Angiotensin converting enzyme and dipeptidyl peptidase-IV inhibitory activities of transglutaminase treated sodium caseinate hydrolysates

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Abstract

The angiotensin converting enzyme (ACE) and dipeptidyl peptidase-IV (DPP-IV) inhibitory activities of cross-linked and non-cross-linked NaCN hydrolysates were studied. Three different samples were generated: NaCN hydrolysed with Prolyve 1000™ (Prolyve), NaCN cross-linked with transglutaminase (TGase) pre-Prolyve hydrolysis (TGase/Prolyve) and NaCN cross-linked post-Prolyve hydrolysis (Prolyve/TGase). Gel filtration and reverse phase HPLC analysis of the resulting samples indicated that the hydrolysates had similar peptide profiles. Hydrolysates showed higher (p<0.05) ACE and DPP-IV inhibitory activity compared with intact protein but no significant (p>0.05) differences in activity were found between cross-linked and non-cross-linked hydrolysate samples. Hydrolysate IC₅₀ values for ACE and DPP-IV inhibition ranged from 0.10-0.17 mg mL⁻¹ and 0.85-1.18 mg mL⁻¹, respectively. Simulated gastrointestinal digestion had no significant (p>0.05) effect on the bioactivities of the hydrolysates. The results demonstrated that incubation with TGase before or after NaCN hydrolysis with Prolyve had no effect on ACE or DPP-IV inhibitory activities.

Keywords

Transglutaminase, Cross-linking, NaCN hydrolysates, ACE inhibitory activity, DPP-IV inhibitory activity.
2.2. Introduction

Enzymatic hydrolysis is a process used to modify the functional and physicochemical properties of food proteins (Flanagan & FitzGerald, 2002; Spellman, Kenny, O'Cuinn, & FitzGerald, 2005). Furthermore, food proteins contain bioactive peptides in their primary structures that can be released during hydrolysis and may subsequently be used as biofunctional ingredients. Many studies have reported that caseins are rich in angiotensin converting enzyme (ACE) inhibitory peptides (Luo, Pan, & Zhong, 2014; Miguel, Contreras, Recio, & Aleixandre, 2009; Norris & FitzGerald, 2013; Robert, Razaname, Mutter, & Juillerat, 2004). Furthermore, casein appears to be the milk protein substrate displaying the highest number of dipeptidyl peptidase-IV (DPP-IV) inhibitory peptide sequences (Lacroix & Li-Chan, 2012; Nongonierma & FitzGerald, 2013, 2015a; Power, Nongonierma, Jakeman, & FitzGerald, 2014). This may be linked, in part, to the high content of Pro residues in caseins, which is related with high DPP-IV and ACE inhibitory activities.

