

1 **Which casein in sodium caseinate is most resistant to *in vitro* digestion? Effect of**  
2 **emulsification and enzymatic structuring**

3  
4 Franziska Böttger<sup>1</sup>, Didier Dupont<sup>1</sup>, Dorota Marcinkowska<sup>2</sup>, Balazs Bajka<sup>3</sup>, Alan Mackie<sup>4</sup>, Adam  
5 Macierzanka<sup>1,2,5\*</sup>

6  
7 <sup>1</sup> STLO, INRA, Agrocampus Ouest, 65 Rue de St. Briec, 35000 Rennes, France

8 <sup>2</sup> Department of Colloid and Lipid Science, Faculty of Chemistry, Gdansk University of Technology,  
9 Narutowicza 11/12, 80-233 Gdansk, Poland

10 <sup>3</sup> Department of Nutritional Sciences, King's College London, London SE1 9NH, UK

11 <sup>4</sup> School of Food Science & Nutrition, University of Leeds, Leeds LS2 9JT, UK

12 <sup>5</sup> Institute of Food Research, Norwich Research Park, Colney Lane, Norwich NR4 7UA, UK

13  
14 \*Corresponding author. E-mail address: adam.macierzanka@pg.edu.pl (A. Macierzanka). Present address:  
15 Gdansk University of Technology.

16 Declarations of interest: none

17  
18 **Abstract**

19 We investigated the resistance of individual constituent casein epitopes ( $\alpha$ S<sub>1</sub>-,  $\alpha$ S<sub>2</sub>-,  $\beta$ - and  $\kappa$ -CN)  
20 in food-grade milk protein sodium caseinate (NaCN) to simulated human gastro-duodenal digestion.  
21 The influence of NaCN adsorption to the surface of oil-in-water emulsion droplets and the effect of  
22 crosslinking of the protein with enzyme transglutaminase (TG) on the proteolysis were studied by  
23 indirect ELISA. TG crosslinking rendered fragments of casein molecules significantly resistant to  
24 digestion. However, it depended on the type of casein and whether NaCN was presented in solution  
25 or emulsion. The crosslinking was found to considerably hinder the digestion of several amino acid  
26 regions in one of the major caseins of NaCN,  $\beta$ -CN. For  $\alpha$ S<sub>1</sub>- and  $\alpha$ S<sub>2</sub>-CN, only limited resistance to  
27 digestive enzymes was observed after NaCN had been crosslinked in solution but not (or to a limited  
28 extent) in emulsion.  $\kappa$ -CN proved to be the least resistant to the enzymatic hydrolysis regardless of  
29 the TG treatment. Our work shows for the first time how the digestibility of individual components of  
30 important food-grade protein ingredients can differ in a complex, colloidal food system. It also shows  
31 an example of how the digestibility can be modulated by chemical and physical structuring.

32  
33 **Keywords:** Digestion; Sodium caseinate; ELISA; Emulsion; Transglutaminase; Casein

34  
35 **1. Introduction**

36 Micro- and macro-structural organisations of proteins in foods are often generated by various food  
37 processing methods (e.g., emulsification, heating, gelation, enzymatic treatment, etc.). Although  
38 required to create desirable, functional structures in food, the processing can render proteins either  
39 significantly less or significantly more accessible for the digestive enzymes of the human

40 gastrointestinal tract and hence modify amino acid bioaccessibility during digestion (Singh & Ye, 2013;  
41 Gan, Bornhorst, Henrick, & German, 2018).

42 The digestion of a single protein leads to the release of hundreds of peptides in the gut lumen that  
43 can be identified by mass spectrometry (Boutrou et al., 2013) but the information is only semi-  
44 quantitative. It is therefore difficult to get a clear picture of the extent of hydrolysis of a specific protein  
45 domain (Dupont, 2017). An alternative to the mass spectrometry has been proposed based on the use  
46 of monoclonal antibodies with known specificity (Dupont, Rolet-Repecaud, & Senocq, 2003). The  
47 underlying idea is that when an antibody binds the epitope of a protein that contains a protease  
48 cleavage site, it means that the epitope has not been cleaved by the enzyme. In contrast, hydrolysis  
49 of the epitope causes a loss of interaction between the antibody and the target protein that can be  
50 easily monitored by immunoassays such as ELISA. This strategy was successfully applied to follow  
51 proteolysis events occurring during cheese ripening (Senocq, Dupont, Rolet-Repecaud, & Leveux,  
52 2002). As a result of their loose structure, caseins (CNs) are particularly adapted to this approach as  
53 most of their epitopes are sequential, allowing the production of a wide collection of monoclonal and  
54 polyclonal antibodies targeting several epitopes of  $\alpha S_1$ -,  $\alpha S_2$ -,  $\beta$ - and  $\kappa$ -CN (Johansson et al., 2009).

