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1 # Interactions in milk suggest a physiological role for β-lactoglobulin

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5 Running title: Interactions of β-lactoglobulin in milk

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6 ## *Abstract*

7 β-Lactoglobulin is the most abundant protein in the whey fraction of ruminant milks, yet is
8 absent in human milk. It has been studied intensively due to its impact on the processing and
9 allergenic properties of ruminant milk products. However, the physiological function of β-
10 lactoglobulin remains unclear. Sedimentation velocity experiments have identified new
11 interactions between fluorescently-labelled β-lactoglobulin and other components in milk.
12 Co-elution experiments support that these β-lactoglobulin interactions occur naturally in milk
13 and provide evidence that the interacting partners are immunoglobulins, while further
14 sedimentation velocity experiments confirm that an interaction occurs between these
15 molecules. Ruminants (e.g. cows and goats) are born without circulating immunoglobulins,
16 which they must obtain from their mothers' milk, whilst humans obtain immunoglobulins
17 both through milk and during gestation via the placenta. We propose that β-lactoglobulin
18 serves to protect immunoglobulins within ruminant milk during digestion, ensuring their
19 efficient transfer from mother to offspring.

20

21 ## *Statement of Significance*

22 β-Lactoglobulin is an abundant protein in the whey fraction of ruminant milks (e.g. cow and
23 goat milk), yet it is completely absent in human milk. While this protein has been extensively
24 studied, due to its impact on the processing and allergenic properties of milk, its
25 physiological function remains unclear. We fluorescently labelled β-lactoglobulin to monitor
26 its interactions with other milk components within its physiological environment, milk.
27 Under these near physiological conditions β-lactoglobulin is capable of interacting with
28 several classes of immunoglobulins. Immunoglobulins are susceptible to digestion, but are
29 required to confer immunity from the mother to the offspring. We propose that β-
30 lactoglobulin serves to protect immunoglobulins within ruminant milk during digestion,
31 ensuring their efficient transfer from mother to offspring.

32

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33 **Introduction**

34 β-Lactoglobulin is the most abundant protein in the whey fraction of ruminant milk,
35 including cow and goat milk, yet is absent in human milk. β-Lactoglobulin is known to affect
36 the processing of ruminant milk, for instance due to heat-induced aggregation during heat
37 treatment (1). It is also one of the main immunogenic proteins that contribute to milk allergies
38 (2). Considerable effort has been made to elucidate the biological and biophysical properties
39 of β-lactoglobulin and as such it is one of the most highly studied proteins, featured in the
40 title or abstract of over 4000 publications in the last 75 years. Despite many conjectures, the
41 physiological role of β-lactoglobulin remains a mystery.

42 β-Lactoglobulin belongs to the lipocalin family, which are a group of small extracellular
43 proteins. Despite considerable diversity at the sequence level, lipocalin proteins share a
44 conserved protein fold: an eight stranded β-barrel enclosing a large hydrophobic cup-shaped
45 cavity (termed a calyx), together with a 3-turn helix between the seventh and eighth β-strands
46 (3). This fold makes lipocalins well suited to binding a range of hydrophobic molecules.
47 While once simply classified as transport proteins, it is now known that lipocalins exhibit
48 great functional diversity (3–10).

49 β-Lactoglobulin was first thought to function as a transporter of retinol between mother and
50 offspring, after it was demonstrated that bovine β-lactoglobulin can bind retinol (vitamin A)
51 (11). Since then, bovine β-lactoglobulin has been shown to bind a range of small hydrophobic
52 molecules, including vitamins, cholesterol and a range of fatty acids, as reviewed by Le
53 Maux et al. (12) and Kontopidis et al. (13). The apparent lack of selectivity makes it less
54 likely that β-lactoglobulin is a specific fatty acid or vitamin transporter. Further, given the
55 absence of β-lactoglobulin in the milk of humans, it is unlikely that this protein participates in
56 a process that is still required in humans.

