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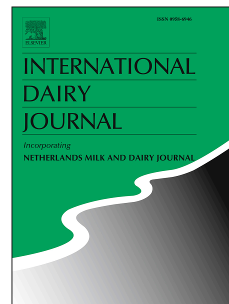

Immunomodulatory activity of 5 kDa permeate fractions of casein hydrolysates generated using a range of enzymes in Jurkat T cells and RAW264.7 macrophages

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2 **generated using a range of enzymes in Jurkat T cells and RAW264.7 macrophages**

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22 ____

23 ABSTRACT

24

25 The in vitro bioactivity of 5 kDa ultrafiltration permeate fractions of casein hydrolysates
26 produced using different enzymes were compared. Reverse phase ultra-performance liquid
27 chromatography and gel permeation chromatography showed that the permeates had different
28 physicochemical properties (molecular mass and degree of hydrolysis). The Flavourzyme®
29 permeate had the highest activity in the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic
30 acid) (ABTS) assay. Cellular antioxidant and immunomodulatory assays showed that none of
31 the permeates exhibited in vitro antioxidant activity, while all permeates significantly ($P <$
32 0.05) decreased interleukin-6 (IL-6) production in ConA-stimulated Jurkat T cells at 0.50%
33 (w/v) and LPS-stimulated RAW264.7 cells at 0.05 and 0.50% (w/v). Three permeates,
34 obtained using Flavourzyme®, Flavorpro Whey and trypsin, also significantly ($P < 0.05$)
35 decreased IL-1β production at 0.05% (w/v) in RAW264.7 cells. Western blot analysis
36 showed that all permeates significantly decreased the expression of the NF-κB subunit, p65,
37 in RAW264.7 cells indicating that anti-inflammatory activity may be associated with this
38 pathway.

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41 **1. Introduction**

42

43 Cardiovascular disease (CVD), in particular atherosclerosis, is associated with
44 elevated inflammation and oxidative stress. Drugs used to treat these conditions may produce
45 unwanted side effects, therefore natural alternatives to synthetic drugs are constantly sought
46 (Chakrabarti, Jahandideh & Wu, 2014). Bioactive peptides are small protein fragments that
47 have the potential to exert beneficial health effects in vivo (Urista, Fernández, Rodriguez,
48 Cuenca & Jurado, 2011). Bioactive peptides, derived from the milk protein casein,
49 demonstrate numerous bioactivities such as antihypertensive, opioid and antimicrobial
50 activity (Di Pierro, O'Keeffe, Poyarkov, Lomolino, & FitzGerald, 2014; Kazlauskaite et al.,
51 2005; Nongonierma, O'Keeffe, & FitzGerald, 2016; Phelan, Aherne-Bruce, O'Sullivan,
52 FitzGerald, & O'Brien, 2009; Tang et al., 2015; Trivedi, Zhang, Lopez-Toledano, Clarke, &
53 Deth, 2016; Yamada et al., 2015). The enzymes used in the generation of these peptides, as
54 well as the length and amino acid sequence of the resultant peptides influence the
55 bioactivities observed (Power, Jakeman, & FitzGerald, 2013).

56 Casein hydrolysates with cellular antioxidant activity have been reported in numerous
57 studies. Garcia-Nebot, Cilla, Alegría, and Barberá (2011) reported that
58 caseinophosphopeptides showed cyto-protective effects against H_2O_2-induced oxidative
59 stress in Caco-2 cells. Xie, Wang, Ao, and Li (2013) reported that an Alcalase® generated
60 hydrolysate protected HepG2 cells from H_2O_2-induced oxidative damage. Hydrolysis of
61 bovine casein glycomacropeptide with papain was also reported to protect against H_2O_2-
62 induced oxidation in RAW264.7 cells, along with increasing the level of cellular antioxidant
63 enzymes (Cheng, Gao, Song, Ren, & Mao, 2015). Treatment of Jurkat T cells with casein
64 hydrolysates generated using different mammalian, plant or bacterial enzymes has previously
65 been reported to increase cellular antioxidant levels (Lahart et al., 2011; Phelan et al., 2009).

66 More recently, the hydrolysis of casein using Prolyve®, generated a hydrolysate which
67 prevented H_2O_2-induced DNA damage in U937 cells (Cermeño, FitzGerald, & O'Brien,
68 2016).

69 Casein hydrolysates have also been studied for their immunomodulatory and anti-
70 inflammatory potential. The tryptic hydrolysis of casein generated a hydrolysate with
71 immune enhancing effects in mouse macrophages (Kazlauskaite et al., 2005), while β-casein
72 and several peptides within β-casein have been reported to decrease proliferation in murine
73 spleen cells (Bonomi et al., 2011). Studies have also reported that the activity of NF-κβ, one
74 of the major inflammatory signalling pathways, may be reduced in cells exposed to casein
75 hydrolysates (Altmann et al., 2016; Malinowski, Klempt, Clawin-Rädecker, Lorenzen, &
76 Meisel, 2014). The hydrolysis of casein using mammalian, bacterial or plant derived enzymes
77 has also been reported to have immunomodulating effects on Jurkat T cells in other studies
78 (Cermeño et al., 2016; Lahart et al., 2011; Phelan et al., 2009). To the best of our knowledge,
79 no study has compared the cellular antioxidant and anti-inflammatory activities of sodium
80 caseinate hydrolysates generated under the same conditions [hydrolysis time and
81 enzyme:substrate (E:S) ratio], using a range of proteolytic preparations.

82 The aims of the present study were: (i) to generate and examine the physicochemical
83 characteristics of 5 kDa permeates of casein hydrolysates generated using seven different
84 proteolytic preparations, (ii) to compare the antioxidant activity of the hydrolysates in vitro
85 and in H_2O_2-challenged U937 cells; (iii) to determine cytotoxicity in RAW264.7 mouse
86 macrophages, Jurkat T cells and U937 lymphocytes and (iv) to compare the
87 immunomodulatory activity of the 5 kDa permeates of the hydrolysates in RAW264.7
88 macrophages and Jurkat T cells.

89

90 **2. Materials and methods**

91

92 *2.1. Materials*

93

94 Human Jurkat T cells, RAW264.7 mouse macrophages and human U937 lymphocytes
95 were purchased from the European Collection of Animal Cell cultures (Salisbury, UK).
96 Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland, UK). Cell
97 culture plastics were supplied by Cruinn Diagnostics (Dublin, Ireland). Sodium caseinate
98 (87.57% (w/w) protein) was from Arrabawn Co-op (Tipperary, Ireland). Protease from
99 *Bacillus licheniformis* (Alcalase® 2.4L), protease from *Aspergillus oryzae* (Flavourzyme®, ≥
100 500 U g^{-1}) and TPCK-trypsin were from Sigma-Aldrich (Wicklow, Ireland). Prolyve 1000
101 was from Lyven Enzymes Industrielles (Caen, France) and Flavorpro Whey, Promod 144MG
102 and Pepsin were all from Biocatalysts (Cefn Coed, Wales, UK). All other cell culture
103 reagents and chemicals including concanavalin A (ConA), lipopolysaccharide (LPS) and
104 Trolox were purchased from Sigma-Aldrich, unless otherwise stated.

