International Journal of Food Science +Technology

Institute of Food Science +Technology ifst

International Journal of Food Science and Technology 2018, **53**, 1865–1870

Original article

Addition of crude tiger nut protein extract affects stiffness of enzymatically cross-linked dairy proteins

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(Received 25 January 2018; Accepted in revised form 7 March 2018)

Summary The influence of crude tiger nut protein extract on the gel properties of enzymatically cross-linked dairy proteins was investigated. Enzymatic cross-linking of dairy proteins in the presence of crude tiger nut proteins caused the formation of larger casein polymers and increased the degree of polymerisation. Gel stiffness of acidified products containing whey proteins was higher when cross-linking occurred in the presence of crude tiger nut proteins. The results are relevant for improving the textural characteristics of acidified aqueous tiger nut extract (tiger nut milk) enriched in dairy proteins.

Keywords Dairy proteins, degree of polymerisation, gel stiffness, tiger nut proteins, transglutaminase.

Introduction

Tiger nut (*Cyperus esculentus* L) is a sweet vegetable nut-size rhizome that is obtained from a perennial cyperaceous plant (Coskuner *et al*., 2002). Tiger nut tubers are rich in carbohydrates, lipids, minerals and contain some proteins (Bado *et al*., 2015). To exploit these nutrients, recent investigations focused on enhancing the functional properties of bakery products by enriching them in fibre from tiger nut powder (Zahra & Ahmed, 2014; Aguilar *et al*., 2015) or using tiger nut oil extracts as an economically important source of essential oils for food applications (Yoon, 2016). Tiger nuts abound in many tropical regions (for instance in West Africa) where its utilisation as a source of nutritious food is largely unexploited (Adejuyitan, 2011). Therefore, exploring aqueous tiger nut extracts (tiger nut milk; TNM) as a base for lactic acid fermented products with acceptable textural properties might be promising. Our recent study showed that enrichment of TNM in globular tiger nut proteins in the range from 10.0 to 20.0 g kg^{-1} reduced phase separation after lactic acid fermentation, but was ineffective in supporting formation of acceptable gel systems (Kizzie-Hayford *et al*., 2016). To utilise TNM for a more acceptable lactic acid fermented product with improved texture, it was necessary to enrich tiger nut milk in dairy proteins, that is sodium caseinate or whey protein isolate to examine their individual contributions to texture of resulting products (Kizzie-Hayford *et al*., 2016). Moreover, enzymatic cross-linking of dairy proteins before their addition to TNM led to a higher viscosity and lower syneresis of resulting lactic acid fermented products (Kizzie-Hayford *et al*., 2017). Mixing dairy proteins with TNM prior to enzymatic cross-linking followed by lactic acid fermentation is a possibility which could allow a simplified process and reduced manufacturing time. However, it is not clear how this alteration might affect the textural characteristics of resulting products. Therefore, investigation of the effects of crude tiger nut protein extract (protein concentrate from tiger nut milk) on the gel properties of enzymatically cross-linked dairy proteins after acidification is relevant for determining the textural properties of lactic acid fermented TNM enriched in dairy proteins and is useful for optimising the manufacturing chain. To achieve this, microbial transglutaminase (mTGase) was used for cross-linking sodium caseinate or whey protein isolates in the presence of crude tiger nut protein. Acidification of cross-linked proteins was achieved by adding glucono-δ-lactone. Protein degree of polymerisation and gel stiffness during acid gelation were measured using size exclusion chromatography and thromboelastometry, respectively.

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Material and methods

Sample collection, preparation and reagents

Tiger nuts were supplied by farmers at Twifo Praso (http://latitude.to/articles-by-country/gh/ghana/189169/twifo-praso) in the Central Region of Ghana, West

doi:10.1111/ijfs.13770

Africa. After removing broken and discoloured nuts and washing the remaining nuts with water, the tubers were dried at room temperature (30 °C) and stored at 6 °C. Sodium caseinate (<98 g protein per 100 g powder) and whey protein isolate (<97 g protein per 100 g powder) were supplied by Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and Sports Supplements Ltd. (Colchester, UK), respectively. Glucono-δ-lactone was obtained from Kampffmeyer Nachf. GmbH (Ratzeburg, Germany). Microbial transglutaminase Activa MP from *Streptomyces mobaraensis* was obtained from Ajinomoto Foods Deutschland GmbH (Hamburg, Germany). The activity of the enzyme preparation was 92 IU g^{-1} (determined using the hydroxamate method by Folk & Cole, 1966).