57 The closest human homologue to β-lactoglobulin is glycodelin (also known as pregnancy
58 protein 14) (14). Unlike β-lactoglobulin and other lipocalins, glycodelin is a glycoprotein.
59 Glycosylation is crucial for the various functions of glycodelin, which include an
60 immunosuppressive activity in the uterus to protect products of the reproductive organs from
61 the immune system. The lack of glycosylation makes it unlikely that β-lactoglobulin is
62 capable of fulfilling a similar role.

63 β-Lactoglobulin may simply act as a source of amino acids for the offspring of the animals
64 that produce it. However, the resistance of β-lactoglobulin to digestion by human proteolytic
65 enzymes (15) and the presence of highly conserved features, including a pH-gated loop
66 movement, among β-lactoglobulin orthologues (16) argue against a simple nutritive function.

67 There have been conflicting reports on the antimicrobial activity of β-lactoglobulin. Chaneton
68 et al. (17) observed that isolated β-lactoglobulin inhibited the growth of *Staphylococcus*
69 *aureus*. Peptides resulting from the enzymatic digestion of β-lactoglobulin appear to possess
70 some antibacterial activity against both *Escherichia coli* and *S. aureus in vitro* (18,19).
71 However, while Fijalkowski et al. (20) observed that incubation of *S. aureus* in whey resulted
72 in growth inhibition, they found no substantial effect using pure β-lactoglobulin.

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73 We reasoned that the native function of β-lactoglobulin might be uncovered if its
74 physiological interactions were known. The use of fluorescence detection analytical
75 ultracentrifugation allowed us to monitor the interactions of β-lactoglobulin in milk, a highly
76 complex colloid, for the first time. The sedimentation of fluorescently-labelled bovine and
77 caprine β-lactoglobulin are both significantly altered in the presence of cow and goat milk, a
78 signature that they interact with other components. Co-elution experiments with milk further
79 support the presence of these interactions. The results lead us to propose a new physiological
80 role for β-lactoglobulin that explains the absence of this otherwise abundant protein from
81 human milk.

82

83 **Methods**

84 ***Protein purification***

85 The cloning, expression and purification of recombinant bovine β-lactoglobulin A and
86 caprine β-lactoglobulin have been described in detail previously (21,22).

87 ***Fluorescent labelling of proteins***

88 Proteins were labelled with fluorescein isothiocyanate (FITC) by reacting proteins at a
89 concentration of 5-10 mg/mL (in 0.1 M sodium carbonate buffer, pH 9) with 100 µL FITC
90 per 1 mL of sample. FITC was dissolved in dimethylformamide at 10 mg/mL immediately
91 prior to use. The solution was rotated to mix for one hour at ambient temperature in the dark,
92 then passed through a 5 mL desalting column (GE Healthcare) to remove the bulk of the free
93 label. β-Lactoglobulin was further purified by gel filtration chromatography (HiLoad
94 Superdex 200 16/60 120 mL). The degree of labelling was calculated by measuring the
95 absorbance at 280 nm and 494 nm using a NanoDrop™ spectrophotometer. The protein
96 concentration was calculated from the absorbance at 280 nm taking into account the
97 contribution of FITC, as per Eq. 1.

98 Equation 1:

99 $$\text{Protein concentration} = A_{280} - (A_{max} \times CF) / \varepsilon$$

100 Where A_{max} is the wavelength of maximum absorbance for the dye molecule (494 nm for
101 FITC), CF is the correction factor which adjusts for the amount of absorbance at 280 nm
102 caused by the dye (0.3 for FITC, as supplied by Thermo Fisher Scientific), and ε is the
103 protein molar extinction coefficient (17210 M^{-1} cm^{-1} for both bovine and caprine β-
104 lactoglobulin, as calculated using ExPasy ProtParam (23)).

105 The degree of labelling was calculated as according to Eq. 2. A degree of labelling between
106 0.3 and 1.2 moles of dye per mole of protein was deemed acceptable.

107 Equation 2:

108 $$\text{Moles dye per mole protein} = A_{max} \text{ of the labelled protein} / \varepsilon' \times \text{protein concentration}$$

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109 Where ε' is the molar extinction coefficient of the fluorescent dye (68000 M^{-1} cm^{-1} for FITC,
110 as supplied by Thermo Fisher Scientific).