105

106 *2.2. Generation of casein hydrolysates, determination of the degree of hydrolysis and*
107 *ultrafiltration*

108

109 Sodium caseinate, reconstituted at 10% (w/v) in distilled water, was equilibrated at 50
110 °C with gentle mixing for 1.5 h and the pH was adjusted to pH 7 (or pH 2 for peptic
111 hydrolysis). Enzymatic hydrolysis was carried out using a pH Stat (718 Stat Titrino,
112 Metrohm, Herisau, Switzerland) as previously described (Spellman, McEvoy, O'Cuinn, &
113 FitzGerald, 2003). Hydrolysis was performed using seven different proteolytic preparations
114 (Alcalase® 2.4L, Prolyve 1000, Flavourzyme®, Flavorpro Whey, Pepsin, TPCK-Trypsin and
115 Promod 144 MG) at industrially relevant E:S ratios (0.23% for Alcalase® 2.4L, Prolyve 1000

116 and Flavourzyme®, Flavorpro Whey and Promod 144 MG, 0.13% for trypsin and 0.25% for
117 pepsin) for 4 h, except for the tryptic hydrolysate that was generated over 3 h.

118 Following hydrolysis, the enzymes were inactivated by heating at 80 °C for 20 min.
119 Control samples were also included; (i) sodium caseinate was incubated at 50 °C without
120 enzyme and (ii) enzyme was incubated at 50 °C without sodium caseinate. These control
121 samples were also subjected to heating at 80 °C for 20 min after 4 h incubation. All samples
122 were freeze-dried (FreeZone 18L, Labconco, Kansas City, USA) and stored at –20 °C until
123 use. The degree of hydrolysis (DH) of the hydrolysates was determined using the 2,4,6-
124 trinitrobenzenesulfonic acid (TNBS) method of Adler-Nissen (1979) and as described by Le
125 Maux, Nongonierma, Barre, and FitzGerald (2016). Ultrafiltration (UF) fractions were
126 generated by passing the hydrolysate through a membrane having a nominal cut-off of 5 kDa
127 using a benchtop ultrafiltration system (Sartoflow Alpha, Sartorius AG, Goettingen,
128 Germany) as described by O'Keeffe and FitzGerald (2014) and were freeze-dried as above.

129

130 *2.3. Reverse phase ultra-performance liquid chromatography and gel permeation high*
131 *performance liquid chromatography analysis of UF permeates of casein hydrolysates*

132

133 Freeze-dried hydrolysates/ultrafiltration permeates were reconstituted at 1 mg mL^{-1} in
134 mobile phase A [0.1% trifluoroacetic acid (TFA) in MS grade H_2O] and 7 µL was separated
135 on an ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters, Milford,
136 Massachusetts, USA) at a flow rate of 0.2 µL min^{-1} using an ACQUITY BEH 300 C18
137 column (2.1 × 50 mm, 1.7 µm; Waters, Dublin, Ireland). Mobile phase B was 0.1% TFA in
138 80% ACN. Separation was achieved using a linear gradient; 0–0.28 min 100% A; 0.28–45
139 min 100–20% A; 45–46 min 20–0% A; 46–48 min 0% A; 48–49 min 0–100% A; 49–51 min
140 100% A. Detector response was measured at 214 nm. Gel permeation high performance

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141 liquid chromatography (GP-HPLC) was performed as previously described (Spellman,
142 O'Cuinn, & FitzGerald, 2009) with separation achieved through isocratic elution (mobile
143 phase: 0.1%TFA in 30% ACN at 1.0 mL min^{-1}) on a TSK G2000 SW column (600 × 7.5 mm
144 ID) connected to a TSKGEL SW guard column (75 × 7.5 mm ID) and the eluent was
145 monitored at 214 nm.
146
147 *2.4. 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) assay*
148
149 The 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)$^{+}$ (ABTS^{+}) radical
150 scavenging assay was carried out as described by Re et al. (1999). The ABTS^{+} radical was
151 prepared by incubating ABTS solution (7 mM) with potassium persulfate (2.45 mM), an
152 oxidizing agent, in a ratio of 1.0:0.5 (v/v) at 20 °C for 16 h in the dark. The radical was then
153 diluted using phosphate buffered saline (5 mM, pH 7.4) until an absorbance of 0.70 ± 0.02 at
154 a wavelength of 734 nm was achieved. Activity was reported based on a standard curve using
155 Trolox and expressed as μmol Trolox equivalents per gram of freeze-dried powder of
156 hydrolysate (FDP). The scavenging activity for each sample was determined by three
157 independent experiments.
158
159 *2.5. Oxygen radical absorbance capacity assay*
160
161 The oxygen radical absorbance capacity (ORAC) assay was performed according to
162 the method of Zulueta, Esteve, and Frígola (2009) with modifications as described by
163 O'Keeffe and FitzGerald (2014). The final ORAC values were expressed as μmol of Trolox
164 equivalents per mg of FDP and were the mean ± the standard error of three independent
165 determinations.

166

167 *2.6. Cell culture*

168

169 Jurkat T and U937 cells were maintained in Royal Park Memorial Institute (RPMI)
170 medium supplemented with 10% (v/v) foetal bovine serum (FBS). RAW264.7 cells were
171 grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v)
172 FBS. All cell lines were cultured in an atmosphere of CO_2-air (5:95, v/v) at 37 °C and were
173 maintained in the absence of antibiotics. The dried 5 kDa permeates of the casein
174 hydrolysates were solubilised to a concentration of 10% (w/v) using distilled deionised water,
175 sterile-filtered using a low protein binding 0.22 μm Durapore^{TM} millex filter unit (Merck
176 KGaA) and diluted with sterile DMEM.

177

178 *2.7. Cell proliferation assay*

179

180 Jurkat T, U937 and RAW264.7 cells were exposed to increasing concentrations of the
181 different 5 kDa permeates (0.05 to 5.0%, w/v) for 24 h in 96 well plates in a final volume of
182 200 μL. Cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-
183 diphenyltetrazolium bromide (MTT) assay (MTT I proliferation kit, Roche Diagnostics;
184 Burgess Hill, West Sussex, UK).

