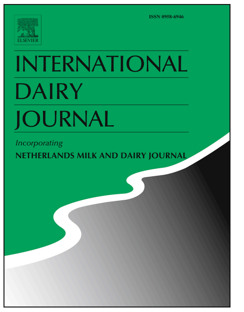

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In vitro antioxidant and immunomodulatory activity of transglutaminase-treated sodium caseinate hydrolysates.

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ABSTRACT

Sodium caseinate (NaCN) was incubated prior to and after hydrolysis with a microbial transglutaminase (TGase) and hydrolysed with Prolyve 1000. The resultant hydrolysates were tested for their immunomodulatory and antioxidant activity. TGase-treated hydrolysates significantly reduced (p < 0.05) the production of IL-6 at 0.5 and 1 mg mL^{-1} and the non-TGase treated hydrolysate reduced the production of IL-6 at 1 mg mL^{-1} in concanavalin (ConA) stimulated Jurkat T cells. None of the samples had an effect on IL-2. The hydrolysates showed higher oxygen radical absorbance capacity assay and ferric reducing antioxidant power activity than unhydrolysed NaCN, but no significant (p > 0.05) differences were found between the TGase-treated and non-TGase-treated samples. In the presence of hydrogen peroxide, the non-TGase-treated sample exhibited the highest DNA protective effect in U937 cells. These findings suggest that NaCN derived hydrolysates with and without treatment with TGase may exert specific antioxidant, genoprotective and anti-inflammatory effects.

1. Introduction

Approximately 30% of occidental population deaths are due to diseases related to cardiovascular problems (WHO, 2011). The continuous exposure to chemicals, unhealthy diets and sedentary life-style may be contributing factors for premature ageing and illness. Oxidative stress is an imbalance between the production of free radicals or reactive oxygen species (ROS) and the neutralisation of these by antioxidant compounds (Lobo, Patil, Phatak, & Chandra, 2010). The excess of ROS produced as a result of oxidative stress is involved in the pathogenesis of neurodegenerative, cardiovascular and inflammatory diseases. For instance, atherosclerotic cardiovascular disease is characterised by the oxidation of low-density lipoproteins (LDL) which induce the adhesion and influx of monocytes and lead to cytokine production, a pro-inflammatory response (Singh, Devaraj, & Jialal, 2005). Some multifactorial diseases such as atherosclerosis or Parkinson's disease are the result of combined inflammatory and oxidative processes (Chen, Lü, Yao, & Chen, 2016). For this reason, there is an increasing interest in studies on the anti-inflammatory and antioxidant potential of bioactive dietary ingredients.

Bioactive peptides (BAPs) are natural protein fragments obtained from food proteins such as dairy, eggs, fish, meat or vegetables. These peptides can be released from proteins by bacterial fermentation, digestion or enzymatic hydrolysis and they may possess potent bioactivities (Korhonen & Pihlanto, 2006; Nongonierma, O'Keeffe, & FitzGerald, 2016). Antioxidant BAPs may inhibit the action of free radicals, reducing oxidation events and thereby contribute to the prevention of inflammatory responses. Antioxidant and anti-inflammatory bioactivities are directly related (Pashkow, 2011). Caseins from bovine milk contain a large number of bioactive peptides encrypted into the parent protein (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014; Nongonierma &

FitzGerald, 2015; Nongonierma et al., 2016; Phelan, Aherne, FitzGerald, & O'Brien, 2009a; Power, Jakeman, & FitzGerald, 2012; Wada & Lönnerdal, 2014). The composition, structure, hydrophobicity, position of amino acid residue and molecular mass are factors directly related with the activity of BAPs (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). The amino acid composition of casein, which is rich in Pro residues, makes it a potential source of bioactive peptides for the production of biofunctional foods (Pihlanto, 2006).

Enzymatic hydrolysis of casein proteins has resulted in the generation of BAPs with demonstrated immunomodulatory and antioxidant activities. Two casein hydrolysates, deriving from digestion with *Lb. helveticus* MIMLh5 and *Lb. acidophilus* ATCC 4356 proteinases, demonstrated anti-inflammatory activity by decreasing NF-κB activity in recombinant Caco-2 cells (Stuknyte, De Noni, Guglielmetti, Minuzzo, & Mora, 2011). A recent study demonstrated that a <5 kDa NaCN hydrolysate was able to reduce IL-8, a pro-inflammatory cytokine, in tumour necrosis factor-alpha (TNF-α) treated Caco-2 cells, and similar results were observed ex vivo in porcine colonic tissue (Mukhopadhya et al., 2015). Similarly, a peptide obtained from β-CN (f 94-98), QEPVL, and its derivative, QEPV, showed the capacity to regulate the inflammatory process not only in vitro but also in vivo in Balb/c mice (Jiehui et al., 2014). Studies using Balb/c mice reported that yak casein hydrolysates possessed radical scavenging activities against 2,2-Diphenyl-1-picrylhydrazyl (DPPH), superoxide and hydrogen peroxide, and also decreased the production of nitric oxide (NO) and the pro-inflammatory IL-6 and IL-1β cytokines (Mao, Cheng, Wang, & Wu, 2011). The antioxidant properties of casein hydrolysates have been widely reviewed (Pihlanto, 2006; Power et al., 2012).