111 ***Analytical ultracentrifugation***

112 Sedimentation velocity experiments were conducted in a Beckman Coulter XL-I analytical
113 ultracentrifuge. Depending on the experiment, sedimentation was monitored utilising one of
114 the three available optical systems (absorbance, interference and fluorescence). Specific
115 experimental conditions, i.e. the buffer used, the protein concentrations, and the wavelengths
116 measured, are specified in each of the figure legends. Reference solution (400 µL) and
117 sample solutions (380 µL) were added to 12 mm double sector cells with quartz or sapphire
118 windows. Cells were mounted in an An-50 Titanium eight-hole rotor. Initial scans were
119 performed at 3,000 rpm to determine the optimal settings, with sedimentation performed at
120 50,000 rpm at 20 °C. Data were collected in step sizes of 0.003 cm with no delay between
121 scans and no averaging.

122 Buffer density and viscosity and an estimate of the partial specific volume of proteins were
123 calculated using SEDNTERP (24). Data were fitted to a continuous sedimentation coefficient
124 [*c*(s)] model or continuous mass [*c*(M)] model using SEDFIT (25). Data were also subjected
125 to two-dimensional spectrum analyses with genetic algorithm optimisation, and van-
126 Holde/Weischet analyses in UltraScan III (26–28).

127 ***Preparation of milk for interaction studies***

128 Fresh, raw, cow milk (Holstein breed) was obtained from the Fairchild Dairy Teaching and
129 Research Centre at the University of New Hampshire, United States of America. Fresh, raw,
130 goat milk was obtained from a local farmer in either New Hampshire, United States of
131 America (Nubian breed) or Canterbury, New Zealand (Nubian/Saanen cross breed). Before
132 use, the milk was spun at 5000 g for 5 mins. The layer of fat that resulted at the top was
133 removed and the liquid underneath was transferred to a new vessel, leaving behind the small
134 pellet of cells that formed at the bottom of the milk sample. Diluted skim milk samples were
135 used for all AUC studies, using 0.1 M sodium phosphate, 0.1 M NaCl, pH 6.7 buffer.

136 ***Size-exclusion chromatography***

137 Samples of skim cow milk were subjected to size-exclusion chromatography using a
138 Superdex 200 PG 1800 column, with 15 mL fractions collected. A dotblot grid (1 x 1 cm)
139 was marked onto a nitrocellulose membrane (Biotrace NT). 2 µL of each eluted fraction was
140 spotted per square, with 2 µL of diluted loading sample loaded as a comparison. The
141 membrane was allowed to air dry, then peroxidase activity was blocked with 3% hydrogen
142 peroxide/1% sodium azide solution at room temperature for 10 minutes. After washing, the
143 membrane was blocked with either 1% polyvinylpyrrolidone-25 (PVP-25) or 1% bovine
144 serum albumin (BSA) in Tris-buffered saline, pH 7.6, containing 0.1% Tween-20 and 0.1%
145 BSA, at room temperature for 2-3 hours. The membrane was incubated with anti-bovine β-
146 lactoglobulin horse radish peroxidase (HRP)-conjugated antibody (Bethyl Lab A10-125P
147 1:100K) for 3 hours and immune-reactivity determined using chemiluminescence. In a
148 similar manner, reactivity of eluted fractions was determined versus anti-bovine IgM (Bethyl

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149 A10-101P 1:40K), anti-bovine IgG (Bethyl Lab A10-118P 1:75K) and anti-bovine IgA
150 (Bethyl Lab A10-131P 1: 40K) antibodies.

151

152 Results

153 *Characterising the behaviour of β-lactoglobulin in cow and goat milk*

154 To identify interactions between β-lactoglobulin and other components in milk,
155 recombinantly expressed and purified bovine and caprine β-lactoglobulin were labelled with
156 fluorescein isothiocyanate (FITC), before being added to samples of diluted cow and goat
157 milk. The sedimentation of β-lactoglobulin, monitored using the fluorescence-optics within
158 the analytical ultracentrifuge, is significantly faster in the presence of cow or goat milk when
159 compared to equivalent studies in simple buffered solutions. This suggests that β-
160 lactoglobulin is forming higher molecular weight species with other milk components.