185

186 *2.8. Alkaline single cell gel electrophoresis (comet) assay*

187

188 U937 cells were used to assess the DNA protective effects of the 5 kDa permeates in
189 oxidant challenged cells. Cells were seeded at a density of 1×10^5 cells mL^{-1} and exposed to
190 the permeates (0.05%, w/v) for 24 h. DNA damage was then initiated by exposing cells to 80

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191 μmol L^{-1} H_2O_2 for 30 min at 37 °C after which DNA damage was assessed using the comet
192 assay as previously described (Phelan et al., 2009).
193
194 *2.9. Cytokine production in Jurkat T and RAW264.7 cells*
195
196 Jurkat T cells were seeded at a density of 2×10^5 cells mL^{-1} and simultaneously
197 incubated with ConA (25μg mL^{-1}) and the 5 kDa permeates (0.50 and 0.05%, w/v) for 24 h.
198 Following incubation, the quantity of interleukin (IL)-6), interferon (IFN)-γ, IL-2 and IL-10
199 in the media was measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience,
200 Insight Biotechnology Ltd, Wembley, UK). RAW264.7 cells were seeded at a density of 0.2
201 $\times 10^5$ cells mL^{-1} and simultaneously incubated with LPS (0.1 μg mL^{-1} for IL-6 and tumour
202 necrosis factor (TNF)-α; 2 μg mL^{-1} for IL-1β) and the 5 kDa permeates (0.050% and 0.005,
203 w/v) for 24 h. Following incubation, the content of each of the three cytokines (IL-6, Il-1β
204 and TNF-α) in the media was measured using ELISA kits. Absorbance was determined at
205 450 nm with a reference wavelength of 570 nm (Varioskan™ Flash Multimode Reader,
206 Thermoscientific, Waltham, MA, USA).
207
208 *2.10. Western blotting*
209
210 RAW264.7 cells were seeded at a density of 8×10^5 cells per dish in 60 mm dishes
211 and allowed to adhere overnight. Cells were then stimulated using LPS (0.1 μg mL^{-1}) and
212 treated with the permeates (0.05%, w/v) for 24 h. After this treatment, the RAW264.7 cells
213 were washed using ice cold PBS and lysed using RIPA buffer containing protease (Halt
214 protease inhibitor, Sigma 78439) and phosphatase inhibitors (1 mM $NaVO_4$, 2.5 mM $Na_4O_7P_2$
215 and 2 mM β-glycerophosphate). Cell lysates were then scraped and transferred to Eppendorf

216 tubes and placed on ice for 20 min. Lysates were centrifuged at 25,155 × *g* for 1 h at 4 °C and
217 the supernatant transferred to fresh Eppendorf tubes. Protein concentration was determined
218 using the BCA method (Smith et al., 1985) and samples were stored at –80 °C until Western
219 blot analysis.

220 Proteins were separated using a 10% sodium dodecyl sulphate polyacrylamide
221 electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene fluoride membrane. The
222 membrane was then blocked overnight at 4 °C using Odyssey Blocking buffer with 0.1%
223 Tween-20. The following day, the membrane was washed using Tris-buffered saline-Tween-
224 20 (TBS-T) and incubated with mouse anti-p65 antibody overnight at 4 °C. Following
225 incubation, the membrane was again washed using TBS-T and incubated for a further hour at
226 room temperature with Infrared-labelled goat anti-mouse secondary antibody (LI-COR Inc.).
227 Finally, the membrane was washed using TBS-T, followed by TBS to remove any remaining
228 Tween-20. The Odyssey Clx Imager was used to visualise protein bands. Protein loading was
229 determined using β-actin as a control.

230

231 *2.11. Statistical analysis*

232

233 Statistical analysis was determined by ANOVA followed by Dunnett's test or Tukey's
234 post-hoc test using Prism 5.0 (GraphPad Inc. San Diego, California, USA). Results are
235 expressed as mean ± SE or mean ± SD. Statistical significance was $P < 0.05$.

236

237 **3. Results**

238

239 *3.1. Physicochemical characterisation, degree of hydrolysis and in vitro antioxidant*
240 *assessment of casein hydrolysates*

241

242 The different proteolytic preparations produced hydrolysates having distinctly
243 different RP-UPLC (Fig. 1) and GPC (Fig. 2) profiles demonstrating the differing
244 specificities of the proteolytic preparations. The higher specificity of TPCK-Trypsin and
245 pepsin resulted in a lower proportion of low molecular mass (< 500 Da) peptides present in
246 these samples (Fig. 2) and in a lower DH (Table 1). Alcalase®, Flavourzyme® and Prolyve
247 hydrolysis resulted in hydrolysates with the highest proportion of low molecular mass
248 peptides (< 500 Da) and the highest degree of hydrolysis (~14–18%).

249 All 5 kDa permeates showed significantly ($P < 0.05$) higher antioxidant activity than
250 intact sodium caseinate or the full hydrolysates in both the ORAC and $ABTS^+$ assays (data
251 not shown). There was no significant ($P < 0.05$) difference in antioxidant activity between the
252 different 5 kDa permeates when measured by the ORAC assay, while the 5 kDa UF permeate
253 of the Flavourzyme® hydrolysate had the highest activity in the ABTS assay (Table 1);
254 significantly ($P < 0.05$) higher than that of the 5 kDa UF permeate of the tryptic hydrolysate.
255 There was no correlation between the DH of the hydrolysates and the antioxidant activity (via
256 ORAC or $ABTS^+$ assays) of the corresponding 5 kDa UF fractions (Table 1).

257

258 *3.2. Effects of casein hydrolysate 5 kDa permeates on cell proliferation in RAW264.7*
259 *mouse macrophages, human U937 lymphocytes and human Jurkat T cells.*

260

261 RAW264.7 mouse macrophages were exposed to increasing concentrations (0–5%
262 w/v) of the different 5 kDa permeates for 24 h. The MTT assay was then used to assess the
263 effect of each hydrolysate on cell proliferation. Proliferation was generally unaffected by the
264 permeates up to a concentration of 1% (w/v) (Table 2). At a concentration of 5% (w/v), cell
265 proliferation declined significantly ($P < 0.05$) in cells incubated with hydrolysate permeates

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266 generated using Flavorpro Whey, trypsin and pepsin. For Jurkat T cells, the 5 kDa permeates
267 obtained from hydrolysates generated using Promod, trypsin and pepsin significantly ($P <$
268 0.05) decreased cell proliferation at 5% (w/v), while the Flavourzyme® hydrolysate permeate
269 significantly ($P < 0.05$) increased cell proliferation at this concentration (Table 3). A similar
270 trend was seen with 5 kDa permeates in U937 cells (Table 4). Concentrations of 0.050 and
271 0.005% (w/v) were, therefore, selected for bioactivity assays involving RAW264.7 cells.
272 Non-cytotoxic concentration of 0.50 and 0.05% (w/v) for Jurkat T cells, and 0.05% (w/v) for
273 U937 were used for bioactivity assays ensuring that all cell viabilities remained greater that
274 85%.
275
276 *3.3. Antioxidant activity of casein hydrolysate 5 kDa permeates*
277
278 The comet assay was used to assess the DNA protective effect of the 5 kDa permeates
279 against H_2O_2-induced DNA damage in U937 cells. Tail DNA was increased to approximately
280 80% in U937 cells exposed to H_2O_2 (80 μmol L^{-1}) for 30 min from a control level of
281 approximately 16% in untreated cells. Pre-incubation of U937 cells with the different
282 permeates at 0.05% (w/v) for 24 h did not protect cells against H_2O_2–induced DNA damage
283 (Fig. 3).
284
285 *3.4. Cytokine production in Jurkat T cells*
286
287 Jurkat T cells were stimulated to produce cytokines using 25 μg mL^{-1} ConA,
288 following which cytokine (IL-6, IFN-γ, IL-2 and IL-10) production was measured. IL-6
289 production was seen to decrease in cells exposed to the 5 kDa permeates (Table 5). All
290 hydrolysate permeates at 0.50% (w/v) produced a significant ($P < 0.05$) decrease in IL-6

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291 production compared with control values. No significant ($P < 0.05$) effects were seen in IL-2,
292 IL-10 and IFN-γ production after exposure to the 5 kDa permeates at either 0.50 or 0.05%
293 (w/v).