Furthermore, the combination of cross-linking and enzymatic hydrolysis in casein may lead to the generation of novel peptides with new bioactivities due to the intra and inter cross-links created within the casein peptide structure. Cross-linking with TGase is known to improve the physicochemical and organoleptic properties of dairy products. The addition of TGase improved the emulsifying and foaming properties of NaCN (Flanagan & FitzGerald, 2003). The application of TGase in yoghurt and cheese is well established leading to improved product quality (Özer, Hayaloglu, Yaman, Gürsoy, & Şener, 2013; Romeih, Abdel-Hamid, & Awad, 2014). However, little is known about the effect of TGase on the bioactivity of peptides. A recent study by Hong, Gottardi, Ndagijimana, and Betti (2014) found that glycopeptides from fish, obtained by glycosylation and proteolytic hydrolysis with Alcalase, improved their cellular antioxidant activity in HepG2 cells and their lipid oxidation inhibition activity with the addition of TGase. Additionally, gluten hydrolysates glycosylated with TGase, have been reported to improve their in vitro antioxidant activity (Gottardi, Hong, Ndagijimana, & Betti, 2014). Preliminary work in our laboratory has shown that samples treated with TGase prior to hydrolysis had an anti-inflammatory activity in LPS induced Jurkat T cells; however, no antioxidant activity was detected (O'Sullivan, Lahart, O'Callaghan, O'Brien, & FitzGerald, 2013).

The aim of the present study was to assess the effect of enzymatic hydrolysis and its combination with TGase cross-linking treatment on the immunomodulatory and antioxidant activity of NaCN hydrolysates.

2. Materials and methods

2.1. Materials

Sodium caseinate (NaCN; 87.57%, (w/w, protein) was provided by Arrabawn Co-op Society Ltd., Tipperary, Ireland. Calcium independent TGase from *Streptoverticillium* spp.

was provided by Forum Products Ltd. (Brighton Rd., Redhill, Surrey, UK). Prolyve 1000™ was kindly provided by Lyven Enzymes Industrielles (Caen, France). U937 and Jurkat T cells were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, Wilts, UK). MTT I proliferation kit was obtained from Roche Diagnostics (Burgess Hill, West Sussex, UK). IL-6 and IL-2 eBioscience enzyme-linked immunoadsorbent assay (ELISA) Ready-SET-Go kits were purchased from Insight Biotechnology (Wembley, UK). All other chemicals and reagents were purchased from Sigma Chemical Company Ltd. (Wicklow, Ireland), unless otherwise stated.

2.2. Generation of cross-linked NaCN hydrolysates

TGase-treated hydrolysates were generated prior to (TGase/Prolyve) and after (Prolyve/TGase) Prolyve hydrolysis. For the generation of the Prolyve/TGase hydrolysate sample a NaCN solution (10%, w/v) was incubated with 0.3% (v/v) Prolyve 1000™ at 50 °C and pH 7 using a pH stat (Titrando 843, Metrohm, Dublin, Ireland). After 240 min of incubation, the enzymatic reaction was stopped by heating at 80 °C for 20 min. An aliquot of this solution was used as a non-TGase-treated hydrolysate (Prolyve). Then the resultant solution was incubated with TGase (2%, v/v) at room temperature and pH 7.0 for 180 min. Inactivation of TGase was carried out by heating at 80 °C for 20 min. For the generation of the TGase/Prolyve hydrolysate sample, NaCN was incubated firstly with TGase (2%, v/v) and subsequently submitted to hydrolysis with Prolyve 1000™ using the same conditions as outlined above. All the hydrolysates generated where further subjected to in vitro digestion with pepsin (enzyme:substrate ratio 1:40, w/w) for 90 min at 37 °C at pH 2.0 and subsequently with Corolase PP® (enzyme:substrate ratio 1:10, w/w) for 180 min at 37 °C at pH 7 to simulate in vitro gastrointestinal digestion (SGID; Walsh et al., 2004).

2.3. Cell culture

A leukaemic monocytic lymphoma cell line, U937 cells, and a human leukaemic T cell line, Jurkat T cells, were maintained at 37 °C in a 5% CO_2 atmosphere, in antibiotic-free medium (RPMI-1640) supplemented with 10% foetal bovine serum (FBS). Reduced serum media (2.5% FBS) was used during experiments.

2.4. Cell viability assay

Cells at a density of 1×10^5 cells mL^{-1} in growth media were seeded in each well of 96-well flat-bottom plates. Cells were incubated with hydrolysates (0–50 mg mL^{-1}) at 37 °C for 24 h. Following incubation, cell viability was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, media was removed (100 µL) and MTT reagent 1 (5 µL) was added and cells were incubated for 4 h at 37 °C. Then, MTT reagent 2 (100 µL) was added to the cells and the plate was incubated overnight at 37 °C. The absorbance was measured on a Varioskan Flash microplate reader (ThermoScientific, Tewksbury, MA, USA) at 570 nm. The half maximal effective concentration (EC_{50}) values were calculated in triplicate ($n = 3$) and expressed as mg mL^{-1} using GraphPad Prism 4.

2.5. Immunomodulatory activity – cytokine production

Jurkat T cells, at a density of 2×10^5 cells mL^{-1}, were seeded in 96-well plates in the presence of concanavalin A (ConA, 50 µg L^{-1}) and were incubated with test samples at 0.5

and 1 mg mL^{-1} for 24 h at 37 °C. Production of the cytokines IL-6 and IL-2 was determined using ELISA kits. Absorbance was read at 450 nm using a microplate reader. Experiments were performed in triplicate (n = 3) and data were expressed as a percentage of the stimulated cell control.