Enzymatic cross-linking is also a widely used method of protein modification which results in the addition of inter- and intra-cross links within and between protein residues. Transglutaminase (TGase; E.C. 2.3.2.13) is an enzyme which can catalyse cross-linking reactions between the amino acids Lys and Gln in proteins leading to the formation of an isopeptide bond (Motoki & Seguro, 1998). The addition of TGase to sodium caseinate (NaCN) modified its structure, and enhanced its emulsifying and foaming properties. Moreover, the combination of cross-linking and enzymatic hydrolysis of NaCN resulted in a further significant improvement in the above mentioned properties (Flanagan, Gunning, & FitzGerald, 2003). However, there is limited information about the biofunctional activities of protein hydrolysates subjected to TGase treatment and some of the reports appear contradictory. Cross-linking of soy protein isolate
hydrolysates (Fan, et al., 2005) and soybean protein hydrolysates (Song, et al., 2013) was reported to have no effect on *in vitro* antioxidant activity. Similarly, Hiller and Lorenzen (2009) demonstrated that TGase did not modify the antioxidant activity of whey protein isolates and total milk proteins. However, a recent study from Bagheri, Madadlou, Yarmand, and Mousavi (2014) reported a significant (p<0.05) improvement in antioxidant activity of TGase cross-linked whey protein hydrolysates using the ferric reducing antioxidant power assay. The addition of TGase in combination with hydrolysis with the proteolytic enzyme Alcalase decreased the immunoreactivity of $\alpha$-lactalbumin and $\beta$-lactoglobulin (Wróblewska, Jędrychowski, Hajós, & Szabó, 2008). However, O’Sullivan and FitzGerald (2012); Sabadin, Villas-Boas, Zollner, and Netto (2012); Stanic, et al. (2010), found no differences in antigenic properties between cross-linked and non-cross-linked casein hydrolysates. Furthermore, O’Sullivan, Lahart, O’Callaghan, O’Brien, and FitzGerald (2013) showed that NaCN hydrolysate samples generated by cross-linking with TGase prior to enzymatic hydrolysis produced an anti-inflammatory response in Jurkat T cells, while TGase treatment following hydrolysis and non-cross-linked hydrolysates showed no response. Although it has been demonstrated that some NaCN hydrolysates may possess *in vitro* bioactivities (Nongonierma & FitzGerald, 2013; Wu, Pan, Zhen, & Cao, 2013) no studies appear to have yet reported on the effect of TGase cross-linking on *in vitro* markers related to glycaemic management and blood pressure effects, i.e., DPP-IV and ACE inhibitory activity, respectively.

Given the limited information and the potentially contradictory results reported on the role of TGase treatments, the aim of the present study was to characterise the effect of combining cross-linking and enzymatic hydrolysis on the physicochemical and *in vitro* ACE and DPP-IV inhibitory properties of NaCN.
2.3. Material and methods

2.3.1. Materials

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, England). Prolyve 1000™ was kindly provided by Lyven Enzymes Industrielles (Caen, France). Trinitrobenzensulphonic acid (TNBS) was from Medical Supply Company (Dublin, Ireland). HPLC grade water and acetonitrile and PTFE syringe filters (0.2 µm) were obtained from VWR International (Dublin, Ireland). Bovine lung for the extraction of ACE was provided by Gaelic Meats and Livestock Ltd. (Limerick, Ireland). Abz-Gly-p-nitro-Phe-Pro-OH and Abz-Gly-OH-HCl were from Bachem Feinchemikalien (Bubendorf, Switzerland). Black 96-well microplates were purchased from Thermo Fisher Scientific (Dublin, Ireland). Trifluoroacetic acid (TFA) and L-leucine were supplied by Sigma–Aldrich (Dublin, Ireland). All other chemicals were obtained from Sigma and were of analytical grade unless otherwise stated.

2.3.2. Generation of cross-linked NaCN hydrolysates

A NaCN solution (10 % (w/v) protein) was incubated with 0.3 % (v/v) Prolyve 1000™ at 50 ºC and pH 7.0 using a pH stat (Titrando 843, Metrohm, Dublin, Ireland) to generate the non-cross-linked hydrolysates (termed Prolyve). Prolyve 1000™ is a non-specific microbial endoproteinase, produced by *Bacillus licheniformis*, which contains subtilisin activity (Spellman, O’Cuinn, & FitzGerald, 2009). Aliquots were taken at several time points. After 240 min of incubation, the enzymatic reaction was stopped by heating at 80 ºC for 20 min. For the production of the cross-linked post-hydrolysis samples (Prolyve/TGase), the hydrolysed sample (Prolyve) was
incubated with TGase (2% (w/v)) at room temperature and pH 7.0 for 180 min. Inactivation of TGase was carried out as mentioned above. For cross-linking pre-hydrolysis samples (TGase/Prolyve), NaCN was incubated first with TGase and subsequently submitted to hydrolysis with Prolyve 1000™ using the same conditions as outlined above. A NaCN solution (10 % (w/v) protein) incubated at 50 °C for 240 min and subjected to the heat treatment conditions used for enzyme inactivation was used as a control (Intact NaCN) in all experiments.