294

295 *3.5. Cytokine production in RAW264.7 cells*

296

297 RAW264.7 cells were stimulated with LPS at 0.1 μg mL^{-1} or 2 μg mL^{-1} and treated
298 with 5 kDa permeates for 24 h before cytokine analysis (Table 6). At 0.05% (w/v), each of
299 the hydrolysate fractions caused a significant ($P < 0.05$) decrease in IL-6 production
300 compared with cells incubated with LPS alone. At 0.005% (w/v), none of the hydrolysate
301 fractions caused significant ($P < 0.05$) decreases in IL-6 production. Hydrolysate permeates
302 generated using Flavourzyme®, Flavorpro Whey and trypsin caused a significant ($P < 0.05$)
303 decrease in IL-1β production in cells incubated with these samples at 0.050% (w/v). No
304 effect was seen in TNF-α production after treatment with the 5 kDa permeates at any
305 concentration.

306

307 *3.6. NF-κB protein expression in RAW264.7 cells*

308

309 RAW264.7 cells were stimulated using 0.1 μg mL^{-1} LPS and treated with the 5 kDa
310 permeates at 0.050% (w/v) for 24 h in 60 mm dishes. Cells were then lysed and cell lysates
311 were examined for protein expression of the NF-κB subunit, p65. All hydrolysate fractions
312 caused a significant ($P < 0.05$) decrease in p65 protein expression compared with cells treated
313 with LPS alone (Fig. 4).

314

315 **4. Discussion**

316

317 It is recognised that utilisation of a variety of analytical techniques is required to
318 capture differing pathways of antioxidant activity. The ORAC assay measures the ability of
319 an antioxidant to prevent peroxyl radical oxidation of a fluorescent probe by means of
320 hydrogen atom transfer (HAT). In the ABTS assay, the antioxidant present in the permeate
321 sample scavenges the ABTS radical cation ($ABTS^{\bullet+}$) by means of electron transfer (Power et
322 al., 2013). In the present study, during initial experimentation it was observed that
323 concentration of the low molecular mass peptides on processing through a UF membrane
324 having a molecular mass cut-off of 5 kDa resulted in an increase in the antioxidant activity as
325 measured by the ORAC assay. However, no further increase in antioxidant activity was
326 achieved on further processing through a 1 kDa membrane (data not shown). Therefore, the 5
327 kDa permeates of each of the hydrolysates were used for all other determinations.
328 Studies have reported that casein hydrolysates possess non-cellular antioxidant
329 activity, mainly radical scavenging or electron donating ability. Alcalase® has been
330 frequently used to produce such hydrolysates (Ao & Li, 2013; Chen & Li, 2012; De Gobba,
331 Tompa, & Otte, 2014; Xie, Liu, Wang, & Li, 2014; Xie, Wang, Jiang, Liu, & Li, 2015).
332 Alcalase® was also used in this study; however, no significant difference was seen in the
333 antioxidant activity of this hydrolysate compared with hydrolysates produced by the other
334 enzyme preparations and, interestingly, Flavourzyme® produced a hydrolysate with higher
335 ABTS activity. This may be due to the presence of a larger number of peptides below 500 Da
336 in the Flavourzyme hydrolysate.
337 No cellular antioxidant activity was observed with the hydrolysates in the present
338 study. Previously, Phelan et al. (2009) reported that non-ultrafiltered casein hydrolysates
339 generated using commercial food-grade enzyme preparations altered glutathione and catalase
340 (CAT) activity in Jurkat T cells, but did not prevent H_2O_2-induced DNA damage in Caco-2

341 cells. Cermeño et al. (2016) reported that a casein hydrolysate, generated using Prolyve,
342 significantly ($P < 0.05$) protected U937 cells from H_2O_2-induced DNA damage. The 5 kDa
343 permeate of the Prolyve hydrolysate herein was generated using similar conditions; however,
344 no cell protective effect was seen. Notably, the full hydrolysate was used in Cermeño et al.
345 (2016) while our study used the 5 kDa permeate fraction; this may imply that the cell
346 protective effect was associated with higher molecular mass peptides. Xie et al. (2013) found
347 that casein hydrolysates produced using Alcalase® or the simulated gastrointestinal digestion
348 of casein showed significant protective effects in challenged HepG2 cells by reducing oxidant
349 induced cell death. A follow-on study reported that the Alcalase® hydrolysis of casein
350 produced hydrolysate fractions which enhanced catalase and superoxide dismutase activity
351 and increased viability in H_2O_2-exposed HepG2 cells. The hydrolysate was fractionated based
352 on charge and negatively charged fractions had greater antioxidant activity (Wang, Xie, & Li,
353 2016). Results herein indicate that the enzymes used did not affect antioxidant activity as
354 hydrolysate 5 kDa permeates with similar activity were produced in all cases.

355 In the present study, hydrolysates significantly decreased pro-inflammatory cytokine
356 production (IL-6 and IL-1β) in T cells and macrophages. Casein hydrolysates, produced using
357 combinations of TGase and Prolyve were previously reported to significantly decrease IL-6
358 production in Jurkat T cells (Cermeño et al., 2016). Malinowski et al. (2014) also reported
359 that a tryptic hydrolysate of bovine β-casein had significant anti-inflammatory activity in
360 kidney cells. In this case, casein was hydrolysed for 4 h and the hydrolysate 1–5 kDa
361 permeate fractions exhibited significant anti-inflammatory activity in human kidney cells. It
362 was suggested that a group of large hydrophobic peptides were responsible for the anti-
363 inflammatory activity. The hydrolysis of β-casein using cod trypsin has also been reported to
364 have anti-inflammatory activity in kidney cells and larger peptides (> 5 kDa) were reported to
365 have higher activity compared with lower molecular mass peptides (Altmann et al., 2016).

366 The hydrolysis of sodium caseinate using a bacterial enzyme has also been reported to
367 reduce IL-8 production in TNF-α stimulated Caco-2 cells, as well as downregulating several
368 pro-inflammatory cytokines expression in LPS-stimulated colonic tissue. Activity was
369 reported to be highest in the 1 kDa retentate fraction in this case (Mukhopadhya et al., 2014).
370 All the hydrolysates in the present study were 5 kDa permeates and had significant anti-
371 inflammatory activity, particularly those produced using trypsin, Flavourzyme® and
372 Flavorpro Whey.