161 A dilution series of cow and goat milk samples was prepared, to which fluorescently-labelled
162 bovine or caprine β-lactoglobulin was added to a final concentration of 0.75 μM. This is
163 much lower than the concentration of β-lactoglobulin usually found in milk (~150 μM (12)).
164 At this concentration (0.75 μM) and pH (6.7), bovine and caprine β-lactoglobulin are
165 predominantly monomeric with a small amount of dimer present (see the black lines in Fig.
166 1). The sedimentation profile changes considerably once milk is added to the solution, with
167 the appearance of a new, larger species with a sedimentation coefficient of ~8-10 S (Fig. 1).
168 As the concentration of milk increases a greater proportion of labelled β-lactoglobulin is
169 found in complex with other components. The sedimentation velocity data were also analysed
170 using the van-Holde/Weischet method: a model-independent analysis that directly assesses
171 the shape of the sedimenting boundary. These analyses support the conclusion made from the
172 *c*(s) analyses that with increasing concentrations of milk the proportion of β-lactoglobulin
173 that is found in complex increases (Fig. 1 E-H).

174

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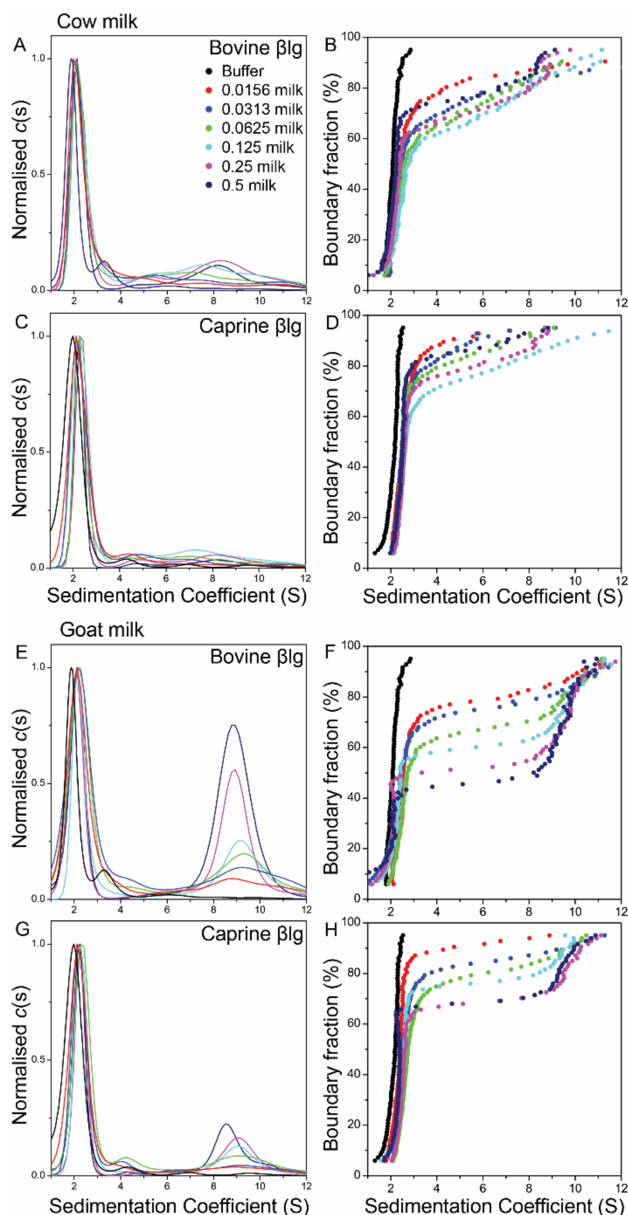
175 

176 Figure 1: Sedimentation velocity analysis of bovine and caprine β-lactoglobulin in cow and
177 goat milk. Each milk was serially diluted from ½ (0.5) to 1/64 (0.0156) dilution. FITC-
178 labelled protein was added to each sample to a final concentration of 0.75 µM. The data were
179 fitted to a continuous sedimentation coefficient distribution ($c(s)$) model for A) FITC-labelled
180 bovine β-lactoglobulin A in cow milk, B) FITC-labelled caprine β-lactoglobulin in cow milk,
181 C) FITC-labelled bovine β-lactoglobulin A in goat milk, D) FITC-labelled caprine β-
182 lactoglobulin in goat milk. E-H) the same data were analysed using the van-Holde/Weischet
183 method.