55 Enzymatic crosslinking of proteins is an attractive and feasible food technology due to the specificity  
56 of enzymes and the mild reaction conditions (Buchert et al., 2010). Modification with crosslinking  
57 enzymes such as transglutaminase (TG) has been extensively used to change the functionality of  
58 proteins and thereby to improve the textural quality, stability and function of protein-based food  
59 products (Dickinson, 1997). The enzyme permanently crosslinks proteins through an acyl transfer  
60 mechanism between glutamine and lysine residues (Griffin, Casadio, & Bergamini, 2002). Monogioudi  
61 et al. (Monogioudi et al., 2011) showed that enzymatically crosslinked purified  $\beta$ -CN was more  
62 resistant to pepsin than a non-crosslinked protein. The crosslinking was also shown to delay the  
63 simulated human gastro-duodenal proteolysis of food-grade protein sodium caseinate (NaCN) in  
64 emulsion, which prevented the emulsion from destabilising under the gastric conditions (Macierzanka  
65 et al., 2012). Our recent *in vivo* human study (Juvonen et al., 2015) showed that even subtle structural  
66 modification of NaCN interfacial layer in emulsion by TG was able to alter the early postprandial profiles  
67 of glucose, insulin, CCK, appetite and satiety through a decreased protein digestion, without  
68 significantly affecting the gastric emptying or an overall lipid digestion. Although we showed significant  
69 differences in the extent of digestion between NaCN crosslinked in emulsion and in solution  
70 (Macierzanka et al., 2012), the detailed roles of constituent casein epitopes of NaCN (i.e.,  $\alpha S_1$ -,  $\alpha S_2$ -,  
71  $\beta$ - and  $\kappa$ -CN) in exerting the resistance to digestion could not be evaluated. This fundamental  
72 knowledge is required for developing novel foods as the nutritional interventions aiming to modulate  
3 dietary protein bioaccessibility and amino acid bioavailability provides the best strategy for preventing  
4 diet-related health problems such as food allergies or sarcopenia.

## 6 2. Materials and methods

### 7 2.1. Materials

78 Food-grade sodium caseinate (NaCN; 90% protein) was obtained from DMV International (The  
79 Netherlands). Microbial transglutaminase (TG) and triglyceride oil were treated as described before  
80 (Macierzanka et al., 2012). Details have also been given in the Supplementary Material (SM; S1.1.).  
81 Eighteen monoclonal antibodies and one polyclonal antibody (SM; Table S1, Fig. S1) were taken from  
82 the INRA's collection in order to cover as much of the sequences of  $\alpha S_1$ -,  $\alpha S_2$ -,  $\beta$ - and  $\kappa$ -CN as possible  
83 (Johansson et al., 2009; Fig. S1). More details have been given in the SM (S1.1.).

## 84 **2.2. NaCN in emulsion and solution; sample preparation and characterisation**

85 The preparation of NaCN-stabilised emulsions and NaCN solutions, TG crosslinking, *in vitro* gastro-  
86 duodenal digestion experiments, and SDS-PAGE characterisation of the digestion samples were done  
87 as described previously (Macierzanka et al., 2012). For convenience, detailed experimental  
88 procedures have also been given in the SM.

## 89 **2.3. Indirect ELISA**

90 The indirect ELISA was performed for selected time-point samples from digestion of NaCN in order  
91 to detect protein regions (in  $\alpha S_1$ -,  $\alpha S_2$ -,  $\beta$ - and  $\kappa$ -CN) resistant to digestion, using the antibodies listed  
92 in Table S1. Detailed experimental procedure has been described in the SM (S1.7.)

## 94 **3. Results and discussion**

### 95 **3.1. SDS-PAGE characterisation**

96 We have investigated the impact of NaCN adsorption to the oil-water interface in an emulsion and  
97 its subsequent crosslinking with TG on the susceptibility of constituent casein polypeptides to  
98 simulated human gastro-duodenal proteolysis. SDS-PAGE was used initially to provide a rapid  
99 screening of the overall behaviour of NaCN during the digestion experiments carried out for the protein  
100 presented in different physical-chemical states (i.e., in solution vs. adsorbed, and non-crosslinked vs.  
101 covalently crosslinked by TG) and under different conditions (i.e., +/- vesicular PC in the gastric  
102 digestion compartment). This initial part of the study was carried out using a similar approach to the  
103 work presented previously (Macierzanka et al., 2012). Therefore, it was important to demonstrate that  
104 the SDS-PAGE characterisations of the digestion products in the present study were consistent with  
105 the results shown in that report. This offers a coherent experimental introduction to the original ELISA  
106 results reported in this paper. The SDS-PAGE results are shown in the Supplementary Material (SM;  
107 Fig. S2). Because of their consistency with the previously published work (Macierzanka et al., 2012),  
108 detailed description and discussion of the results have only been given in the SM (S2.1.).