373 Two enzyme-only controls were generated based on the high activity of the
374 corresponding hydrolysates and these controls were screened for anti-inflammatory activity
375 in RAW264.7 cells to rule out reagent/enzyme related activity. Enzyme-only controls had
376 little anti-inflammatory activity (data not shown), therefore it was determined that the
377 observed anti-inflammatory activity was due to the hydrolysis of casein by these enzymes.
378 Flavourzyme® has previously been shown to generate hydrolysates from brewer's spent
379 grain that were capable of decreasing IFN-γ production in Jurkat T cells (McCarthy et al.,
380 2013a,b). Bamdad, Shin, Suh, Nimalaratne, and Sunwoo (2017) also reported that a
381 Flavourzyme® generated casein hydrolysate decreased nitric oxide production and TNF-α
382 mRNA expression in RAW264.7 cells; however, this hydrolysate was produced using a
383 combined treatment of high hydrostatic pressure and enzymatic digestion. To the best of our
384 knowledge, no other studies have previously reported anti-inflammatory activity with
385 Flavorpro Whey generated casein hydrolysates.

386 In the present study, while hydrolysate treatment resulted in a decrease in IL-6 and
387 IL-1β production, TNF-α production was unaffected. Yak milk casein hydrolysates, produced
388 using Alcalase®, were reported to decrease the production of IL-6, IL-1β and TNF-α in
389 macrophages (Mao, Cheng, Wang, & Wu, 2011). Hydrolysates used in the study by Mao et
390 al. (2011) had significant in vitro antioxidant activity that may have contributed to the

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391 enhanced anti-inflammatory response; IL-6 and IL-1β production in macrophages was
392 decreased by ~70% and 60%, respectively, in the study by Mao et al. (2011) compared with
393 ~55% and 30%, respectively, in our study. The differing amino acid compositions of milk
394 from different species may also have affected activity, as reported by a study which reported
395 higher antioxidant activity in camel milk casein hydrolysates compared with bovine milk
396 casein hydrolysates (Moslehishad et al., 2013).

397 The NF-κB pathway is a major transcription pathway in cells linked to inflammation
398 and chronic inflammatory diseases such as atherosclerosis, inflammatory bowel disease and
399 cancer (Yamamoto & Gaynor, 2001). In the current study, the NF-κB subunit p65 (also
400 known as RELA), which is involved in nuclear translocation and activation, was studied after
401 treatment with hydrolysate fractions in LPS-stimulated RAW264.7 cells. The anti-
402 inflammatory activity of all seven hydrolysate 5 kDa permeates may be linked to NF-κB
403 activation as the protein expression of p65 was inhibited in all cases. Milk-derived
404 hydrolysates with anti-inflammatory activity have previously been reported to act through the
405 NF-κB pathway in different cell lines (Altmann et al., 2016; Malinowski et al., 2014;
406 Marcone, Haughton, Simpson, Belton, & FitzGerald, 2015; Nielsen, Theil, Larson, & Parup,
407 2012). To the best of our knowledge, our study is the first to examine the molecular
408 mechanism behind the anti-inflammatory activity of bovine casein hydrolysates in
409 macrophages. A study of rice protein hydrolysates, prepared using trypsin, yielded similar
410 results to our study, where IL-6 and IL-1β expression in LPS-stimulated RAW264.7 cells
411 were decreased after 24 h hydrolysate treatment and p65 translocation to the nucleus was also
412 decreased (Wen et al., 2016).

413

414 5. Conclusions

415

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416 The 5 kDa UF permeates of casein hydrolysates produced using mammalian, plant,
417 fungal and bacterially-derived proteolytic preparations showed significant in vitro anti-
418 inflammatory activity in Jurkat T cells and RAW264.7 macrophages. These hydrolysate
419 permeates had a greater anti-inflammatory effect on IL-6 production in RAW264.7 cells
420 compared with Jurkat T cells. Three of the hydrolysate permeates also significantly decreased
421 IL-1β production in RAW264.7 cells and were produced using either fungal or mammalian
422 derived enzymes. Based on the ability of the hydrolysate 5 kDa permeates to significantly
423 inhibit the expression of the NF-κB subunit, p65, our results also indicate that this anti-
424 inflammatory activity may be dependent on the NF-κB inflammatory pathway. Future studies
425 on the in vivo anti-inflammatory activity of hydrolysate permeates, generated using fungal or
426 mammalian derived enzymes, may be of interest.
427
428
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434
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Figure legends

Fig. 1. Reversed phase ultra-performance liquid chromatography profiles of (a) sodium caseinate and (b–h) casein hydrolysate 5 kDa permeates where the enzymatic preparations employed were: (b) Alcalase® 2.4L; (c) Flavourzyme®; (d) Prolyve 1000; (e) Flavorpro Whey; (f) Promod 144MG; (g) Trypsin; (h) Pepsin.

Fig. 2. Molecular mass distribution profiles of intact sodium caseinate and 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates generated with different enzymatic preparations using gel permeation chromatography; ■, < 500 Da; ■, 1000–500 Da; ▥, 2000–1000 Da; ▧, 5000–2000 Da; ▩, 10,000–5,000 Da; ⊟, > 10,000 Da.

Fig. 3. The ability of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0.05%, w/v) to protect against H_2O_2 induced DNA damage in U937 lymphocytes; H, cells treated with H_2O_2 only. Tail DNA damage was measured using the comet assay and expressed as a percentage relative to hydrogen peroxide control values (untreated cells). Data are means ± SD of 2 independent experiments; significance was measured using ANOVA followed by Dunnett's test.

Fig. 4. Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0.05%, w/v) on NF-κB (p65) protein expression in RAW264.7 cells relative to cells treated with LPS alone (Control, assigned at 100%); p65 protein expression was assessed in LPS stimulated RAW264.7 cells by Western Blot after 24 h sample treatment. The data show one of three independent experiments, which yielded similar results and are the means ± SE of 3 independent experiments; significance was measured using ANOVA followed by Dunnett's test; * denotes $P < 0.05$.

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Table 1

Oxygen radical absorbance capacity (ORAC) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activities of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates generated with different enzymatic preparations. [a]

Enzyme	Degree of hydrolysis (%)	ORAC value (µmol TE mg^{-1} FDP)	ABTS value (µmol TE g^{-1} FDP)
Alcalase®	18.01 ± 1.59[a]	1.072 ± 0.07[a]	52.86 ± 3.46[a,b]
Flavourzyme®	14.43 ± 0.61[b]	1.130 ± 0.076[a]	71.18 ± 7.65[a]
Prolyve	15.65 ± 0.60[a,b]	1.120 ± 0.088[a]	58.38 ± 3.48[a,b]
Flavorpro Whey	12.86 ± 0.89[b]	1.154 ± 0.007[a]	67.59 ± 4.91[a,b]
Promod 144MG	5.23 ± 0.42[c,d]	1.080 ± 0.085[a]	52.14 ± 2.19[a,b]
Trypsin	7.21 ± 0.72[c]	1.080 ± 0.041[a]	44.23 ± 4.83[b,c]
Pepsin	2.31 ± 0.61[d]	1.120 ± 0.044[a]	59.00 ± 4.50[a,b]

[a] Abbreviations are: TE, Trolox equivalents; FDP, freeze dried powder of hydrolysate The degree of hydrolysis was calculated for the full hydrolysates prior to ultrafiltration. Data are the mean ± SEM of 3 independent experiments; values with different superscript letters are significantly different at $P < 0.05$ within each assay.