2.6. Antioxidant activity

2.6.1. Intracellular reduced glutathione (GSH)

U937 cells (1×10^5 cells mL^{-1}, 5mL) were incubated with NaCN and its hydrolysates (0.5%, v/v) in a 96 well plate for 24 h at 37 °C. Following incubation, cells were harvested, sonicated on ice at 13 mA for 30 s, centrifuged (14,000 × *g*, 30 min, 4 °C) and the supernatant was collected. An aliquot (100 µL) of sample was mixed with 0.01 M sodium phosphate-0.005 M ethylenediamine tetraacetic acid buffer (1.8 mL) and *o*-phthaldialdehyde (0.1 mg). The fluorescence was determined at 350 nm (absorption) and 420 nm (emission). The GSH content was determined from a standard curve using known concentrations of GSH and the results were expressed relative to the protein content. The protein content of the samples was determined by the bicinchoninic acid (BCA) protein assay as previously described by Smith et al. (1985). The assay was performed in triplicate (n = 3).

2.6.2. Comet assay

U937 cells (1×10^5 cells mL^{-1}) were treated with 5 mg mL^{-1} (0.5%, v/v) of test sample for 24 h in a 6-well plate (final volume 2 mL) at 37 °C. After incubation, cells were treated with 50 µM H_2O_2 or 400 µM tert-butyl hydroperoxide (t-BOOH) for 30 min. The comet assay, previously described by McCarthy et al. (2012), was then used to measure oxidative DNA damage. Cells were harvested and transferred to microscope slides (prepared with normal gelling agarose; NGA) and covered with low melting point agarose (LMP). The slides were placed in lysis solution for 1 h at 4 °C, followed by electrophoresis at 300 mA, 20 V for 25 min. The slides were then neutralised using 0.4 M Tris-base at pH 7.5 and stained with ethidium bromide (20 µg mL^{-1}). Cells were visualised under a fluorescence microscope and Komet 5.5 image analysis software was used to score 50 cells per slide. The DNA damage was performed in quadruplicate (n = 4) and expressed as percentage of tail DNA.

2.6.3. Oxygen radical absorbance capacity assay

The oxygen radical absorbance capacity (ORAC) assay was performed as described by Zulueta, Esteve, and Frígola (2009) using a Synergy™ HT plate reader (BioTek Instruments Limited, Bedfordshire, UK). An aliquot (50 µL) of test sample (0.1 mg mL^{-1}), standard or phosphate buffer (75 mM) and 50 µL of fluorescein (0.78 µM) were added into a microtitre plate incubated at 37 °C. The reaction was started with the addition of 25 µL of 2.2'-azobis-2-methyl-propanimidamide (AAPH) to each well. Fluorescence readings were recorded every 5 min for 120 min at excitation and emission wavelengths of 485 and 520 nm, respectively. The ORAC values, expressed as µmoles trolox equivalents (TE) per mg freeze dried sample, were calculated using trolox as a standard. Experiments were performed in triplicate (n = 3).

2.6.4. Ferric reducing antioxidant power activity

The ferric reducing antioxidant power (FRAP) value of hydrolysate samples was determined using the method described by Benzie and Strain (1999) with some modifications. Briefly, 2 mL of freshly prepared FRAP reagent [150 µL; 0.3 M acetate buffer (pH 3.6), 0.01 M 2,4,6-tripyridyl-s-triazine (TPTZ), 0.02 M $FeCl_3 \cdot 6H_2O$ 10:1:1] heated to 37 °C was added into a cuvette and the absorbance was read at 590 nm. Test sample

(100 µL), $FeSO_4$ (standard) and MeOH (blank) was then added and the absorbance (590 nm) was read after 30 min incubation at 37 °C. The experiment was performed in triplicate (n = 3) and the FRAP values (µM) were calculated from the standard curve.

2.6.5. 2.2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonate) radical scavenging assay

The 2.2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS+) radical scavenging activities were measured using the previously described method by Re et al. (1999). The ABTS•+ solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulphate for 16 h to generate the radicals. The radical solution was then diluted to an absorbance of 0.70 ± 0.02 at 734 nm. Test samples (10 µL) at a final concentration 1 mg mL^{-1} were added in a 96 well plate with the radical solution (200 µL) and kept in the dark at room temperature for 5 min. Absorbance was then measured at 734 nm. Known concentrations of trolox solutions were used to plot a standard curve and ABTS+ values were calculated. All samples were analysed in triplicate (n=3) and the final inhibitory activity was expressed as %ABTS inhibition using the following equation:

$$\text{ABTS inhibitory activity (\%)} = [(Abs_{blank} - Abs_{sample}) / Abs_{blank}] \times 100$$

2.6.6. DPPH radical scavenging assay

The DPPH assay was carried out according to the method described by Brand-Williams, Cuvelier, and Berset (1995). Concentrations of trolox ranging from 0.04 to 0.40 µM were used to prepare a standard curve for calibration. Hydrolysate test samples (100 µL) at a final concentration 1 mg mL^{-1} were diluted with methanol and incubated with 3.9 mL of 6 µM DPPH reagent for 30 min. Absorbance was read at 515 nm at 0 and 30 min. All samples were analysed in triplicate (n=3) and the results were expressed as % DPPH inhibition using the following equation:

$$\text{DPPH inhibition (\%)} = [(\text{Abs}_{blank} - \text{Abs}_{sample}) / \text{Abs}_{blank}] \times 100$$

2.7. Statistical analysis

All data were determined as the mean and standard error values of at least three independent experiments. Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's test (or Tukey's multiple comparison test, where appropriate), using Graph-Pad Prism 4 (Graph-Pad software, California, U.S.A.).