### 110 **3.2. ELISA study**

1 An important consideration before analysing ELISA results is an effect that crosslinking might have  
2 on the binding properties of antibodies, i.e., whether the crosslinking could block antibodies even  
3 though the peptides they are specific to remain intact during the digestion. Crosslinking could  
4 theoretically affect antibody binding the target protein, causing a decrease in immunoreactivity due to  
5 steric hindrance. Nevertheless, in the present study, ELISA results were expressed as residual  
6 immunoreactivity (RI) normalised against the immunoreactivity detected for undigested protein (native

117 i.e. non-crosslinked, or crosslinked), thereby accounting for potential changes in antibody binding  
118 efficiency resulting from crosslinking. A loss of signal, therefore, means a hydrolysis of the epitope and  
119 not steric hindrance.

120 After crosslinking NaCN with TG, significant RIs of several  $\beta$ -CN fragments were observed in  
121 digestion samples (Fig. 1). This suggests that the crosslinking restricted hydrolysis by digestive  
122 enzymes. The RI was significantly lower for the non-crosslinked protein. The fragment f4-28 was the  
123 only one, for which the RI of over 80% persisted until the end of the gastric phase and was still up to  
124 ca. 60% during the first 5 min of the duodenal proteolysis (Fig. 1B,D). In emulsion, approximately 70%  
125 of the adsorbed  $\beta$ -CN is closely associated with the oil-water interface (Mackie, Mingins, & North,  
126 1991), with one exception being the sequence of 40–50 residues at the N-terminus. The sequence is  
127 predominantly hydrophilic and thus oriented into the aqueous phase (Dickinson, 2006). It contains four  
128 phosphoserine residues (Table S1). The electrostatic repulsion produced by this part of the protein is  
129 crucial for preventing coalescence of emulsion droplets (Caessens, Gruppen, Slangen, Visser, &  
130 Voragen, 1999). All the above suggests that the fragment f4-28 might remain exposed to the TG, not  
131 only in solution but also after the protein had been adsorbed to oil droplets in emulsion. This fragment  
132 contains one lysine (Table S1) that is the likely residue crosslinked and responsible for the high RI  
133 observed during the gastric phase of digestion (Fig. 1B,D). In the absence of crosslinking, the fragment  
134 was much more susceptible to pepsinolysis, and the RI fell to ca. 10% after 60 min of gastric digestion  
135 (Fig. 1A,C).

136 Another segment of  $\beta$ -CN, which expressed increased resistance to pepsin after crosslinking was  
137 the fragment f94-113 (Fig. 1F,H). At the end of the gastric digestion, its RI was up to ca. 40%  
138 depending on the crosslinking and digestion conditions (i.e., solution vs. emulsion, +/- PC). This short  
139 region of  $\beta$ -CN contains five lysine residues (Table S1) that could be crosslinked, and hence restrict  
140 access of pepsin during the digestion. However, in the absence of PC, relatively high RI (up to ca.  
141 30% under the gastric conditions) of this fragment was also seen for the non-crosslinked protein  
142 digested in emulsion (Fig. 1G). This suggests that adsorption to the interface alone might have  
143 contributed to restricting access of pepsin. Much higher resistance to pepsinolysis (RI of ca. 95% in  
144 the absence of PC) was recorded for the adjacent fragment f133-150, regardless of the TG pre-  
145 treatment in emulsion (Fig. 1K,L), but not in solution (Fig. 1 I,J), indicating protection must have been  
146 limited to the protein segment adsorbed at the oil–water interface. Both, f133-150 and f94-113 are  
147 parts of the  $M_r$  6 kDa peptide, which can persist during the pepsinolysis of purified  $\beta$ -CN in emulsion  
148 (Macierzanka et al., 2009). The f133-150 contains several aliphatic residues and a tryptophan (Table  
149 S1), which may be closely associated with the oil phase (Dickinson, Horne, Pinfield, & Leermakers,  
0 1997). Such a close interaction of the  $M_r$  6 kDa peptide with the oil phase was suggested to be the  
1 reason for its protection from pepsinolysis (Macierzanka et al., 2009). Here, such behaviour has been  
2 confirmed by ELISA for  $\beta$ -CN adsorbed to the interface in the presence of several other constituent  
3 caseins of a food-grade NaCN. In the presence of PC, the protective effect of the interface was  
4 completely abolished for the f133-150 (Fig. 1K,L) and significantly reduced for the f94-114 (Fig. 1G),

155 so their resistance to digestion was similar to that observed in solution (Fig. 1I,J and 1E, respectively).  
156 Vesicular PC introduced to the gastric digestion mix is very efficient in displacing protein (including  
157 NaCN) from the oil–water interface into the surrounding aqueous phase of emulsion as the lipid is  
158 more surface active (Macierzanka et al., 2009; Macierzanka et al., 2012) After rapid desorption, the  
159 protein is then digested with the kinetics similar to those observed in solution. Here, it has been clearly  
160 seen for both f133-150 and f94-113.

161 The crosslinking also improved the RI of f167-178 (Fig. 1N), although to a lesser extent in emulsion  
162 (Fig. 1P). This short protein fragment contains two lysine and two glutamine residues (Table S1), which  
163 could have been crosslinked and therefore contributed to restricting the hydrolysis. Other fragments  
164 of  $\beta$ -CN (i.e., f33-49 and f184-202) showed very little RI (SM; Fig. S3).