2.3.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a Mini-Protean® Tetra Cell system (Bio-Rad, Hemel Hempstead, UK) according to the method of Laemmli (1970) using Mini-PROTEAN TGX precast gels (4-20 % resolving gel). The quantity of protein added to each well was between 15-20 µg. The molecular mass of the proteins was estimated by reference to the relative migration of the molecular weight standards SigmaMarker™ Wide Range (M.W. 6,500-200,000). The gel was stained with a Coomassie Blue R-250 solution and destained in an aqueous solution of 40 % (v/v) methanol and 10 % (v/v) acetic acid.

2.3.4. Determination of degree of hydrolysis (DH)

The DH was determined using the TNBS method as outlined by Adler-Nissen (1979) using L-Leucine as standard. The percentage degree of hydrolysis was calculated using the formula as described previously by Spellman, et al. (2009).

2.3.5. Gel filtration and reversed-phase high performance liquid chromatography

Gel permeation high performance liquid chromatography (GP-HPLC) and analytical reversed-phase (RP-) HPLC were performed as described previously by Spellman, et al. (2009)
with minor modifications. For GP-HPLC, 20 μL of sample (0.8 % (w/v)) was injected on a TSK G2000 SW separating column (600 × 7.5 mm ID). Separation was performed by isocratic elution using 0.1% (v/v) TFA, 30% (v/v) acetonitrile in H2O, at a flow rate of 1 mL min⁻¹. For RP-HPLC samples (20 μL at 0.8% (w/v)) were injected on a Jupiter Proteo column (C18, 250 mm x 4.6 mm ID, 5 μm particle size, 300 Å pore size, Phenomenex, Cheshire, UK). The column was equilibrated using solvent A: 0.1% (v/v) TFA in H2O at a flow rate of 1 mL min⁻¹. Samples were eluted using solvent B: 0.1% (v/v) TFA, 80% acetonitrile (v/v) in H2O at a flow rate of 1 mL min⁻¹ employing a linear gradient from 0% at 0 min to 100% at 60 min.

2.3.6. ACE inhibitory activity

The ACE used was extracted from fresh bovine lung following the method by Meng, Balcells, Dell'Italia, Durand, and Oparil (1995). ACE inhibitory activity was measured using the fluorometric method of Sentandreu and Toldrá (2007) with some minor modifications. An aliquot (50 μL) of test sample was added to 50 μL of assay buffer (100 mM sodium borate buffer, 300 mM NaCl, pH 8.3) and 200 μL of substrate (0.45 mM Abz-Gly-Phe-(NO₂)-Pro). The reaction was started with the addition of 50 μL of enzyme extract (8 mU) and fluorescence was determined using a Synergy HT Multi-Mode Microplate Reader (BioTek, Mason Technology, Dublin, Ireland) for 30 min at excitation and emission wavelengths of 360 and 400 nm, respectively. Fluorescence from the release of product (Abz-Gly) was quantified at time zero and time 30 min. Experiments were carried out as independent triplicates assayed in triplicate. IC₅₀ values (concentration of inhibitor that inhibits 50% of ACE activity) were calculated using GraphPad® Prism 4.0 sigmoidal dose response plots of inhibitor concentration (mg mL⁻¹) versus % inhibition. The values were expressed as the mean IC₅₀ ± standard deviation (n=3). Captopril was used as a reference positive
2.3.7. **DPP-IV inhibitory activity**

DPP-IV inhibition was assayed by the method of Nongonierma and FitzGerald (2014a) with minor modifications. In brief, 10 μL of serial dilutions of protein hydrolysate (5-0.05 mg mL⁻¹) was mixed with 30 μL 20 mM Tris–HCl buffer, pH 8.0 containing 100 mM NaCl and 1 mM EDTA, and 50 μL 200 μM H-Gly-Pro-AMC. Following 5 min incubation at 37 °C the reaction was initiated by addition of 10 μL DPP-IV (8 mU mL⁻¹). The change in fluorescence was monitored over a 30 min period using a plate reader Varioskan Flash (ThermoScientific, Tewksbury, MA, USA) at excitation and emission wavelengths of 360 and 460 nm, respectively. One unit (U) of DPP-IV activity was defined as that amount of enzyme which hydrolyses 1 μmol of H-Gly-Pro-AMC per min at 37 °C. Diprotin A was used as a reference DPP-IV inhibitory substance. The inhibition was expressed as IC₅₀ values. IC₅₀ values are reported as the mean of three independent replicate assays ± standard deviation.