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Table 2

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0–5%, w/v) generated using different enzyme preparations on proliferation in RAW264.7 mouse macrophages. [a]

Enzyme	Cell proliferation (% control) with 5 kDa ultrafiltration permeates (%, w/v)				
	0.05	0.10	0.50	1.00	5.00
Alcalase®	109.8± 6.7	116.8±6.5	122.2±7.2	120.0±7.6	87.0±10.5
Flavourzyme®	106.8±5.8	106.7±4.3	111.8±5.7	110.6±4.6	74.0±16.8
Prolyve	107.7±5.5	107.7±7.0	109.5±5.6	109.3±6.1	77.8±13.2
Flavorpro Whey	106.7±9.8	106.6±5.7	103.6±1.2	97.9±1.6	40.4±9.6*
Promod 144MG	99.5±3.1	99.2±4.0	92.0±9.5	90.3±9.3	68.9±13.5
Trypsin	68.7±9.5	64.0±15.7	91.2±5.8	89.9±7.7	59.2±8.7*
Pepsin	107.1±3.5	107.1±6.7	112.3±5.0	110.8±8.2	33.6±6.9*

[a] RAW264.7 mouse macrophages were seeded at a density of 0.2×10^5 cells mL^{-1} and treated with increasing concentrations (0–5%, w/v) of samples for 24 h. Cell proliferation was determined using the MTT assay and values are expressed as a percentage relative to untreated cells. Data are the mean ± SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test; $P < 0.05$) in cell proliferation, compared with untreated RAW264.7 cells.

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Table 3

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0–5%, w/v) on proliferation in human Jurkat T cells. [a]

Enzyme	Cell proliferation (% control) with 5 kDa ultrafiltration permeates (%, w/v)				
	0.05	0.10	0.50	1.00	5.00
Alcalase®	89.3±7.5	84.0±10.4	77.9±9.1*	77.2±8.2*	84.3±4.3
Flavourzyme®	91.1±7.5	96.1±1.2	96.1±4.0	117.6±9.1*	242.8±12.4*
Prolyve	96.3±3.8	94.1±4.1	90.9±6.4	94.7±5.1	98.6±5.1
Flavorpro Whey	95.1±2.8	92.7±1.8	90.0±1.6*	101.2±4.8	102.0±7.7
Promod 144MG	98.3±5.9	94.5±5.8	81.6±1.3*	78.8±2.4*	83.8±4.4*
Trypsin	96.9±1.7	90.7±2.6	76.2±9.9*	79.0±3.6*	78.4±5.2*
Pepsin	95.8±7.3	94.6±3.4	78.9±2.8*	77.8±2.4*	13.4±0.5*

[a] Jurkat T cells were seeded at a density of 1×10^5 cells mL^{-1} and treated with increasing concentrations (0–5%, w/v) of samples for 24 h. Cell proliferation was determined using the MTT assay and values are expressed as a percentage relative to untreated cells. Data are the mean ± SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test; $P < 0.05$) in cell proliferation, compared with untreated human Jurkat T cells.

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Table 4

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0–5%, w/v) on proliferation in human U937 lymphocytes. [a]

Enzyme	Cell proliferation (% control) with 5 kDa ultrafiltration permeates (%, w/v)				
	0.05	0.10	0.50	1.00	5.00
Alcalase®	102.7±3.3	96.3±4.3	84.9±4.3	84.4±4.4	76.3±9.8*
Flavourzyme®	107.0±7.0	101.2±2.0	94.6±3.6	103.8±10.4	159.4±5.3*
Prolyve	102.8±3.6	97.5±3.5	87.5±2.3	92.4±4.8	91.4±10.4
Flavorpro Whey	95.3±3.1	97.6±1.3	94.3±1.6	94.1±6.8	56.8±6.8*
Promod 144MG	101.5±6.6	89.1±8.3	87.6±1.2	82.5±5.4	71.4±6.3*
Trypsin	99.4±5.0	94.2±2.7	85.8±2.5	80.6 ±5.2	62.8±9.8*
Pepsin	93.7±4.4	94.6±1.9	83.1±3.9*	81.2±3.2*	12.2±0.9*

[a] U937 lymphocytes were seeded at a density of 1×10^5 cells mL^{-1} and treated with increasing concentrations (0–5%, w/v) of samples for 24 h. Cell proliferation was determined using the MTT assay and values are expressed as a percentage relative to untreated cells. Data are the mean ± SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test; $P < 0.05$) in cell proliferation, compared with untreated human U937 cells.

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Table 5

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0.50 and 0.05%, w/v) on IL-6, IFN-γ, IL-2 and IL-10 production in Jurkat T lymphocytes. [a]

Sample	Cytokine production (% control)							
	IL-6		IFN-γ		IL-2		IL-10	
	0.50%	0.05%	0.50%	0.05%	0.50%	0.05%	0.50%	0.05%
Control	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±.0.0	100.0±0.0	100.0±0.0	100.0±0.0
Alcalase®	76.7±2.8*	87.8±2.9	91.0±3.9	97.5±2.8	103.4±3.8	103.4±3.8	105.9±4.0	103.8±2.9
Flavourzyme®	81.7±2.1*	87.9±2.1	89.1±3.9	94.2±2.4	101.7±4.7	101.7±4.7	111.2±3.3	104.2±5.0
Prolyve	79.5±1.0*	92.8±4.1	94.2±3.2	100.3±1.5	95.0±5.9	95.0±5.9	102.7±5.1	105.2±0.4
Flavorpro Whey	83.6±0.8*	92.0±2.0	103.6±3.6	100.7±5.2	103.3±5.9	103.3±5.9	106.9±3.8	107.2±4.8
Promod 144MG	79.4±1.5*	99.9±3.2	93.5±2.6	103.8±3.6	100.9±3.3	100.9±3.3	111.2±7.6	108.1±6.3
Trypsin	80.9±1.5*	102.7±4.1	88.8±2.7	105.7±3.5	116.7±8.4	116.7±8.4	116.0±6.1	104.3±9.3
Pepsin	87.2±2.1*	102.2±2.6	97.2±1.4	110.0±7.6	98.2±3.2	98.2±3.2	102.1±5.0	99.6±1.2

[a] Jurkat T cells were seeded at a density of 2×10^5 cells mL^{-1}, stimulated with ConA (25 µg mL^{-1}) and treated with 0.50 and 0.05% (w/v) 5 kDa UF permeates of sodium caseinate hydrolysates for 24 h. Cytokine production was measured using the enzyme-linked immunosorbent assay (ELISA) and values were expressed as a percentage relative to cells treated with ConA alone (control): IL-6 concentration, control = 0.004 ng mL^{-1}; IFN-γ concentration, control = 0.099 ng mL^{-1}; IL-2 concentration, control = 0.147 ng mL^{-1}; IL-10 concentration, control = 0.156 ng mL^{-1}. Data are the mean ± SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test; $P < 0.05$) in cytokine production, compared with Jurkat T cells treated with ConA alone.