165 We have observed a rapid degradation of  $\alpha$ S<sub>1</sub>-CN in non-crosslinked NaCN (Figs. 2, S4). The TG  
166 crosslinking improved resistance of two protein fragments (i.e., f56-74 and f75-92) to hydrolysis by  
167 pepsin, however the protection was predominantly observed for the protein crosslinked in solution  
168 (Fig. 2B,F) than in emulsion (Fig. 2D,H). The adsorbed  $\alpha$ S<sub>1</sub>-CN molecule is depicted as a tri-block  
169 polymer, with a hydrophobic region at each end and a hydrophilic central loop containing several  
170 phosphoserines (Dickinson, 2006). Thus, one can expect that in both emulsion and solution the TG  
171 should have accessed and crosslinked the central region of the protein more easily than the terminal  
172 regions. Interfacial rheology studies (Faergemand, Murray, Dickinson, & Qvist, 1999) demonstrated  
173 that the structural build-up for adsorbed  $\alpha$ S<sub>1</sub>-CN was slower than for either  $\beta$ -CN or NaCN. This was  
174 assumed to be caused by slower adsorption of  $\alpha$ S<sub>1</sub>-CN and/or possibly faster crosslinking of the other  
175 proteins. A significant decrease in crosslinking kinetics (calculated from the loss of monomeric caseins  
176 during the incubation with TG) upon protein adsorption to lipid droplet was found to be a general  
177 phenomenon for all constituent caseins of NaCN (Macierzanka et al., 2011). However, crosslinking of  
178  $\alpha$ S<sub>1</sub>-CN was reduced much more significantly than other caseins. Hence, the limited crosslinking of  
179 adsorbed  $\alpha$ S<sub>1</sub>-CN might have accounted for the low RI of f56-74 and f75-92 observed here (Fig. 2D,H).

180 Increased RI has been recorded for another fragment of  $\alpha$ S<sub>1</sub>-CN, f133-151, although similar results  
181 were observed for both non-crosslinked and crosslinked samples, and only after the protein had been  
182 adsorbed at the oil–water interface (Fig. 2K,L). This segment of  $\alpha$ S<sub>1</sub>-CN contains 7 hydrophobic  
183 residues (i.e., Val, Ile, Met, 2× Phe, 2× Met), and was previously shown to reside very close to the  
184 interface after protein adsorption (Dickinson et al., 1997). This close interaction with the oil might have  
185 offered protection from proteolysis in a similar way as for fragments f133-150 and f94-113 of  $\beta$ -CN  
186 (Fig. 1G,H,K,L), although, to a more limited extent. As with the  $\beta$ -CN fragments, the protection was  
187 reduced when the digestion was carried out in the presence of PC (Fig. 2K,L), suggesting that also in  
8 this case PC might have displaced the protein from the oil-water interface, so the protein was digested  
9 mainly in the aqueous phase of emulsion. Other fragments of  $\alpha$ S<sub>1</sub>-CN (i.e., f1-19, f19-37, Nat f125-  
0 132 and f149-166) showed very little RI (SM; Fig. S4).

1 We have also investigated the digestibility of the two minor constituents of NaCN:  $\alpha$ S<sub>2</sub>-CN and  $\kappa$ -  
2 CN. For the digested emulsion samples, all of the  $\alpha$ S<sub>2</sub>-CN-specific antibodies returned very low RI,



193 regardless of the pre-treatment with TG (data not shown).  $\alpha$ S<sub>2</sub>-CN is the most hydrophilic of all caseins,  
194 which is the result of three clusters of anionic groups in the amino acid sequence, composed of  
195 phosphoserine and glutamyl residues (Farrell et al., 2004). The overall hydrophilic nature of  $\alpha$ S<sub>2</sub>-CN  
196 could make it more exposed to the aqueous phase of emulsion than  $\beta$ -CN and  $\alpha$ S<sub>1</sub>-CN after NaCN  
197 had been adsorbed to the oil droplets, therefore making  $\alpha$ S<sub>2</sub>-CN more vulnerable to the digestive  
198 enzymes. This, coupled with its lower crosslinking rate in emulsion than in solution (Macierzanka et  
199 al., 2011), would possibly explain that the limited resistance of the protein to digestion was only seen  
200 after the crosslinking in solution (Fig. S5). The most pronounced effect was observed for f96-114 (Fig.  
201 S5 F). This region of  $\alpha$ S<sub>2</sub>-CN contains one lysine and three glutamine residues (Table S1) that offer  
202 potential sites for TG. However, it remains unclear why the other two epitopes (f16-35 and f76-95)  
203 showed more modest resistance to digestion after the incubation with TG (Fig. S5 B,D) despite the  
204 fact that they contain 5-6 TG amino acid substrates each (Table S1).