3. Results and discussion

3.1. Effect of casein hydrolysates on cell viability

Cell viability of Jurkat T (Table 1) and U937 (Table 2) cells was measured by the MTT assay to determine non-toxic concentrations of hydrolysates to be used for subsequent experiments. The MTT assay measures cellular mitochondrial activity by assessing the activity of mitochondrial reductase. Cells were incubated with increasing concentrations of NaCN and its hydrolysates (0–5%, v/v, equivalent to 0–50 mg mL^{-1}). The EC_{50} values were calculated and represent the concentration required that inhibits cell viability by 50%. Based on the EC_{50} values obtained, the hydrolysates seemed to have similar cytotoxic effects on Jurkat T and U937 cells (Table 3). McCarthy et al. (2013) reported the cytotoxic effect of brewers' spent grain (BSG) hydrolysates on U937 cells was higher than on Jurkat T cells. In the present study, samples at 0.5% (v/v) showed significant ($p < 0.05$) inhibition of the viability of Jurkat T cells compared with control (non-treated cells) supporting the previous results from Lahart et al. (2011). Lahart et al. (2011) reported that a NaCN hydrolysate at

0.25% (v/v), obtained with Alcalase (A4), decreased the viability of Jurkat T cells to 61.2% compared with untreated cells (100%). All the hydrolysates studied herein induced a significant ($p < 0.05$) cytotoxic activity at concentrations of 0.5% (v/v) in Jurkat T cells. In a study by Phelan, Aherne-Bruce, O'Sullivan, FitzGerald, and O'Brien (2009b), NaCN hydrolysates prepared using different food-grade enzyme preparations were cytotoxic to Jurkat T cells at a concentration of 0.5% (v/v). The addition of 100 µg mL^{-1} of a glycomacropeptide from bovine milk was reported to significantly inhibit the viability of U937 cells (Li & Mine, 2004). In contrast, in our study concentrations of the hydrolysates up to 5 mg mL^{-1} (corresponding to 0.5%, v/v), showed no inhibition in the viability of U937 cells. The conformation, degree of hydrolysis and the source of the proteolytic enzyme used to generate the hydrolysates are key factors that may affect the cytotoxicity of hydrolysates (Lahart et al., 2011; Zou, He, Li, Tang, & Xia, 2016). TGase treated hydrolysates showed similar results in Jurkat T cells to those reported by O'Sullivan et al. (2013). Sample concentrations that showed a cell viability < 75% of control were considered toxic. Therefore, non-toxic test sample concentrations of 0.5 and 1 mg mL^{-1} were used for subsequent immunomodulatory and antioxidant activities in both cell lines.

3.2. Immunomodulatory effects of NaCN hydrolysates

The anti-inflammatory activity of intact NaCN, the NaCN hydrolysate (Prolyve) and the cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase) was screening by measuring their potential to supress the production of IL-2 and IL-6 in ConA stimulated Jurkat T cells. ConA is a lectin mitogen known for its ability to stimulate the T-cell receptor and the subsequent activation of signalling pathways involving nuclear factor of activated T-cells (NFAT) and mitogen-activated protein kinase (MAPK) pathways resulting in the production of cytokines (Takahashi et al., 2009; Tanaka, Akaishi, Hosaka, Okamura, & Kubohara, 2005). The results showed that all hydrolysates tested significantly reduced ($p < 0.05$) IL-6 production (Table 4). This effect was dose dependent. IL-6 production was significantly decreased ($p < 0.05$) by NaCN up to 41.85 and 30.21% of the control ConA-stimulated cells at concentrations of 0.5 and 1 mg mL^{-1}, respectively. In contrast, a study using yak casein showed that intact casein did not produce a decrease in IL-6, whereas its hydrolysates decrease cytokine production in LPS-stimulated macrophages (Mao et al., 2011). The production of cytokines in LPS-induced RAW cells incubated with yak casein hydrolysates has been previously reported by Mao et al. (2011). The study reported that at a concentration of 0.5 mg mL^{-1} the hydrolysates significantly inhibited the production of the pro-inflammatory cytokines IL-6, IL-1β and TNF-α. A recent study showed similar results in ConA induced Jurkat T cells incubated with NaCN hydrolysates whereby IL-6 cytokine production was significantly decreased compared with the control whereas IL-2 production was unchanged (O'Sullivan, O'Callaghan, O'Keeffe, FitzGerald, & O'Brien, 2015). In an earlier study, Phelan et al. (2009b) studied the effect of eight distinct casein hydrolysates generated with several food-grade enzyme preparations on IL-2 production in Con-A induced Jurkat T. The study demonstrated that six of the hydrolysates enhanced the secretion of IL-2. The authors suggest that this pro-inflammatory effect might be useful on regulation of deficient immune processes. Yak casein hydrolysates were also reported to increase IL-2 production in ConA stimulated spleen cells (Mao, Yang, Song, Li, & Ren, 2007). Nevertheless, in the present study, NaCN and its hydrolysates did not have any effect on ConA stimulated IL-2 production in Jurkat T cells. NaCN at 1 mg mL^{-1} significantly reduced the production of IL-2 (79%) compared with the control (Table 4). Similarly, Lahart et al. (2011) found no difference in the secretion of IL-2 in Jurkat T cells incubated with 0.5% (v/v) of intact NaCN or 0.5% (v/v) of NaCN hydrolysates generated with Alcalase and