2.3.8. **In vitro simulated gastrointestinal digestion**

The two stage digestion process was performed according to the method of Walsh, et al. (2004). For gastric digestion, the Prolyve, Prolyve/TGase and TGase/Prolyve samples were incubated with pepsin (enzyme:substrate ratio 1:40 (w/w)) for 1.5 h at 37°C and pH 2.0 followed by intestinal digestion with Corolase PP® (enzyme:substrate ratio 1:10 (w/w)) for 3 h at 37 °C and pH 7.0. Both enzymes were inactivated by heating the sample at 80 °C for 20 min. Aliquots of the samples were taken at several time points. The DH, ACE and DPP-IV inhibitory activities of the digested samples were performed as mentioned above.
2.3.9. Statistical analysis

Data were reported as means ± standard deviation of three independent replicates. ANOVA one way analysis of variance with Bonferroni or Dunnett test was used to analyse the results using Graphpad Prism, version 4.00 for Windows. Significant levels were defined as p < 0.05.

2.4. Results and discussion

2.4.1. Characterisation of the hydrolysates

Three different samples were generated in the present study. NaCN cross-linked pre-hydrolysis (TGase/Prolyve), NaCN cross-linked post-hydrolysis (Prolyve/TGase) and NaCN hydrolysed with Prolyve 1000™ (Prolyve). Figure 1A shows the SDS-PAGE profile of NaCN incubated with TGase. After 30 min of incubation (Fig 1A, lane 3), the profile showed the appearance of bands with higher molecular masses in the 65 kDa region and a decrease in the intensity of the casein bands (24-30 kDa region) suggesting that polymerization occurred. Following 180 min of incubation with TGase (Fig 1A lane 8), reductions in the intensity of the casein bands were evident with higher molecular bands appearing around 220 kDa. Furthermore, at the later stages of TGase incubation (120-180 min, Fig 1A lanes 6-8) it was evident that large molecular mass polymers were present which do not enter the gel. Figure 1B represents the SDS-PAGE profile of NaCN incubated with the proteolytic enzyme Prolyve. The NaCN control (Fig 1B lane 2) shows bands representing α-, β- and κ-casein, eluting at 34, 29 and 24 kDa, respectively. After 5 min of hydrolysis (Fig 1B lane 3) the caseins were degraded and low molecular weight
bands appeared on the gel. After 180 and 240 min of hydrolysis (Fig 1B lanes 8,9) no bands were detected in the gel indicating that the molecular masses of the peptides in the samples were less than 6 kDa. Figure 2 a and b shows the molecular mass distribution profile, obtained by GPC-HPLC, and the DH (%) as a function of time during incubation with TGase and Prolyve. As expected, the addition of TGase prior to or after hydrolysis did not alter the DH of the cross-linked samples, whereas the addition of Prolyve increased the extent of hydrolysis and therefore the release of peptides from NaCN. At the end of the hydrolysis process, 90 % of the samples were < 2 kDa and the DH obtained for the three samples, Prolyve, TGase/Prolyve and Prolyve/TGase reached similar (p>0.05) values, i.e. 17.4 ± 0.4, 16.7 ± 0.7 and 16.9±0.5%, respectively (Table 1). The RP-HPLC profiles (Figure 3) showed no discernable differences in the peptide profiles obtained for the Prolyve, TGase/Prolyve and Prolyve/TGase samples. O’Sullivan, et al. (2013) previously reported that the profiles of similar NaCN hydrolysates showed some residual intact protein (at ~53 min) in the Prolyve sample that did not appear in Prolyve/TGase and TGase/Prolyve samples. However, in this study a minor peak of intact NaCN at ~53 min was seen in all the cross-linked and non-cross-linked samples (Fig.3). It was previously reported that TGase treatment after hydrolysis of β-lactoglobulin resulted in a lower DH in comparison to the non-treated hydrolysates indicating that polymerization had occurred (Sabadin, et al., 2012). In contrast, the results found in this study showed a lower final mean DH value for the cross-linked samples compared to the non-cross-linked sample, however, these values were not significantly (p>0.05) different. The differences in the results observed between these studies could be attributed to the study differences, e.g., protein substrate, proteolytic preparation and the concentration of TGase used. Overall, the results found herein show that no major differences were detectable in the physicochemical properties (SDS-PAGE, % DH, RP- and GP-HPLC profiles) of the hydrolysates
produced regardless of the order of cross-linking, i.e., prior to or after hydrolysis. These findings concur with those of (O’Sullivan & FitzGerald, 2012) which reported no differences in physicochemical properties between samples hydrolysed pre or post TGase cross-linking.