205 The antibodies specific to  $\kappa$ -CN only showed insignificant RI of this protein in NaCN samples  
206 digested in solution or in emulsion (data not shown). The  $\kappa$ -CN contains lowest proportion of lysine  
207 and glutamine residues, and less phosphoserine than other caseins (Farrell et al., 2004). It also  
208 comprises a considerable amount of  $\beta$ -structure (Huppertz, Fox, & Kelly, 2018). Both of these factors  
209 have been used to explain much poorer crosslinking of  $\kappa$ -CN compared to the other caseins in NaCN  
210 (Macierzanka et al., 2011). In general, caseins in NaCN solutions exist as a dynamic system of casein  
211 monomers, complexes, and aggregates (Lucey, Srinivasan, Singh, & Munro, 2000), depending on  
212 conditions such as protein concentration, pH, ionic strength, temperature, etc. For example, at low  
213 ionic strength (3 mM) NaCN was found to be present as individual molecules (HadjSadok, Pitkowski,  
214 Nicolai, Benyahia, & Moulai-Mostefa, 2008) but formed small aggregates (hydrodynamic radius = 11  
215 nm) at high ionic strength (>100 mM). In dilute aqueous solutions at neutral pH, NaCN consists  
216 predominantly of protein nanoparticles (up to 20 nm) in equilibrium with free casein molecules, and  
217 some supramolecular species composed largely of  $\kappa$ -CN (Dickinson, 2010). Recent discussion on  
218 NaCN suspensions and casein micelles (Huppertz et al., 2017) proposed a model where NaCN  
219 particle suspension consist of assembled non-spherical primary casein particles (PCPs, which are  
220 naturally present in casein micelles). The  $\kappa$ -CN rich domains are likely to be located on the surface of  
221 the assembled structures. The above characteristics may reflect conditions of the NaCN solutions  
222 used in our present study. The possible easy access of digestive enzymes to  $\kappa$ -CN together with its  
223 poor ability to crosslinking may therefore account for the rapid hydrolysis of the protein under the *in*  
224 *vitro* digestion conditions.

225 For the  $\alpha$ S<sub>1</sub>-CN and the  $\alpha$ S<sub>2</sub>-CN, the crosslinking seemed to offer more protection to digestion after  
6 the incubation of NaCN with TG in solution than in emulsion (Figs 2 and S5). Apart from the  
7 aforementioned higher rate/degree of crosslinking of the caseins in solution (Macierzanka et al., 2011),  
8 the reason might also lie in the type of the crosslinking observed in the two systems. In the same  
9 studies, it was shown that incubation of NaCN with TG in solution might have led to some  
0 intramolecular crosslinking as the oligomers formed were found to be more mobile on SDS-PAGE than  
1 their counterparts formed from NaCN crosslinked at the oil-water interface, indicating that



232 intermolecular crosslinking might have prevailed at the interface. Therefore, the higher extent of  
233 crosslinking and more compact structuring of the proteins offered by the intramolecular crosslinks  
234 might account for some fragments of  $\alpha$ S<sub>1</sub>-CN and  $\alpha$ S<sub>2</sub>-CN incubated with TG in solution being more  
235 resistant to digestion than those crosslinked in emulsion. This leads to the conclusion that the group  
236 of oligomers of  $M_r$  ca. 50-100 kDa formed during the gastric digestion of crosslinked NaCN in emulsion  
237 (Fig. S2 F), might have been mainly composed of the  $\beta$ -CN fragments that showed significant  
238 resistance to pepsinolysis (Fig. 1).

239 Our results suggest that the TG crosslinking can improve resistance of casein molecules to  
240 gastrointestinal digestion, if, for example, this is required for modulating phase behaviour of protein-  
241 stabilised emulsions in the stomach and the rate of nutrients release (van Aken et al., 2011). The  
242 findings might then be useful for optimising protein structuring in personalised nutrition in order to  
243 modulate specific physiological responses to food, such as the ileal brake, which could in turn  
244 determine satiety and calorie intake.

245

## 246 Acknowledgements

247 The work at INRA-Agrocampus Ovest (UMR STLO 1253) was funded by INRA internal funding.  
248 The work at the IFR was supported by the BBSRC through an Institute Strategic Programme Grant  
249 (BB/J004545/1), and the work at GUT was funded through an internal grant (DS/032403T016). The  
250 authors are participants of the EU funded COST action INFOGEST (COST FA 1005).

251

## 252 Supplementary Material

253 Detailed description of the materials and methods used as well as additional data and discussion  
254 of the results obtained.