Preparation of substrates and enzymatic cross-linking

Tiger nut protein extract was prepared by aqueous extraction of tiger nut milk (TNM) according to Kizzie-Hayford *et al.* (2016). Briefly, tiger nuts were soaked at 40 °C for 24 h and after washing with water, they were wet-milled using a Kult pro mixer (WMF AG, Geislingen, Germany). The resulting material was filter-pressed using a pneumatic press (6.55×10^5 N m^{-2}) to separate TNM from the pressing residue. To concentrate tiger nut proteins, the obtained TNM was vacuum-evaporated to reach approx. 300 g kg^{-1} total solids using a R-124 rotational vacuum evaporator coupled to a B-172 vacuum controller (BÜCHI Labortechnik AG, Flawil, Switzerland) at 70 °C. Next, the concentrated TNM was centrifuged (SIGMA 3–30 K, Laborzentrifugen GmbH, Osterode, Germany) at 20 000 ***g*** at 4 °C for 20 min to remove starch and fibre (Kizzie-Hayford *et al.*, 2015). After separating solid fat from the supernatant, the remaining aqueous portion served as the crude tiger nut protein extract. Crude tiger nut protein extract was analysed for the protein content using the Kjeldahl method ($N \times 6.25$) and the ash content by combustion of predried and preweighed samples in a muffle furnace at 550 °C for 6 h according to Matissek *et al.* (1992).

Solutions of 50 g kg^{-1} sodium caseinate and 50 g kg^{-1} whey protein isolate were separately prepared by dispensing the required amount in distilled water and dispersing it with a magnetic stirrer at 200 rpm, 25 °C for approx. 2 h. Sodium azide was added at 0.3 g kg^{-1} to prevent deterioration due to microbial growth. Where applicable, whey protein isolate solutions were heated for denaturation in a water bath at 80 °C for 10 min and subsequently cooled in ice water. The protein mixtures were divided into three aliquots for cross-linking with mTGase according to Jaros *et al.* (2014a, 2014b): one part was solely treated with mTGase (sample codes: Ce and We), another part was treated with mTGase and subsequently blended with tiger nut protein extract (CeT and WeT) and the last part was first blended with tiger nut protein extract and treated with mTGase afterwards (CTe and WTe). To obtain proteins of various cross-linking degrees, dairy proteins were incubated at 40 °C for 2, 5 or 24 h and subsequently heated at 80 °C to inactivate enzyme, and finally cooled in ice water. Concentrations of dairy proteins and, if applicable, tiger nut proteins were 30 and 10 g kg^{-1}, respectively. For comparison, mixtures of 10 g kg^{-1} tiger nut proteins and 30 g kg^{-1} non-cross-linked sodium caseinate (CT) or 30 g kg^{-1} whey protein isolate (WT) were prepared. All samples were produced in duplicate.

Size exclusion chromatography of proteins

To investigate the effects of tiger nut protein on the degree of polymerisation after mTGase cross-linking of dairy proteins, size exclusion chromatography (AZURA Assistant ASM 2.1L, Knauer Wissenschaftliche Gerate GmbH, Berlin, Germany) was used to analyse the molecular weight distribution of proteins. The elution buffer, which comprised 1.0 g L^{-1} 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 6.0 mol L^{-1} urea, 0.1 mol L^{-1} NaCl, and 0.1 mol L^{-1} Na_2HPO_4, was adjusted to pH 6.8 using 6 mol L^{-1} HCl. The protein solutions were diluted with elution buffer (dilution factor, 1/13) and treated with 0.15 g L^{-1} dithiothreitol to dissociate protein aggregates and reduce disulphide bonds (Wingfield, 1995). Samples were separated by 0.5 mL min^{-1} isocratic elution using a Superdex 200 increase 10/300 column (GE Healthcare, Uppsala, Sweden) and detected at 280 nm using a UVD 2.1S detector (Knauer Wissenschaftliche Gerate GmbH, Berlin, Germany) at ambient temperature (20 °C). Chromatographic data were acquired using the ClarityChrom v.3.07 software (Knauer Wissenschaftliche Gerate GmbH, Berlin Germany), and corresponding peak areas (PA) were calculated for monomer, dimer and polymer fractions. Degree of polymerisation (DP, %) was calculated according to Bönisch, Lauber and Kulozik (2004) with the following equation (eqn 1).

$$\mathrm{DP} = 100 \sum \frac{(\mathrm{Area[dimers + trimers + polymers]})}{(\mathrm{Area[monomers + dimers + trimers + polymers]})} \quad (1)$$

Acid gelation

Because of the small sample quantities of the tiger nut protein extract (approx. 10.0 g), acid-induced gelation was studied by thromboelastometry (Raak *et al.*, 2015) instead of rheometry. Acidification was induced by

adding 40 mg glucono-δ-lactone to 1 g protein solution at 30 °C, and gelation was monitored using the automated thromboelastometer MultiTEM (Framar Hemologix SRL, Roma, Italy) with disposable cup-and-pin-vessels (6 mm inner diameter, 8 mm outer diameter, 7 mm height). Samples were equilibrated at 30 °C, and temperature was maintained with a heated steel block placed around the cup. An initial angular displacement of the cup of $\varphi = 4.75°$ was maintained, and the increase in pin displacement over time as a consequence of increasing sample stiffness during gelation was converted into an arbitrary amplitude parameter ($A = 0$–100 mm) by the software TEMAwin (Version 1.7.1; Framar Hemologix SRL, Rome, Italy). The maximum amplitude of displacement, MA (mm), which was directly obtained from the software was taken as indicator for gel stiffness. Values represent arithmetic mean of three independent determinations.