184

185 Different behaviours were observed for both the type of milk used and the orthologue of β-
186 lactoglobulin examined. In each milk a greater amount of complex is formed for bovine β-
187 lactoglobulin than for caprine β-lactoglobulin (compare Fig. 1A and 1C to Fig. 1B and 1D),
188 despite the proteins having a very high level of sequence and structural identity (22). As the

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189 same concentration of β-lactoglobulin was added to each sample, this suggests that the A
190 isoform of bovine β-lactoglobulin (used in these interaction studies) has a higher binding
191 affinity for the interacting component than caprine β-lactoglobulin. In goat milk, a greater
192 amount of complex is formed with both bovine and caprine β-lactoglobulin as compared to
193 that seen in cow milk (compare Fig. 1C and 1D to Fig. 1A and 1B). As the same volume of
194 milk was added to each sample, this suggests that the interacting component has a higher
195 affinity for β-lactoglobulin or is present in goat milk at a higher concentration.

196 The sedimentation of free FITC dye is not altered in the presence of milk (Fig. 2A). This
197 demonstrates that FITC is not interacting with other components in milk, and is thus not
198 responsible for the peak seen at 8-10 S in Fig. 1. We expect that fluorescently-labelled β-
199 lactoglobulin is capable of mixing with endogenous β-lactoglobulin already present in milk,
200 forming hetero-dimers, which would effectively increase the concentration of fluorescently-
201 labelled β-lactoglobulin. Therefore, it is possible that the larger species formed in the
202 presence of milk is a product of the self-association of β-lactoglobulin into an even higher-
203 order oligomer. However, sedimentation velocity experiments of bovine β-lactoglobulin and
204 caprine β-lactoglobulin at high concentrations (up to 400 μM) in buffered solutions
205 unequivocally show a single species with a sedimentation coefficient of 2.6 S (Fig. 2B). This
206 demonstrates that β-lactoglobulin does not form any higher-order species above a dimer, even
207 at the concentrations that may be encountered in milk.

208

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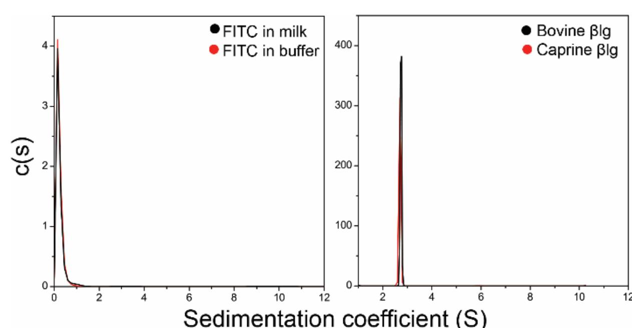

210 Figure 2: A) Sedimentation velocity analysis (using absorbance optics) of FITC in buffer and
211 in goat milk (1/20 dilution). B) Sedimentation velocity analysis (using interference optics) of
212 bovine and caprine β-lactoglobulin at 400 μM in buffer (0.1 M sodium phosphate, 0.1 M
213 NaCl, pH 6.7).

214

215 Unlabelled β-lactoglobulin, added to milk that already contains FITC-labelled β-
216 lactoglobulin, can compete for the interacting site that leads to the higher molecular mass
217 species. This can be seen in Fig. 3 as a reduction in peak size at 8 S (Fig. 3A and 3B) and a
218 decrease in the fraction of the boundary corresponding to the larger species (Fig. 3C and 3D).
219 This demonstrates that the formation of the higher molecular mass species at 8 S is not due to
220 any structural changes in β-lactoglobulin induced by the addition of FITC. Curiously, the
221 sedimentation coefficient of the larger species appears to decrease, with increasing
222 concentration of unlabelled β-lactoglobulin (Fig. 3), suggesting that the complex is