255

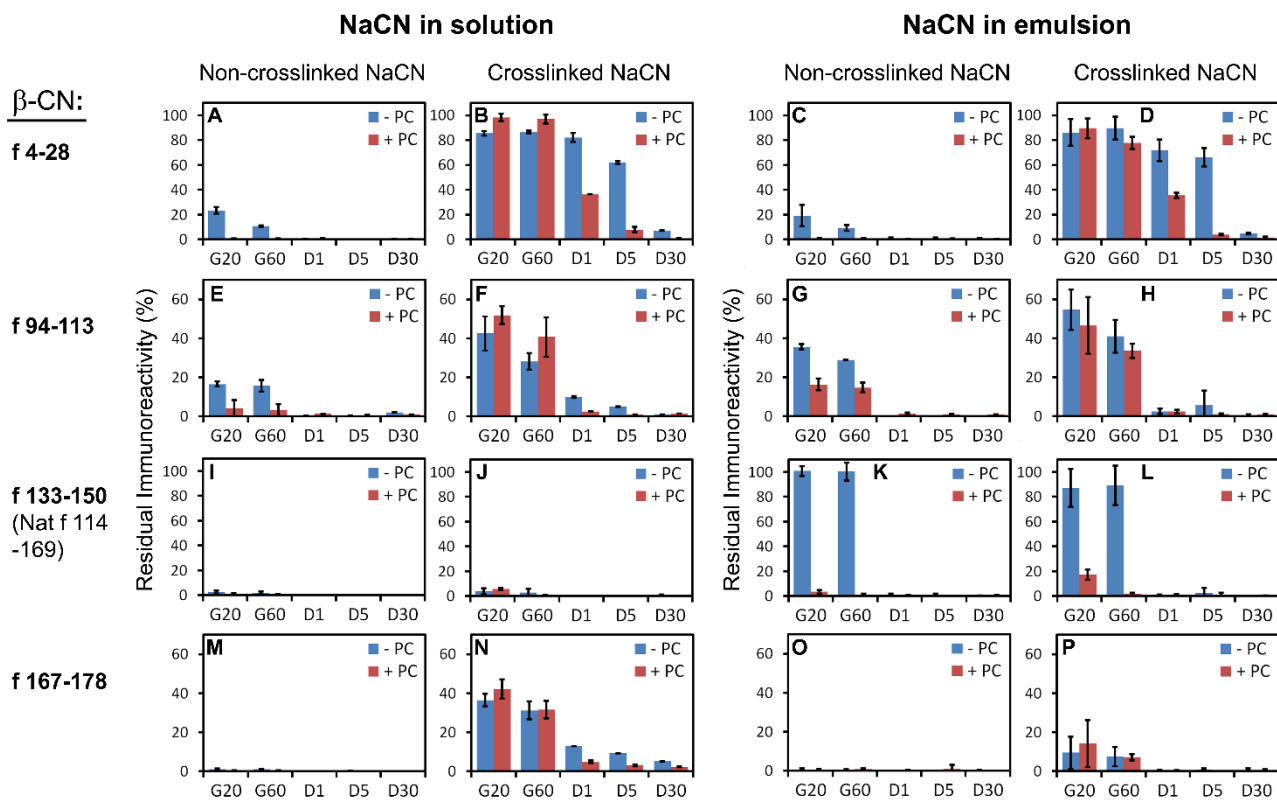
## 256 References

- 257 Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., Benamouzig, R., Tome, D.,  
258 & Leonil, J. (2013). Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy  
259 humans. *American Journal of Clinical Nutrition*, 97, 1314-1323.
- 260 Buchert, J., Ercili Cura, D., Ma, H., Gasparetti, C., Monogioudi, E., Faccio, G., Mattinen, M., Boer, H., Partanen,  
261 R., Selinheimo, E., Lantto, R., & Kruus, K. (2010) Crosslinking food proteins for improved functionality. *Annual*  
262 *Review of Food Science and Technology*, 1, 113–138.
- 263 Caessens, P. W. J. R., Gruppen, H., Slangen, C. J., Visser, S., & Voragen, A. G. J. (1999). Functionality of  $\beta$ -  
264 casein peptides: Importance of amphipathicity for emulsion-stabilizing properties. *Journal of Agricultural and*  
265 *Food Chemistry*, 47, 1856–1862.
- 266 Dickinson, E. (1997). Enzymatic crosslinking as a tool for food colloid rheology control and interfacial  
267 stabilization. *Trends in Food Science and Technology*, 8, 334-339.
- 268 Dickinson, E. (2006). Colloid science of mixed ingredients. *Soft Matter*, 2, 642–652.
- 269 Dickinson, E. (2010). Flocculation of protein-stabilized oil-in-water emulsions. *Colloids and Surfaces B:*  
0 *Biointerfaces*, 81, 130–140.
- 1 Dickinson, E., Horne, D. S., Pinfield, V. J., & Leermakers, F. A. M. (1997). Self-consistent-field modelling of  
2 casein adsorption. Comparison of results for  $\alpha$ <sub>s1</sub>-casein and  $\beta$ -casein. *Journal of the Chemical Society,*  
3 *Faraday Transactions*, 93, 425-432.
- 4 Dupont, D. (2017). Peptidomic as a tool for assessing protein digestion. *Current Opinion in Food Science*, 16,  
5 53-58.
- 6 Dupont, D., Rolet-Repecaud, O., & Senocq, D. (2003). A new approach to monitoring proteolysis phenomena  
7 using antibodies specifically directed against the enzyme cleavage site on its substrate. *Analytical*  
8 *Biochemistry*, 317, 240-246.