2.4.2. In vitro simulated gastrointestinal digestion (SGID)

The ability of bioactive peptides to exert their function may depend on their resistance to digestion by gastrointestinal enzymes and to their capability to pass through the intestinal barrier in order to reach target organs (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011). An in vitro study with digestive enzymes was performed herein with the aim of evaluating the stability of the cross-linked NaCN hydrolysates to gastrointestinal digestion. This two stage process was performed using pepsin and Corolase PP® as gastric and pancreatic enzyme preparations, respectively. The extent of hydrolysis obtained after the two stages of the SGID process is shown in Figure 4. As mentioned previously, the Prolyve, TGase/Prolyve and Prolyve/TGase samples had a DH of ~17%. After incubation with pepsin there was a small increase in the DH (from 0.0 h to 1.5 h). However, incubation with Corolase PP® produced a significant increase (p<0.05) in the extent of hydrolysis (from 1.5 h to 4.5 h) reaching values of 31.4 ± 5.2, 31.6 ± 6.2 and 33.4 ± 4.2 % DH for Prolyve, TGase/Prolyve and Prolyve/TGase, respectively (Figure 4 and Table 1). Pepsin is an aspartic proteinase with broad substrate specificity ( Vance, LeBlanc, & London, 1997) and Corolase PP® is a porcine proteolytic preparation of pancreatic enzymes with broad specificity and activities (e.g., trypsin, chymotrypsin, elastase and carboxypeptidase) (Mullally, O'Callaghan, FitzGerald, Donnelly, & Dalton, 1994). This may explain the further hydrolysis observed after SGID treatment. This change was also observed in the RP-HPLC profiles as shown in Fig. 3 a, b and c. Hydrolysates appeared to be resistant to pepsin
digestion and no major changes were observed in the chromatographic profiles in comparison with the non-SGID samples. Following incubation with Corolase PP®, the samples displayed further hydrolysis where more hydrophilic peptides were generated. The same trend has been found in other studies such as by Guo, et al. (2015) who reported a small increase in DH after incubation of Alaska Pollock skin collagen with pepsin and a large increase in DH when the sample was incubated with Corolase PP®. The RP-HPLC profile of the samples before and after incubation with Corolase PP® showed minor differences between the samples (Fig. 3 a, b and c). The results herein demonstrate that no significant comparable differences in DH (p>0.05) or gross peptide profile were observed between the cross-linked and non-cross-linked samples following SGID.