Flavourzyme. However, O'Sullivan et al. (2013) reported a decrease in IL-2 production in ConA-stimulated Jurkat T cells incubated with NaCN cross-linked with TGase pre-hydrolysis. The extent of hydrolysis reached and the enzymatic preparation used to generate the hydrolysates are mainly responsible for the final sequences of peptides within the hydrolysates and could induce different cell reactions. It is interesting to note a study where the substitution of proline in short peptides had a negative effect on their immunomodulatory activity, but the substitution of proline with proline analogues did not have an impact on the final bioactivity. The study reported on an immunomodulatory peptide from β-casein (191–209) (LLYQEPVLGPVRGPFPIIV) which was synthesised with modifications around Pro residues. In particular, substitution of the last proline (P206) with D-Pro produced an inhibition in the in vitro immunosuppressory effects in α-CD3 and α-CD28 stimulated murine spleen cells (Bonomi et al., 2011). Hence, the structure and sequence of peptides is a crucial factor which directly affects their anti-inflammatory activity.

3.3. Cellular antioxidant assays

Reduced GSH, an important antioxidant, is produced within the cells to prevent cell damage induced by ROS. Incubation with the NaCN hydrolysates led to a small increase in GSH concentration in U937 cells, whereas the parent protein, unhydrolysed NaCN, produced a reduction of the GSH content (Table 5). However, none of the results was statistically significant compared with the untreated cells. These results are in agreement with those reported by O'Sullivan et al. (2013) where no difference in GSH content was found in TGase-treated NaCN hydrolysates in Jurkat T cells. In contrast, GSH content and catalase activity were increased by NaCN hydrolysates in Jurkat T cells (Lahart et al., 2011; Phelan et al., 2009b). Some studies suggest that the peptide profile affects its antioxidant activity. For instance, peptides from whey protein hydrolysates had a more effective protecting ability against oxidative stress in PC12 cells as their hydrophobicity increased (Zhang et al., 2015). The hydrophobicity of the peptide residues enhances the accessibility of the peptide to the fatty acids in cell membranes, which are subjected to oxidation by free radicals and ROS (Aluko, 2012). The proteolytic enzyme used to obtain the hydrolysates is another key factor in hydrolysate bioactivity. For instance, Alcalase hydrolysates from casein efficiently increased the intracellular antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) in H_2O_2 treated HepG2 cells (Xie, Wang, Ao, & Li, 2013). Casein phosphopeptides (CPP), obtained by SGID, produced an increase in GSH and CAT activity in H_2O_2 stimulated Caco-2 cells (García-Nebot, Cilla, Alegría, & Barberá, 2011). Prolyve and Alcalase, are food-grade proteolytic enzyme preparation obtained from *Bacillus licheniformis* and both have subtilisin activity. However, only Alcalase possesses glutamyl endopeptidase activity and is consequently able to yield higher extents of hydrolysis than Prolyve (Spellman, Kenny, O'Cuinn, & Fitzgerald, 2005). Thus, the generation of peptides with different proteinases produces distinctive peptide profiles and therefore this may explain the differing results.

3.4. Genoprotective effect of casein hydrolysates

The ability of the samples to protect against oxidant-induced DNA damage was determined by the comet assay also called single cell gel electrophoresis. This method measures deoxyribonucleic acid (DNA) strand breaks in the cells. The oxidants used, *t*-BOOH (Fig.1a) and H_2O_2 (Fig. 1b), significantly increased the percentage of DNA damage (% DNA tail). None of the samples decreased the DNA damage induced by t-BOOH (Fig.1a). The Prolyve hydrolysate protected against the genotoxic effects of H_2O_2 ($p < 0.05$).

The rest of the hydrolysates and NaCN were not significantly different from the mean value obtained for H_2O_2 treatment (Fig.1b).

This result may be due to the different mechanisms of action of the two oxidants. H_2O_2 induced cell oxidation is produced by the release of hydroxyl radicals (OH·) and it is an iron dependent reaction, whereas t-BOOH produces lipid peroxidation and it is not iron dependent. Previous studies have shown that casein hydrolysates had no effect on DNA damage induced by H_2O_2 in Caco-2 cells (Phelan et al., 2009b). However, several investigations have shown the genoprotective results of food-derived hydrolysates. The enzymatic extracts from a brown seaweed *Ecklonia cava* showed potent DNA protection in rat lymphocytes using the comet assay (Heo, Park, Park, Kim, & Jeon, 2005). Another study using fish gelatine hydrolysates demonstrated that DNA damage was decreased in a dose-response manner in H_2O_2 challenged U937 cells (Karnjanapratum, O'Callaghan, Benjakul, & O'Brien, 2016). A fractionated protein hydrolysate from brewers' spent grain (BSG) with a molecular mass <5 kDa was reported to decrease the DNA damage in U937 cells treated with H_2O_2 (McCarthy et al., 2013). The authors stated that the genoprotective effect of unfractionated BSG samples was lower than their correspondent fractionated samples. This suggests that further fractionation of the present hydrolysate samples may be of interest to assess specific peptide effects on cellular DNA damage.