- 279 Faergemand, M., Murray, B. S., Dickinson, E., & Qvist, K. B. (1999). Cross-linking of adsorbed casein films with  
280 transglutaminase. *International Dairy Journal*, 9, 343-346.
- 281 Farrell, H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., Hicks, C. L., Hollar,  
282 C. M., Ng-Kwai-Hang, K. F., & Swaisgood, H. E. (2004). Nomenclature of the proteins of cows' milk - Sixth  
283 revision. *Journal of Dairy Science*, 87, 1641-1674.
- 284 Gan, J. A., Bornhorst, G. M., Henrick, B.M., & German, J.B. (2018) Protein digestion of baby foods: Study  
285 approaches and implications for infant health. *Molecular Nutrition & Food Research*, 62, Article no: 1700231
- 286 Griffin, M. A., Casadio, R., & Bergamini, C. M. (2002). Transglutaminases: nature's biological glues. *Biochemical*  
287 *Journal*, 368, 377-396.
- 288 HadjSadok, A., Pitkowski, A., Nicolai, T., Benyahia, L., & Moulai-Mostefa, N. (2008). Characterisation of sodium  
289 caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering. *Food*  
290 *Hydrocolloids*, 22, 1460-1466
- 291 Huppertz, T., Gazi, I., Luyten, H., Nieuwenhuijse, H., Alting, A., & Schokker, E. (2017). Hydration of casein  
292 micelles and caseinates: Implications for casein micelle structure. *International Dairy Journal*, 74, 1-11.
- 293 Huppertz, T., Fox, P. F., & Kelly, A. L. (2018). The caseins: Structure, stability, and functionality. In R. Y. Yada  
294 (Ed.), *Proteins in Food Processing (Second Edition)* (pp. 49-92). Duxford: Woodhead Publishing.
- 295 Johansson, A., Lugand, D., Rolet-Repecaud, O., Molle, D., Delage, M. M., Peltre, G., Marchesseau, S., Leonil,  
296 J., & Dupont, D. (2009). Epitope characterization of a supramolecular protein assembly with a collection of  
297 monoclonal antibodies: The case of casein micelle. *Molecular Immunology*, 46, 1058-1066.
- 298 Juvonen, K. R., Macierzanka, A., Lille, M. E., Laaksonen, D. E., Mykkänen, H. M., Niskanen, L. K., Pihlajamäki,  
299 J., Mäkelä, K., Mills, C. E. N., Mackie, A. R., Malcolm, P., Herzig, K.-H., Poutanen, K. S., & Karhunen, L. J.  
300 (2015). Cross-linking of sodium caseinate structured emulsion with transglutaminase alters the postprandial  
301 metabolism and appetite responses in healthy young individuals. *British Journal of Nutrition*, 114, 418-429.
- 302 Lucey, J. A., Srinivasan, M., Singh, H., & Munro, P. A. (2000). Characterization of commercial and experimental  
303 sodium caseinates by multiangle laser light scattering and size-exclusion chromatography. *Journal of*  
304 *Agricultural and Food Chemistry*, 48, 1610-1616.
- 305 Macierzanka, A., Sancho, A. I., Mills, E. N. C., Rigby, N. M., & Mackie, A. R. (2009). Emulsification alters  
306 simulated gastrointestinal proteolysis of  $\beta$ -casein and  $\beta$ -lactoglobulin. *Soft Matter*, 5, 538-550.
- 307 Macierzanka A., Bordron F., Rigby N. M., Mills E. N. C., Lille M., Poutanen K., & Mackie A. R. (2011).  
308 Transglutaminase cross-linking kinetics of sodium caseinate is changed after emulsification. *Food*  
309 *Hydrocolloids*, 25, 843-850.
- 310 Macierzanka, A., Böttger, F., Rigby, N. M., Lille, M., Poutanen, K., Mills, E. N. C., & Mackie A. R. (2012).  
311 Enzymatically structured emulsions in simulated gastrointestinal environment: Impact on interfacial  
312 proteolysis and diffusion in intestinal mucus. *Langmuir*, 28, 17349-17362.
- 313 Mackie, A. R., Mingins J., & North, A. N. (1991). Characterisation of adsorbed layers of a disordered coil protein  
314 on polystyrene latex. *Journal of the Chemical Society, Faraday Transactions*, 87, 3043-3049.
- 315 Monogioudi, E., Faccio, G., Lille, M., Poutanen, K., Buchert, J., & Mattinen, M.-L. (2011). Effect of enzymatic  
316 cross-linking of  $\beta$ -casein on proteolysis by pepsin. *Food Hydrocolloids*, 25, 71-81.
- 317 Senocq, D., Dupont, D., Rolet-Repecaud, O., & Leveux, D. (2002). ELISA for monitoring the cleavage of beta-  
318 casein at site Lys(28)-Lys(29) by plasmin during Comte cheese ripening. *Journal of Dairy Research*, 69, 491-  
319 500.
- 320 Singh, H., & Ye, A. Q. (2013). Structural and biochemical factors affecting the digestion of protein-stabilized  
321 emulsions. *Current Opinion in Colloid & Interface Science*, 18, 360-370.
- 322 van Aken, G. A., Bomhof, E., Zoet, F. D., Verbeek, M., Oosterveld, A. (2011). Differences in in vitro gastric  
323 behaviour between homogenized milk and emulsions stabilised by Tween 80, whey protein, or whey protein  
324 and caseinate. *Food Hydrocolloids*, 25, 781-788.
- 325  
326

