic/aromatic amino acids (Lopez-Fandino *et al*., 2006; Corrêa *et al*., 2014).

269 The ACE inhibitory activity of the whey hydrolysates after 8 h incubation is presented

270 in Fig. 4. The corresponding IC_{50} (mg/mL) values follow the order: 20% (0.087) <5%

271 (0.109) < 10% (0.117) <0% (0.118) and indicate that fortification with salal fruits at 20%

272 increases ACE inhibitory activity. This effect could be due to the extent of hydrolysis

273 of whey proteins by Pronase E, which was significantly higher ($P<0.05$) for 20% SB

274 compared to the control (0%) after 4 h of incubation and until the end of the incubation

275 period (Fig. 5). This data suggest that whey protein hydrolysis increases with incubation

276 time for all samples and the degree of hydrolysis is also affected by the addition of SB

277 extract at high concentration (20%). Previous studies reported a positive correlation

278 between the increase in hydrolysis time or the decreasing size of peptides and ACE

279 inhibitory activity (Hernández-Ledesma *et al.,* 2002; Chobert *et al*., 2005). Peptides of

280 low molecular mass (<3 kDa) are considered potent inhibitors of ACE and may also

281 play a physiological antihypertensive role in vivo (Mullaly et al., 1997). Thus, the

282 beneficial effect against ACE inhibition observed in this study may be attributed to the

283 extensive hydrolysis of whey proteins at high SB concentration (20%).

284 A peptidomic database-search approach was employed to investigate if peptides of

285 known bioactivity are present in the hydrolysates (Giacometti and Buretić-Tomljanović,

286 2017). The list of bioactive peptides released from whey hydrolysates (0% and 20%)

287 after 8 h incubation is presented in Table 1. According to the literature, the peptides

288 identified have predominantly ACE and DPP-IV inhibitory activity. Many of these

289 peptides, which are mainly derived from β-lactoglobulin, contain hydrophobic amino

290 acids (i.e. Tre, Tyr, Phe, Pro) at the C-terminal position and branched-chain amino acids
291 (i.e Ile, Val) at the N-terminal position (Wu and Ding, 2002; Yust *et al*., 2003). Bioactive
292 peptides from β-casein were also identified, which confirms the presence of casein
293 fractions in the whey protein isolate mixture. These are mainly associated with ACE-
294 inhibitory activity and may contribute to the observed effects in this study. Numerous
295 peptides with no documented bioactivity were identified exclusively in the whey
296 sample with 20% SB extract (supplementary material). Overall the number of peptides
297 identified from the sample with SB extract (20%) and particularly deriving from β-
298 lactoglobulin after 4 h of incubation (data not shown) was higher compared to the
299 control (0%). This finding agrees with the data from the degree of hydrolysis and
300 indicate that the addition of SB extract may facilitate the hydrolysis of whey proteins
301 by Pronase E. A limitation of the methodology adopted for peptide identification lies in
302 the fact that any peptides with a mass lower than 750 Da would not have been detected.
303 Hence, although it is very likely that peptides of 2-6 amino acids in length are generated
304 in high numbers, peptide sequences of 7 residues or above are only listed. Further work
305 is needed to investigate the bioactivity of the identified peptides from this study.
306

307 **Conclusions**

308 Whey proteins are commonly mixed with plant phenols in various food formulations.
309 The addition of plant phenolics in the diet is likely to have an impact on health-
310 promoting properties of composite foods beyond the well documented antioxidant
311 effects. Particle size analysis of the incubated samples indicates extensive interactions
312 between whey proteins and phenolic compounds naturally present in salal fruits.
313 Fluorescence data suggests that α-lactalbumin is primarily involved in interactions with
314 phenols from salal fruits. Hydrolysates from whey protein incubated with salal fruits
315 extracts exhibited higher ACE inhibitory activity compared to whey protein
316 hydrolysates. Peptides of documented bioactivity (ACE and DPP-IV inhibition) were
317 identified by analyzing the MS/MS data using a protein sequence database approach.
318 The susceptibility to enzymatic hydrolysis may be altered because of protein

319 rearrangements, which are induced by interactions of α-lactalbumin with phenolic
320 compounds. This may facilitate the release of peptides with ACE-inhibitory activity
321 under controlled hydrolysis conditions. Casein-derived peptides with ACE-inhibitory
322 activity were also identified in the hydrolysates, which suggests that whey protein
323 isolates may contain casein fractions. Composite foods containing milk proteins with
324 plant dietary phenols can be sources of bioactive components with potential therapeutic
325 applications for the prevention and management of diet-related noncommunicable
326 diseases.

327

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332

333 **Conflicts of interest**

334 The authors declare that there are no conflicts of interest.

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