2.4.3 ACE and DPP-IV inhibitory activity

ACE is a carboxydipeptidase which plays a major role in the regulation of blood pressure by activation of the renin-angiotensin-aldosterone system. It catalyses the conversion of angiotensin I into the vasoconstrictor, angiotensin II. Some peptides with hypotensive properties act as natural inhibitors of ACE (FitzGerald, Murray, & Walsh, 2004). The different hydrolysates and associated control sample were tested for their ACE inhibitory activity and the results are summarised in Table 1. The IC₅₀ for Captopril, the positive control, was 3.5 ngmL⁻¹, this concurs with the values reported elsewhere in the literature. As expected, unhydrolysed NaCN had a high ACE IC₅₀ value (>10 mg mL⁻¹) which indicates that the parent protein had minimal inhibitory activity. NaCN hydrolysis with Prolyve showed the highest mean inhibitory activity (IC₅₀ = 0.10 ± 0.02 mg mL⁻¹) followed by Prolyve/TGase and TGase/Prolyve with IC₅₀ = 0.15 ± 0.04 mg mL⁻¹ and IC₅₀ = 0.17 ± 0.05 mg mL⁻¹, respectively. However, TGase cross-linking before and after hydrolysis had no significant effect (p>0.05) on ACE inhibitory activity. After SGID the mean
TGase/Prolyve IC$_{50}$ value decreased while that of Prolyve/TGase increased. However, the changes were not significantly (p>0.05) different. ANOVA analysis showed no significant differences between the hydrolysates, whereas all hydrolysates were different from the control. The ACE IC$_{50}$ values for the hydrolysates observed in this study are similar to those of Hayes, et al. (2007), i.e., 0.8 mg mL$^{-1}$ for bovine casein fermentates generated with Lactobacillus animalis and were lower than those reported by Wu, et al. (2013), (2.36 mg mL$^{-1}$) in bovine casein fermentates obtained with Lactobacillus casei. The ACE inhibitory activity of a hydrolysate depends on the type of proteinase and the protein used as the substrate (Xie, Kim, Ha, Choung, & Choi, 2014). Connolly, Piggott, and FitzGerald (2014) reported that Prolyve and Alcalase were the enzymes that yielded the most potent ACE inhibitory hydrolysates in brewers’ spent grain (BSG) protein hydrolysates. This was probably due to the broad specificity of the enzymes compared with other proteolytic preparations tested such as Flavourzyme, Protamex or Promod. Prolyve and Alcalase, obtained from Bacillus licheniformis, have subtilisin activity but only Alcalase possesses an additional minor glutamyl endopeptidase activity (Spellman, et al., 2005). The results show that potent in vitro SGID resistant ACE inhibitory hydrolysates can be generated from NaCN using Prolyve; furthermore, the cross-linking treatment has no effect on the activity.

DPP-IV is a peptidase that regulates the degradation of GLP-1 and GIP, incretins that activate the release of insulin from the pancreas to the circulation. Casein hydrolysates have been associated with DPP-IV inhibitory properties (Nongonierma & FitzGerald, 2013). However, to the best of our knowledge, there appears to be no information in the literature in relation to the inhibition of DPP-IV by cross-linked casein hydrolysates. The DPP-IV inhibitory activity of the NaCN hydrolysates generated herein was therefore investigated. The results obtained are summarised in Table 1. The IC$_{50}$ for diprotin A, the positive control, was 1.2 µg mL$^{-1}$, this value
concurrency with those reported elsewhere in the literature. Hydrolysis of NaCN released potent DPP-IV inhibitory hydrolysates with IC$_{50}$ values of $1.20 \pm 0.13$, $1.05 \pm 0.20$ and $0.97 \pm 0.18$ mg mL$^{-1}$ for Prolyve, TGase/Prolyve and Prolyve/TGase, respectively, whereas the parent protein was not able to inhibit DPP-IV. The DPP-IV inhibitory values obtained in this study were within the same range to those reported previously for casein hydrolysates (0.88-1.11 mg mL$^{-1}$, Nongonierma and FitzGerald (2013)). However, as observed with the results for ACE inhibitory activity, no significant differences ($p>0.05$) were found between the three different sets of samples. Furthermore, while SGID decreased the mean IC$_{50}$ values, no significant ($p>0.05$) differences were found compared to the non-SGID samples. These results indicate that the peptides responsible for the DPP-IV inhibitory activity are stable to SGID enzymes. These results are very similar to those reported by Nongonierma and FitzGerald (2015b), in plant based derived hydrolysates, where no significant differences on DPP-IV activity were shown before and after SGID treatment. The lack of significant differences in bioactivity following SGID may be the result of two different events. SGID treatment (a) had no effect on those peptides responsible for DPP-IV inhibition or alternatively (b) SGID treatment resulted in the degradation of DPP-IV inhibitory peptides and the release of new inhibitory sequences. A similar outcome may have arisen for the ACE inhibitory activity following SGID treatment. The inhibitory potencies of the hydrolysates for ACE and DPP-IV were significantly lower than that of synthetic drugs such as Captopril or the synthetic peptide diprotin A. However, the use of naturally derived ACE and DPP-IV inhibitors has the advantage of not presenting the side-effects associated with synthetic drug inhibitory compounds.