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Table 6

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0.050 and 0.005%, w/v) on IL-6, IL-1β and TNF-α production in RAW264.7 mouse macrophages. [a]

Sample	Cytokine production (% control)					
	IL-6		IL-1β		TNF-α	
	0.050%	0.005%	0.050%	0.005%	0.050%	0.005%
Control	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
Alcalase®	55.4±7.4*	100.7±21.1	78.8±5.3	118.4±7.8	109.8±5.1	114.5±5.2
Flavourzyme®	55.0±12.4*	82.1±7.1	74.7±8.2*	121.4±9.3	109.6±4.7	110.4±3.9
Prolyve	54.2±3.8*	87.2±13.3	77.6±4.1	107.4±13.9	104.3±4.8	110.6±3.3
Flavorpro Whey	47.2±8.9*	79.5±4.6	76.4±3.7*	113.2±14.5	107.5±5.1	112.4±4.7
Promod 144MG	58.4±12.0*	87.9±8.1	96.1±2.9	115.0±13.0	108.1±5.6	109.4±4.0
Trypsin	60.3±9.8*	99.8±19.7	74.8±2.1*	105.1±16.7	112.4±6.0	114.0±4.9
Pepsin	55.7±4.0*	79.0±9.7	86.2±7.2	111.0±15.6	102.0±10.8	104.7±3.4

[a] RAW264.7 mouse macrophages were seeded at a density of 0.2×10^5 cells mL^{-1}, stimulated with LPS (0.1 µg mL^{-1} or 2µg mL^{-1}) and treated with 0.050 and 0.005% (w/v) 5 kDa UF permeates of sodium caseinate hydrolysates for 24 h. Cytokine production was measured using the enzyme-linked immunosorbent assay (ELISA) and values were expressed as a percentage relative to cells treated with LPS alone (Control): IL-6 concentration, control = 0.219 ng mL^{-1}; IL-1β concentration, control = 0.180 ng mL^{-1}; TNF-α concentration, control = 0.721 ng mL^{-1}. Data are the mean ± SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test; $P < 0.05$) in cytokine production, compared with RAW264.7 mouse macrophages treated with LPS alone.

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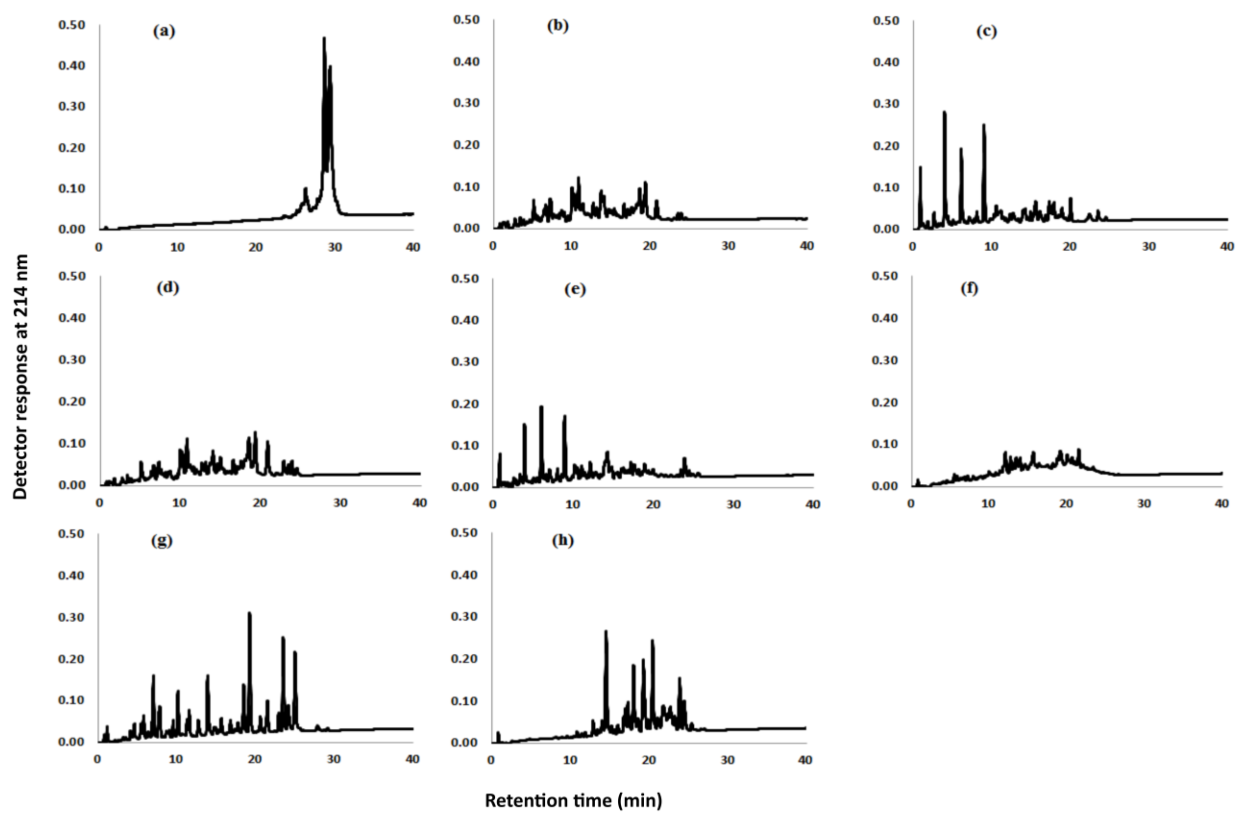

Figure 1:

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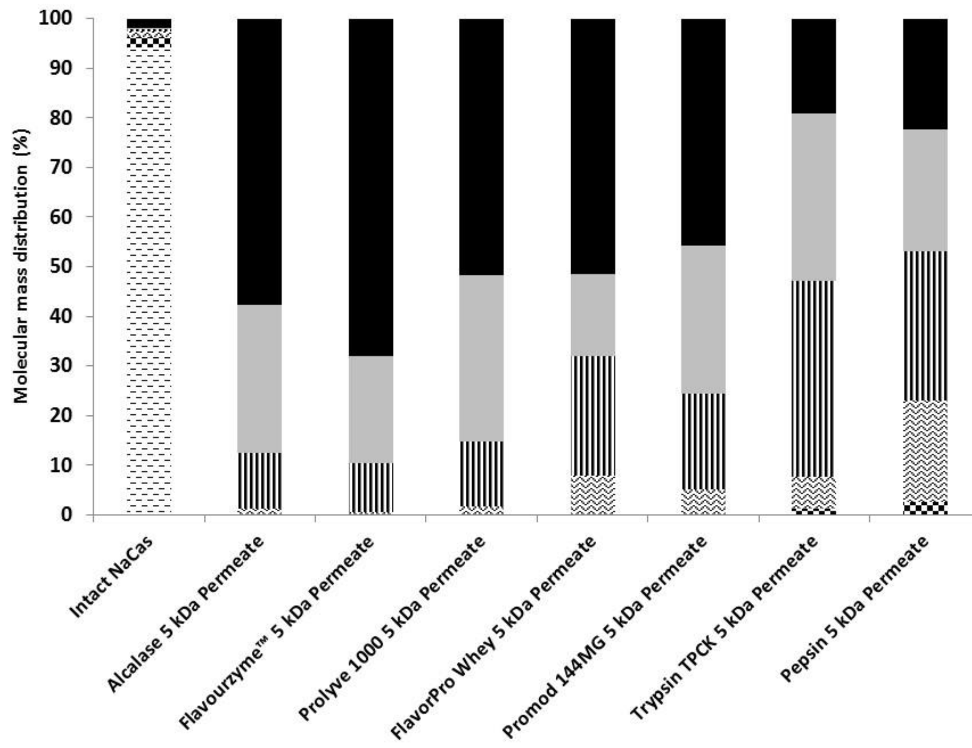