3.5. In vitro antioxidant assays

The chemical antioxidant activity of intact NaCN, the TGase treated NaCN and non-TGase-treated NaCN hydrolysates was determined using four different assays. ORAC, DPPH and ABTS are radical scavenging assays whereas FRAP is based on the ability of the test compound to reduce ferric ions. The results obtained are shown in Fig. 2. The three hydrolysate preparations (Prolyve, TGase/Prolyve and Prolyve/TGase) had significantly higher ($p < 0.05$) ORAC activity than unhydrolysed NaCN. The highest mean ORAC value (887.1 ± 52.6 µmol TE g^{-1}) was found in Prolyve/TGase although no significant differences were found between the three hydrolysates. A similar trend was observed using the FRAP assay. The NaCN hydrolysates showed significantly higher FRAP values (23.02, 24.56 and 22.95 µM for Prolyve, TGase/Prolyve and Prolyve/TGase, respectively) than untreated casein (6.29 µM). But again, no differences were found whether the samples were TGase treated or not. FRAP is an antioxidant assay that measures the ability of the hydrolysates to reduce Fe^{3+} to Fe^{2+}. The ion is captured and the chain reaction of the oxidation process does not occur. FRAP values are relatively high in whey proteins. Bagheri, Madadlou, Yarmand, and Mousavi (2014) reported, using the ferric reducing power assay, that cross-linked whey hydrolysates had higher antioxidant activity than those non-cross-linked or intact whey protein. According to Bagheri et al. (2014) cross-linking was responsible for creating peptide structures with the ability of neutralise the ion radicals. However, the results herein demonstrate that the TGase treatment did not affect the FRAP values obtained for TGase-treated NaCN hydrolysates. The FRAP and H_2O_2-induced DNA damage (Comet assay) assays are both related to an iron-dependent mechanism. Although it was previously shown that the Prolyve hydrolysate had a significant effect on the protection of H_2O_2-induced DNA damage, the FRAP results showed no difference between non-TGase-treated (Prolyve) and TGase-treated hydrolysates (TGase/Prolyve and Prolyve/TGase). This may be caused by the participation of different components or behaviors of the cells such as enzymatic complexes or cell uptake that could influence the antioxidant response and show diverse results than the in vitro chemical assays (López-Alarcón & Denicola, 2013). DPPH and ABTS inhibition showed no significant differences ($p > 0.05$) between the hydrolysates and intact casein. DPPH is a proton-radical scavenging assay. Some studies have previously shown the potential of casein hydrolysates to scavenge DPPH radical ions (Suetsuna, Ukeda, & Ochi, 2000). However, the hydrolysates generated in the present study were not found to possess DPPH scavenging activity in comparison with Trolox and ascorbic acid. Similar results for NaCN hydrolysates were reported by Lahart et al. (2011). The DPPH assay uses methanol or ethanol as solvent. Previous studies determined that the hydrolysate samples generated herein had a hydrophilic profile (data not shown), which may be the reason for the negative results. A method based on an aqueous system, $ABTS^+$ assay, was then performed. However, the results showed that the activity of the hydrolysates against $ABTS^+$ radical ranged between 6.4 and 8.4 % inhibition and none of the hydrolysates showed significant differences with NaCN. A study on the antioxidant activity of different amino acids assessed by the ABTS+ assay, reported that Cys was the most active amino acid followed by Trp, Tyr and His (Aliaga & Lissi, 2000). These specific amino acids are not present in large amounts in caseins. Therefore, based on the results and the amino acid composition of casein it would appear that the DPPH and $ABTS^+$ assays may not be adequate methods to quantify the antioxidant potential of casein hydrolysates. It is been reported that the combination of glycosylation/glycation and TGase may increase the antioxidant properties of fish gelatin hydrolysates (Hong et al., 2014). The TGase/treated glycopeptides were shown to inhibit lipid oxidation of linoleic acid and to increase the cellular antioxidant activity in HepG2 cells using the DCFH-DA method. However, the study was performed using guinea pig TGase and Alcalase. The authors also explained that the glycosylation/glycation process could be an enhancer of the antioxidant properties of the hydrolysates.

The results shown herein demonstrate that casein hydrolysates may be a good source of antioxidant hydrolysates. However, treatment with TGase prior to or post hydrolysis with Prolyve does not seem to exert any significantly difference on the antioxidant activity of the hydrolysates. This was consistent across the in vitro antioxidant assays employed herein.

4. Conclusion

The findings from the present study show that casein derived peptides may exert specific antioxidant and anti-inflammatory effects. The hydrolysates possessed a higher ORAC and FRAP antioxidant activity in comparison with the unhydrolysed NaCN but the results suggest that the addition of TGase prior to or followed hydrolysis does not change the antioxidant activity of the hydrolysates. The non-TGase-treated hydrolysate sample Prolyve, demonstrated genoprotective activity against H_2O_2 induced DNA damage. On the other hand, the hydrolysates generated with the addition of TGase prior to and after hydrolysis showed a significant decrease in the release of IL-6 cytokine at low concentrations, corresponding to an anti-inflammatory activity. The fractionation and isolation of peptides from these bioactive hydrolysates is the next step to obtain potent immunomodulatory or antioxidant peptides and to incorporate them into functional foods.

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Figure legends

Fig. 1. DNA damage (%) in the U937 cell line after incubation with (a) t-BOOH (400 μM) and (b) hydrogen peroxide (50 μM). In each graph, different letters denote significant differences between samples at $p < 0.05$ (n=4). Control - : non-treated cells.

Fig. 2. In vitro antioxidant activities of sodium caseinate (NaCN) hydrolysates against: (a) oxygen radical absorbance capacity assay (ORAC, in μmol TROLOX eq g^{-1}); (b) ferric reducing antioxidant power (FRAP) activity; (c) 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition; (d) 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS+) inhibition. Different letters denote significant differences between samples within each graph at $p < 0.05$ (n=3).