The bioactivities of the hydrolysates are directly related with their primary structure. It is well known that NaCN is rich in the hydrophobic amino acid Pro which is correlated with peptides displaying high DPP-IV and ACE inhibitory activities (Nongonierma & FitzGerald, 2014b; Norris
Some studies suggest that the use of TGase prior to or after hydrolysis of NaCN could modify the structure of the parent protein and therefore lead to the generation of new bioactive peptide entities. However, in the present study it appears that the potent ACE and DPP-IV bioactive hydrolysates obtained are a result of the hydrolysis process rather than due to cross-linking.

2.5. Conclusion

The results reported herein showed that minor differences were found in the physicochemical properties of TGase cross-linked and non-cross-linked NaCN Prolyve hydrolysates. TGase cross-linked and non-cross-linked NaCN hydrolysates had significantly higher DPP-IV and ACE inhibitory activity in comparison to intact NaCN. However, no significant differences in in vitro bioactivities were observed between cross-linked and non-cross-linked hydrolysates. The effects of TGase cross-linking and hydrolysis with other proteolytic enzyme preparations on the in vitro bioactivities is a yet unknown. Furthermore, the in vitro bioactivities of the TGase cross-linked Prolyve hydrolysates were maintained following SGID. This indicates that TGase cross-linking had no effect on the overall bioactivity of the Prolyve hydrolysates. Analysis of the peptide sequences within the different hydrolysates should help further elucidate the structural differences between the cross-linked hydrolysates. Previous analysis of the hydrolysates demonstrated no effect of the order of cross-linking on hydrolysate antioxidant properties (Cermeño, FitzGerald, & O'Brien, 2016). However, the non-cross-linked Prolyve NaCN hydrolysate was shown to have a DNA protective effect against H$_2$O$_2$ induced U937 cells when grown in culture, an effect not observed with the TGase cross-linked hydrolysate samples. These
results indicate that the effects of cross-linking may be dependent on the biomarker being analysed.

2.6. Acknowledgements

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**FIGURE CAPTIONS**

**Figure 1.** (A) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profile of sodium caseinate (NaCN) incubated with transglutaminase (TGase, 2 % (w/v)) for different durations. Lane 1, Wide range molecular weight marker; Lane 2, Intact NaCN; Lane 3, NaCN incubated with TGase for 30 min; Lane 4, NaCN incubated with TGase for 60 min; Lane 5, NaCN incubated with TGase for 90 min; Lane 6, NaCN incubated with TGase for 120 min; Lane 7, NaCN incubated with TGase for 150 min; Lane 8, NaCN incubated with TGase for 180 min. The amount of protein added to each well was 20 µg. (B) SDS-PAGE profile of NaCN incubated with Prolyve (0.3 % (v/v)) for different durations. Lane 1, Wide range molecular weight marker; Lane 2, NaCN; Lane 3, NaCN incubated with Prolyve for 5 min; Lane 4, NaCN incubated with Prolyve for 30 min; Lane 5, NaCN incubated with Prolyve for 60 min; Lane 6, NaCN incubated with Prolyve for 90 min; Lane 7, NaCN incubated with Prolyve for 120 min; Lane 8 NaCN incubated with Prolyve for 180 min; Lane 9 NaCN incubated with Prolyve for 240 min. The amount of protein added to each well was 15 µg.