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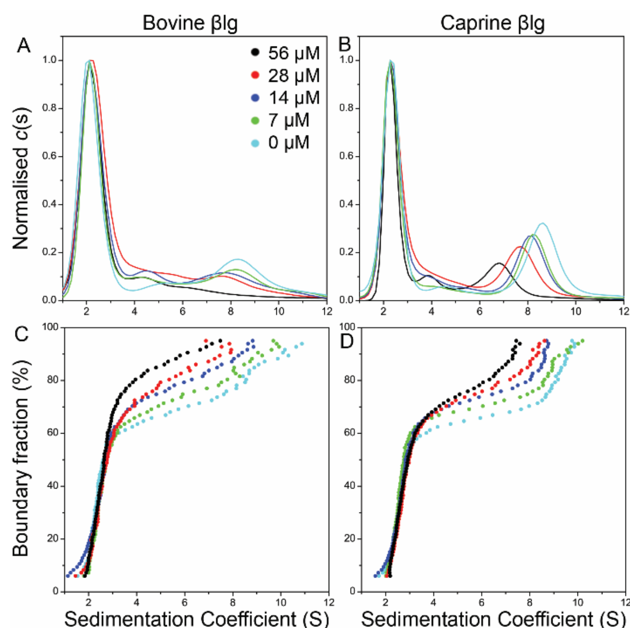
223 decreasing in size. This could be attributed to a higher viscosity and density of the solution as
224 the concentration of unlabelled protein is increased. Alternatively, it may suggest a network
225 is formed between β-lactoglobulin and the interacting partner (which would imply multiple
226 binding sites on both β-lactoglobulin and the interacting partner), where excess of either
227 partner would disrupt the network and result in a reduced average complex size.

228



229

230 Figure 3: Sedimentation velocity analysis of FITC-labelled β-lactoglobulin proteins (0.75
231 μM) in milk (1/4 dilution) with increasing concentrations of unlabelled β-lactoglobulin (0–56
232 μM). Continuous sedimentation coefficient distributions of A) FITC-labelled bovine β-
233 lactoglobulin A in cow milk with unlabelled bovine β-lactoglobulin A, and B) FITC-labelled
234 caprine β-lactoglobulin in goat milk with unlabelled caprine β-lactoglobulin. C and D)
235 enhanced van-Holde/Weischet analyses.

236

237 The higher molecular mass species associated with β-lactoglobulin can also be observed in an
238 unmodified sample of milk when analysed by gel-filtration chromatography. β-Lactoglobulin
239 was identified in the eluted fractions by means of a Western dot-blot assay using antibodies
240 specific for bovine β-lactoglobulin. It is apparent that β-lactoglobulin elutes in two places
241 consistent with our sedimentation experiments: late in the elution consistent with free β-
242 lactoglobulin (see Box A in Fig. 4A), and earlier in the elution at a location expected for a
243 much higher molecular mass species (Box B, Fig. 4A). This suggests that β-lactoglobulin is
244 present in milk as two populations of different sized species, which agrees directly with what
245 is seen in the AUC experiments. The importance of this result lies in the fact that the size-
246 exclusion chromatography separation involves endogenous β-lactoglobulin that has not been
247 recombinantly expressed or modified in any way, yet the same outcome is seen.