Table 1

Effect of sodium casein (NaCN), NaCN non-cross-linked hydrolysate (Prolyve) and NaCN transglutaminase (TGase) cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase) on cell viability in the Jurkat T cell line.[a]

Concentration (%, v/v)	NaCN	Prolyve	TGase/Prolyve	Prolyve/TGase
Control	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
0.025	94.36±6.42	91.40±2.06	95.24±5.56	86.14±3.53
0.05	88.18±5.65	89.61±1.27	87.29±3.49	85.22±2.70*
0.1	80.60±8.65*	90.34±1.17	83.78±2.28	75.69±3.51*
0.5	68.47±4.55*	77.59±5.40*	68.22±4.02*	76.10±4.16*
1.0	72.35±5.56*	82.80±6.65	80.80±7.82	81.07±4.34
2.0	59.79±0.55*	59.38±7.50*	47.46±8.71*	54.16±8.73*
5.0	18.85±5.53*	25.70±16.18*	8.56±0.32*	10.55±1.67*

[a] Cells were exposed to increasing concentrations (0.25–50 mg mL^{-1}) of samples for 24 h and cell viability was determined by the MTT assay. Data represent the mean ± SE of at least three independent experiments expressed as a percentage relative to untreated cells. An asterisk indicates statistically significant difference ($p < 0.05$) in cell viability between control (untreated) and treated cells.

Table 2

Effect of sodium casein (NaCN), NaCN non-cross-linked hydrolysate (Prolyve) and NaCN transglutaminase (TGase) cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase) on cell viability in the U937 cell line. [a]

Concentration (%, v/v)	NaCN	Prolyve	TGase/Prolyve	Prolyve/TGase
Control	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
0.025	99.46±2.02	108.88±1.74*	102.04±2.04	95.19±2.81
0.05	102.21±4.00	112.35±3.47	107.29±7.43	95.47±9.26
0.1	93.52±3.64	116.15±5.50	103.78±3.03	105.37±6.25
0.5	100.70±4.46	117.87±2.35	106.96±8.12	106.22±4.26
1.0	91.99±1.53	81.93±4.16	98.39±8.39	79.97±5.44*
2.0	70.60±3.37*	15.34±3.90*	51.98±12.01*	25.02±7.78*
5.0	28.65±2.45*	12.69±1.46*	14.09±1.43*	13.94±1.48*

[a] Cells were exposed to increasing concentrations (0.25–50 mg mL^{-1}) of samples for 24 h and cell viability was determined by the MTT assay. Data represent the mean ± SE of at least three independent experiments expressed as a percentage relative to untreated cells. An asterisk indicates statistically significant difference ($p < 0.05$) in cell viability between control (untreated) and treated cells.

Table 3

EC50 values of sodium casein (NaCN), NaCN non-cross-linked hydrolysate (Prolyve) and NaCN transglutaminase (TGase) cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase). [a]

Sample	EC_{50} (mg mL^{-1})	
	Jurkat T	U937
NaCN	18.65^{ac}	31.55^{a}
Prolyve	30.55^{b}	13.96^{b}
TGase/Prolyve	16.02^{ac}	21.58^{c}
Prolyve/TGase	22.92^{abc}	14.94^{bc}

[a] Values are mean of at least three independent experiments. Different superscript letters denote significant difference ($p < 0.05$) for each cell line. EC_{50} values represent the concentration of sample that inhibits 50% of cell proliferation.

Table 4

Effect of sodium casein (NaCN), NaCN non-cross-linked hydrolysate (Prolyve) and NaCN transglutaminase (TGase) cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase) on IL-2 and IL-6 cytokine production in Concanavalin (ConA) stimulated Jurkat T cells. [a]

Sample	Cytokine production (% of control)							
	IL-2				IL-6			
	0.5 mg mL^{-1}		1 mg L^{-1}		0.5 mg mL^{-1}		1 mg mL^{-1}	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	100.00 [a]	0.00	100.00 [a]	0.00	100.00 [a]	0.00	100.00 [a]	0.00
NaCN	107.41 [a]	9.41	79.00 [b]	3.35	41.85 [b]	2.82	30.21 [b]	6.90
Prolyve	98.49 [a]	2.72	93.18 [a]	4.71	88.78 [ac]	2.05	80.79 [c]	2.03
TGase/Prolyve	106.17 [a]	3.14	98.38 [a]	5.21	85.45 [c]	2.85	78.12 [c]	1.94
Prolyve/TGase	101.91 [a]	2.98	96.94 [a]	3.44	83.91 [c]	3.71	79.80 [c]	4.81

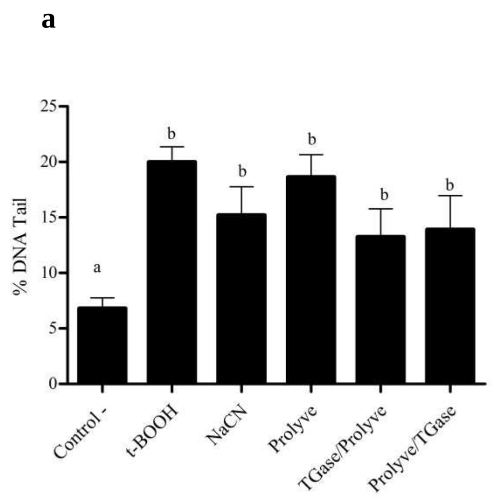
[a] Values are mean ± SE of at least 3 independent experiments, expressed as a percentage relative to the control (non-treated ConA stimulated cells). Different superscript letters denote significant differences ($p < 0.05$) in cytokine production between samples.