Figure 2:

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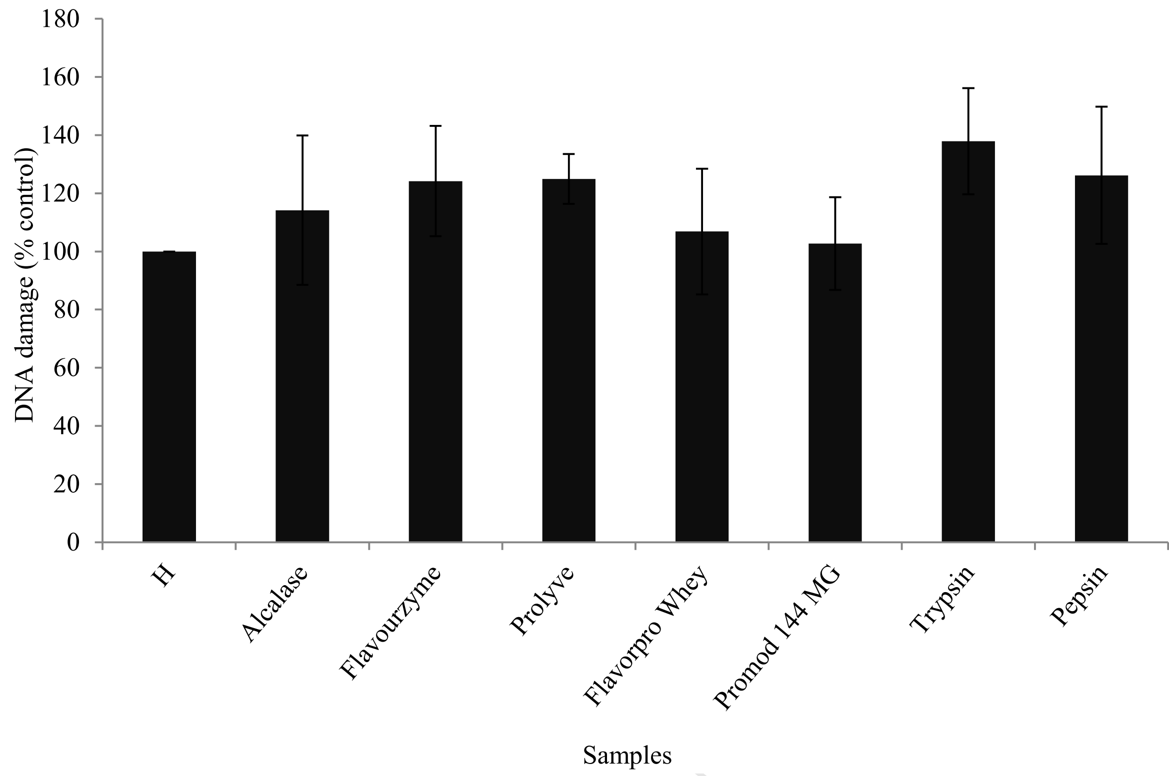

Figure 3:

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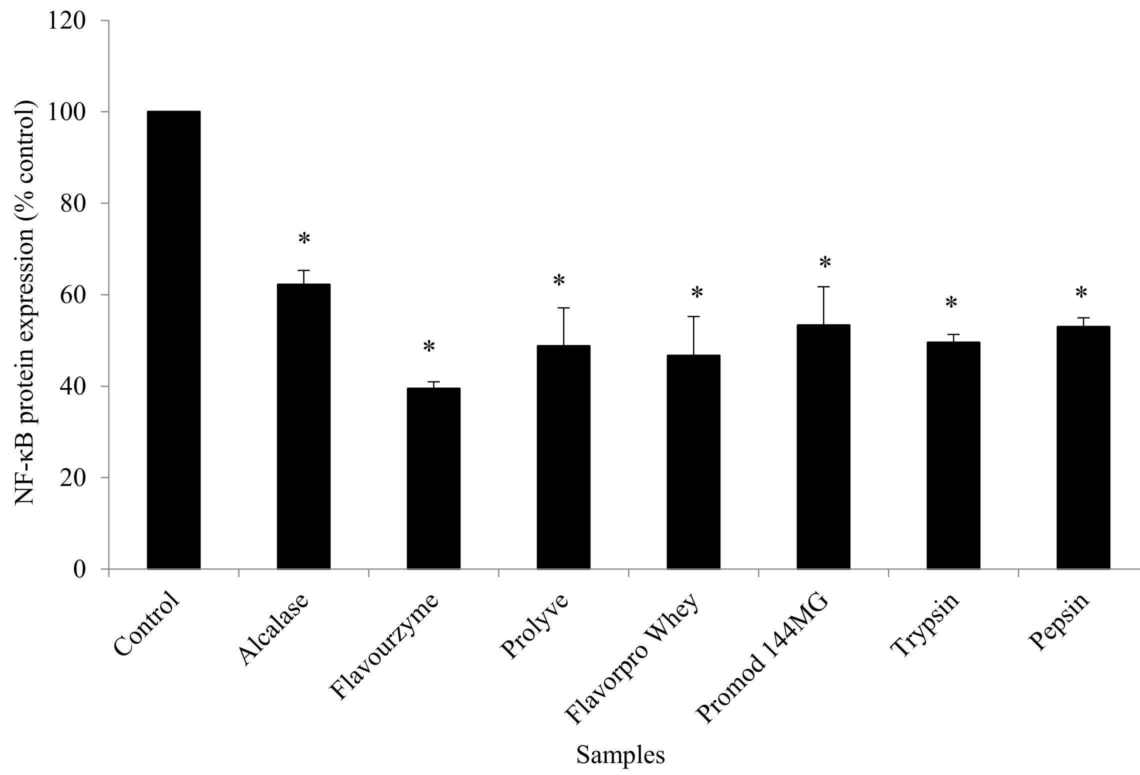

Figure 4:

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