248

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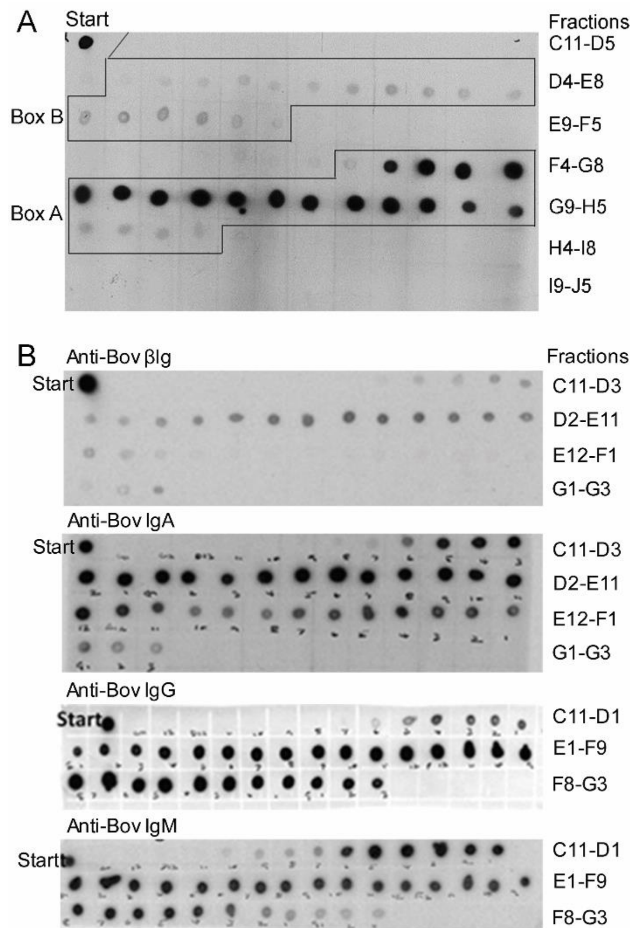

249

250 Figure 4: A) Western dot-blot of cow milk fractions following size-exclusion
251 chromatography, utilising anti-bovine β-lactoglobulin antibodies. B) Western dot-blot of the
252 same milk fractions utilising anti-bovine β-lactoglobulin, anti-bovine IgA, anti-bovine IgG
253 and anti-bovine IgM.

254

255 In summary, the formation of a higher molecular mass species has been observed between
256 bovine and caprine β-lactoglobulin, and other components within cow and goat milk. The
257 possibility of self-association of β-lactoglobulin into a higher order species is ruled out. FITC
258 molecules attached to the surface of β-lactoglobulin proteins are not responsible for complex
259 formation, demonstrated by the fact that FITC does not interact with any milk components
260 and that unlabelled β-lactoglobulin proteins can successfully compete for binding with
261 labelled β-lactoglobulin. Even more convincingly, a higher molecular mass species of β-
262 lactoglobulin can be seen in milk that contains β-lactoglobulin that has not been altered in
263 any way.

264 ***Identifying the interacting component***

265 While bovine and caprine β-lactoglobulin can both form associations with kappa-casein in
266 milk, this interaction is heat-induced and involves a disulfide-exchange reaction (29). Thus,
267 the 8 S species identified in our sedimentation velocity and size-exclusion experiments is
268 unlikely to be an association between β-lactoglobulin and kappa-casein. Given the size of the

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269 complex (8-10 S), we hypothesised that β-lactoglobulin interacts with immunoglobulin
270 proteins present in milk.

271 The fractions of milk eluted from size exclusion chromatography, as described above, were
272 also probed with anti-bovine IgG, IgA and IgM antibodies. It is apparent that these
273 immunoglobulins co-elute from milk in the same fractions as the higher molecular mass
274 bovine β-lactoglobulin (Fig. 4B). This is consistent with bovine β-lactoglobulin binding
275 immunoglobulins in milk to form the higher molecular mass species seen in the
276 sedimentation velocity experiments.

277 To confirm that β-lactoglobulin interacts with immunoglobulins, the sedimentation of bovine
278 and caprine β-lactoglobulin was analysed in the presence of bovine and caprine IgG, purified
279 from the serum of non-immunised cows and goats. For this experiment the sedimentation of
280 FITC-labelled β-lactoglobulin was monitored using absorbance at 495 nm, using the AUC
281 absorbance optical system. It can be seen that the sedimentation of both FITC-labelled bovine
282 and caprine β-lactoglobulin is indeed altered in the presence of IgG molecules (Fig. 5), with a
283 larger species appearing in the $c(s)$ distribution at 6 S. While this is not as large as the 8-10 S
284 species observed in the milk interaction experiments, this is likely due to these experiments
285 being carried out on purified proteins in a simple buffered solution rather than within the
286 complex milieu of milk.