Table 5

Glutathione (GSH) content of U937 cells exposed to sodium casein (NaCN), NaCN non-cross-linked hydrolysate (Prolyve) and cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase). [a]

Sample	GSH content
Control	100.0±0.0
NaCN	72.1±14.7
Prolyve	111.1±18.6
TGase/Prolyve	134.1±21.8
Prolyve/TGase	130.8±21.7

[a] Data are the mean of three independent experiments ± SE; none of the results was statistically significant compared with the untreated cells.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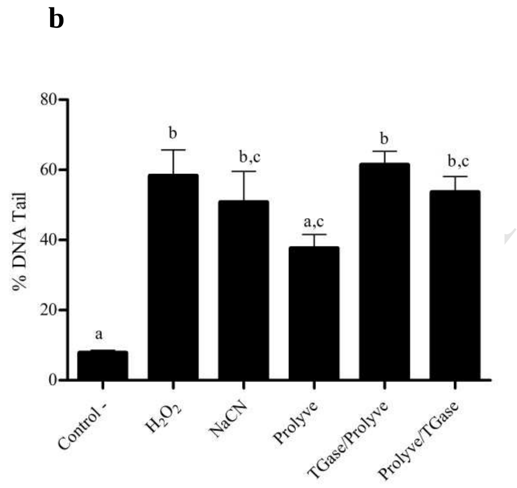
Control -
t-BOOH
NaCN
Prolyve
TGase/Prolyve
Prolyve/TGase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20
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80
a
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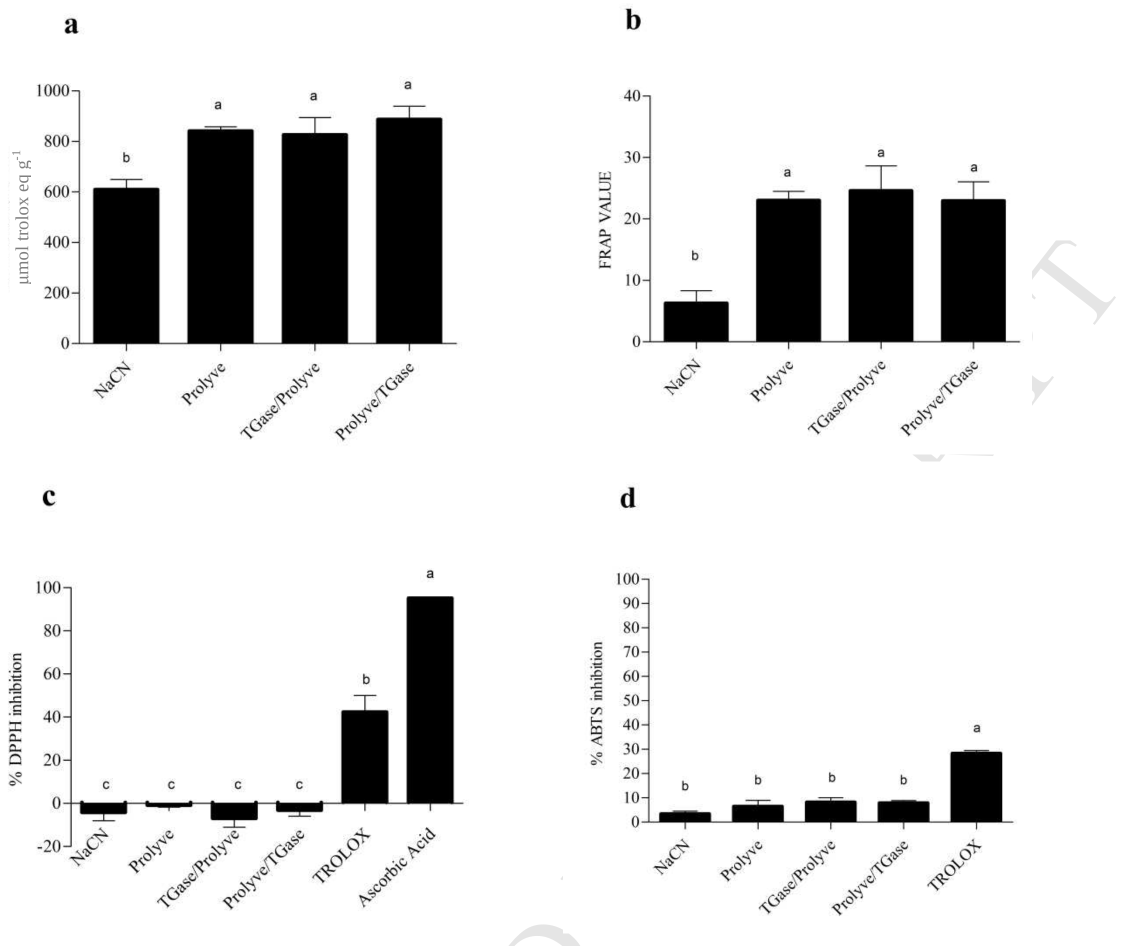
b
b,c
Control -
H_2O_2
NaCN
Prolyve
TGase/Prolyve
Prolyve/TGase

Figure 1.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Prolyve/TGase
c
% DPPH inhibition
100
80
60
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a
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TGase/Prolyve
Prolyve/TGase
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TROLOX

Figure 2.