**Figure 2.** Molecular mass distribution profiles of (a) cross-linked pre-hydrolysis sodium caseinate (NaCN) and (b) cross-linked post-hydrolysis NaCN at different sampling points (≥10 kDa, ≥10-5kDa, ≥5-2kDa, <2 kDa) along with the degree of hydrolysis (DH) of the hydrolysates (---).

**Figure 3.** Reverse phase (RP) HPLC chromatograms obtained at 214 nm for the different sodium caseinate Prolyve hydrolysates with and without transglutaminase (TGase) treatments before and after in vitro simulated gastrointestinal digestion. (a) Prolyve, (b) TGase/Prolyve, (c) Prolyve/TGase. The arrows indicate differences between samples after incubation with Corolase PP. The arrows at ~ 53 min indicate remained intact casein.

**Figure 4.** Change in degree of hydrolysis (DH%) values of non-cross-linked and transglutaminase (TGase) cross-linked hydrolysates of sodium caseinate during simulated gastrointestinal digestion. Prolyve (---), TGase/Prolyve (---), Prolyve/TGase (---). Mean ± SE (n=3). Different letters denote statistically significant change in DH% at p<0.05.
Figure 1

(A)

(B)
Figure 2

(a) Cross-linking time (min) vs. Hydrolysis time (min)

(b) Hydrolysis time (min) vs. Cross-linking time (min)
Figure 3

(a) Corolase PP, Pepsin, Prolyve

(b) Corolase PP, Pepsin, TGase/Prolyve

(c) Corolase PP
Figure 4

![Graph showing DH (%) vs. Time of digestion (h) with Pepsin and Corolase PP interventions.](image)

- DH (%) is plotted on the y-axis, ranging from 10 to 40.
- Time of digestion (h) is plotted on the x-axis, ranging from -0.5 to 5.0.
- Arrows indicate the addition of Pepsin and Corolase PP.
- The graph shows two curves, one with a dotted line and another with a solid line.
- The curves are labeled with letters (a, b) indicating different groups or conditions.

The graph visually represents the effects of Pepsin and Corolase PP on DH over time.
Table 1

Degree of hydrolysis (DH (%)), angiotensin converting enzyme (ACE) and dipeptidyl peptidase-IV (DPP-IV) inhibition of the Prolyve hydrolyzed and transglutaminase (TGase) cross-linked sodium caseinate (NaCN) hydrolysates before and after simulated gastrointestinal digestion (SGID).

<table>
<thead>
<tr>
<th>Sample</th>
<th>DH (%)</th>
<th>ACE IC₅₀ (mg protein mL⁻¹)</th>
<th>DPP-IV IC₅₀ (mg protein mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact NaCN</td>
<td>-</td>
<td>&gt;10ᵃ</td>
<td>&gt;10ᵃ</td>
</tr>
<tr>
<td>Prolyve</td>
<td>17.46±0.43ᵃ</td>
<td>0.10±0.02ᵇ</td>
<td>0.10±0.02ᵇ</td>
</tr>
<tr>
<td>TGase/Prolyve</td>
<td>16.79±0.77ᵃ</td>
<td>0.17±0.05ᵇ</td>
<td>0.14±0.14ᵇ</td>
</tr>
<tr>
<td>Prolyve/TGase</td>
<td>16.87±0.47ᵃ</td>
<td>0.15±0.04ᵇ</td>
<td>0.23±0.08ᵇ</td>
</tr>
</tbody>
</table>

Mean ± SD (n=3). For each assay, samples with different letters are significantly different at p<0.05. SGID: simulated gastrointestinal digestion. IC₅₀ values represent the concentration of hydrolysates that inhibit 50 % of ACE or DPP-IV activity.