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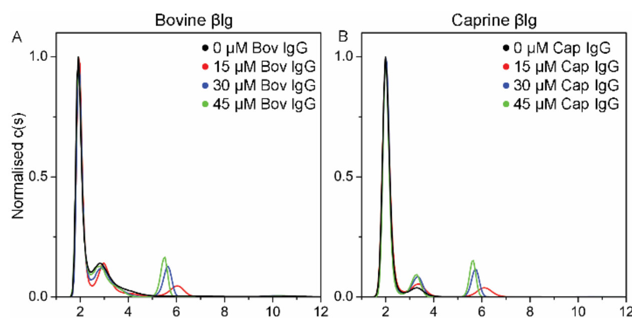
288 

289 Figure 5: Sedimentation velocity analysis of A) FITC-labelled bovine β-lactoglobulin (10
290 μM) and bovine IgG (0-45 μM) and B) FITC-labelled caprine β-lactoglobulin (10 μM) and
291 caprine IgG (0-45 μM).

292

293 To summarise, the co-elution of β-lactoglobulin with immunoglobulins IgG, IgA and IgM
294 from cow milk following size-exclusion chromatography supports the hypothesis that β-
295 lactoglobulin interacts with immunoglobulins within milk. The sedimentation of β-
296 lactoglobulin is altered in the presence of IgG, strong evidence of an interaction between
297 these two species.

298

299 **Discussion**

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300 We propose that the physiological function of β-lactoglobulin within ruminant milks is to
301 protect immunoglobulins, particularly IgG, from digestive enzymes as they traverse the
302 digestive tract, leaving them available to provide immune protection to the ruminant
303 offspring.

304 Given the abundance of β-lactoglobulin in ruminant milk, and its absence in the milk of
305 humans, it is unlikely that this protein would fulfil a role that is necessary in humans. In
306 primates and lagomorphs (*e.g.* rodents and rabbits), species that lack β-lactoglobulin in their
307 milk, IgG is transferred to the foetus *via* the placenta, and the offspring is born with
308 circulating IgG (30). Conversely, the offspring of ruminants (such as cows and goats) are
309 born agammaglobulinemic (*i.e.* with no circulating antibodies) and thus fully rely on the
310 uptake of IgG from colostrum and milk to provide immune protection (30). Ruminant milk is
311 therefore high in IgG (31).

312 We have shown here that bovine and caprine β-lactoglobulin are capable of interacting with
313 immunoglobulins within cow and goat milk. Importantly, the resistance of β-lactoglobulin to
314 gastric digestion is well known (15). The binding of β-lactoglobulin to immunoglobulins may
315 serve to increase their resistance toward proteolysis during transit through the gastrointestinal
316 tract. This would be particularly relevant for IgG, given its importance for transferring
317 immunity in ruminants.

318 A structure of the complex between β-lactoglobulin and these immunoglobulins is necessary
319 to fully understand the nature of this binding interaction. The crystal structure of bovine β-
320 lactoglobulin in complex with an IgE Fab fragment has been reported (32). This complex
321 demonstrates the interaction between an allergen and the antigen-binding region of the
322 antibody, which provides structural insight into the recognition of this milk antigen by the
323 human immune system. Immunoglobulins and β-lactoglobulin within milk would be expected
324 to bind using a different mechanism. This could involve the heavy chain region of the
325 immunoglobulin, rather than the Fab region, as the latter mechanism would likely elicit
326 unwanted immune responses.

327 In conclusion, an interaction between β-lactoglobulin and immunoglobulins within both cow
328 and goat milk has been identified for the first time, using analytical ultracentrifugation and
329 size-exclusion chromatography experiments. We propose that this interaction with protease-
330 resistant β-lactoglobulin protects immunoglobulins, essential to immunity of the neonate calf
331 or kid, from proteolytic attack.

332

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333 ***Author contributions***

334 Designed research: JMC, TL, GBJ, AJH, RCJD; performed research: JMC, MB; analyzed
335 data: JMC, MB, TL, GBJ, AJH, RCJD; contributed analytic tools: TL; wrote the manuscript:
336 JMC; edited the manuscript: TL, GBJ, AJH, RCJD.

337

338 ***Acknowledgements***

339 The authors declare no conflicts of interest.

340

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428 ***Competing interests:***

429 All authors declare no competing interests.