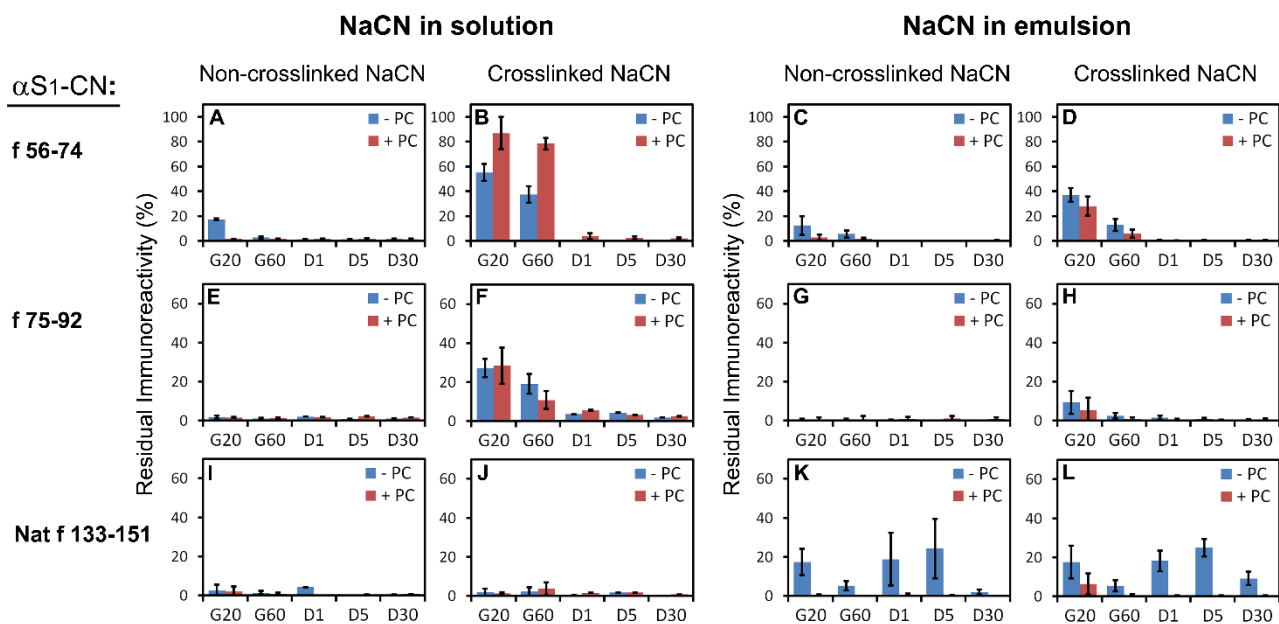






**Fig. 1.** Residual immunoreactivity (RI) of  $\beta$ -CN fragments (f) determined in time-point samples collected during the *in vitro* digestion of NaCN (results were normalised against the immunoreactivity detected for undigested protein sample; native i.e. non-crosslinked, or crosslinked). Effect of (i) presenting NaCN in aqueous solution (1 mg/mL) or emulsion (1 mg/mL), (ii) crosslinking of the protein with TG before digestion, and (iii) carrying out the digestion experiments in the presence or absence of vesicular phosphatidylcholine (PC) in the gastric phase of digestion. Gastric samples have been marked with G and duodenal with D, followed by a number corresponding to the digestion time (min) after which the samples were taken. Extended version of Fig. 1 has been shown in the Supplementary Material (Fig. S3).

329  
330  
331  
332  
333  
334  
335  
336  
337  
338  
339  
340  
341



343  
344  
345  
346  
347

**Fig. 2.** Residual immunoreactivity (RI) of  $\alpha$ S<sub>1</sub>-CN fragments (f) determined in time-point samples collected during the *in vitro* digestion of NaCN. For more details see caption of Fig. 1. Extended version of Fig. 2 has been shown in the Supplementary Material (Fig. S4).

Postprint of: Böttger F., Dupont D., Dulko D., Bajka B., Mackie A., Macierzanka A.: Which casein in sodium caseinate is most resistant to *in vitro* digestion? Effect of emulsification and enzymatic structuring. *FOOD HYDROCOLLOIDS*. Vol. 88, (2019), pp. 114-118. DOI: 10.1016/j.foodhyd.2018.09.042