Statistical analysis

Data were evaluated using one-way analysis of variance. Tukey HSD or Games–Howell post hoc analyses were used to compare the mean values. SPSS software package version 16.0 was used for performing the statistical analyses (SPSS Inc., Chicago, IL, USA). All significance statements refer to $P < 0.05$.

Results and discussion

Crude tiger nut protein extract contained 25.0 ± 0.3 g kg^{-1} crude protein and 7.0 ± 0.04 g kg^{-1} ash on wet basis. The high content in ash was expected as tiger nuts are known to contain substantial amounts of minerals, with the more abundant ions being potassium (556.0–845.0 mg per 100 g), silicon (220.0–242.0 mg per 100 g), phosphorus (229.0–236.0 mg per 100 g), sulphur (164.0–194.0 mg per 100 g) and magnesium (100.0–102.0 mg per 100 g) (Bado *et al.* (2015). Size exclusion chromatograms displayed in Fig. 1 illustrate the effect of tiger nut protein extract on the polymerisation of sodium caseinate (a) and whey proteins (b) after 2, 5 and 24 h enzymatic cross-linking. Generally, protein polymerisation increased with duration of enzymatic cross-linking. Incubation of proteins with mTGase is known to generate intermolecular ε-(γ-*glutamyl*) lysine isopeptide bonds between γ-carboxyamide of a protein-bound glutamine and ε-amino groups of lysine residues, leading to protein polymers of high molecular weight (Gaspar & de Góes-Favoni, 2015). Previously, it was shown that mTGase continuously forms isopeptide bonds in casein during 24 h incubation under the same conditions (Jaros *et al.*, 2014a, 2014b; Raak *et al.*, 2017, 2018). There was no change in the main fraction of tiger nut proteins (elution times from 35–40 min, determined from previous chromatographic analysis of tiger nut protein extract) data not shown, suggesting that they are not sensitive to intermolecular cross-linking by mTGase. Nevertheless, it can be seen from earlier elution and higher peak intensities (elution time of about 15 min) that larger casein polymers were formed in the presence of tiger nut protein extracts. A similar observation was made in a previous study where the formation of larger casein polymers was triggered by the addition of ions (Raak *et al.*, 2018). The presence of ions during enzymatic cross-linking is known to screen protein net charge, favouring casein self-association and thus, the formation of larger polymers as a consequence of cross-linking molecules within particular aggregates (O'Connell & de Kruif, 2003; Raak *et al.*, 2018). Ash content of the tiger nut extract containing 10 g kg^{-1} protein was 2.8 g kg^{-1} and thus, may have contributed to the observed effects. Polymers of enzymatically cross-linked whey proteins in the presence of tiger nut proteins (WTe) did not significantly differ from the enzymatically cross-linked whey proteins produced before tiger nut proteins addition (WeT) (Fig. 1b) in terms of hydrodynamic size. Probably, the low accessibility of denatured whey proteins by enzymatic cross-linking might contribute to the marginal effects of tiger nut protein ions on its polymerisation.

Effects of enzymatic cross-linking of sodium caseinate in the presence of crude tiger nut proteins on the degree of polymerisation, DP (Fig. 2) were similar to that on polymer sizes. For instance, 2 h enzymatic cross-linking of sodium caseinate in the presence of tiger nut proteins (CTe, $73.14 \pm 0.02\%$) resulted in significantly higher DP than in the absence of tiger nut proteins (CeT, $68.49 \pm 1.55\%$). Again, ions of TNP extract may have contributed to a higher DP of enzymatically cross-linked sodium caseinate. However, the presence or absence of tiger nut protein extracts during enzymatic cross-linking of whey proteins did not show any significant differences in their degree of polymerisation.

Crude TNP extract did not show any remarkable gel formation upon acidification (data not shown). However, stiffness of acid gels from enzymatically cross-linked dairy proteins mixed with tiger nut proteins shown in Fig. 3 depicts different effects: enzymatic treatment of sodium caseinate enriched in tiger nut proteins (CeT and CTe) resulted in significantly higher gel stiffness than that of the counterpart without the enrichment (Ce) after 2 h incubation. However, further increase in duration of enzymatic cross-linking resulted in stiffer gels of sodium caseinate (Ce) whilst gels from systems enriched in tiger nut proteins became weaker. Increasing gel stiffness upon enzymatic cross-linking of sodium caseinate was attributed to the formation of covalent isopeptide bonds. However, larger casein polymers may lack in the molecular flexibility that is necessary to form stiffer

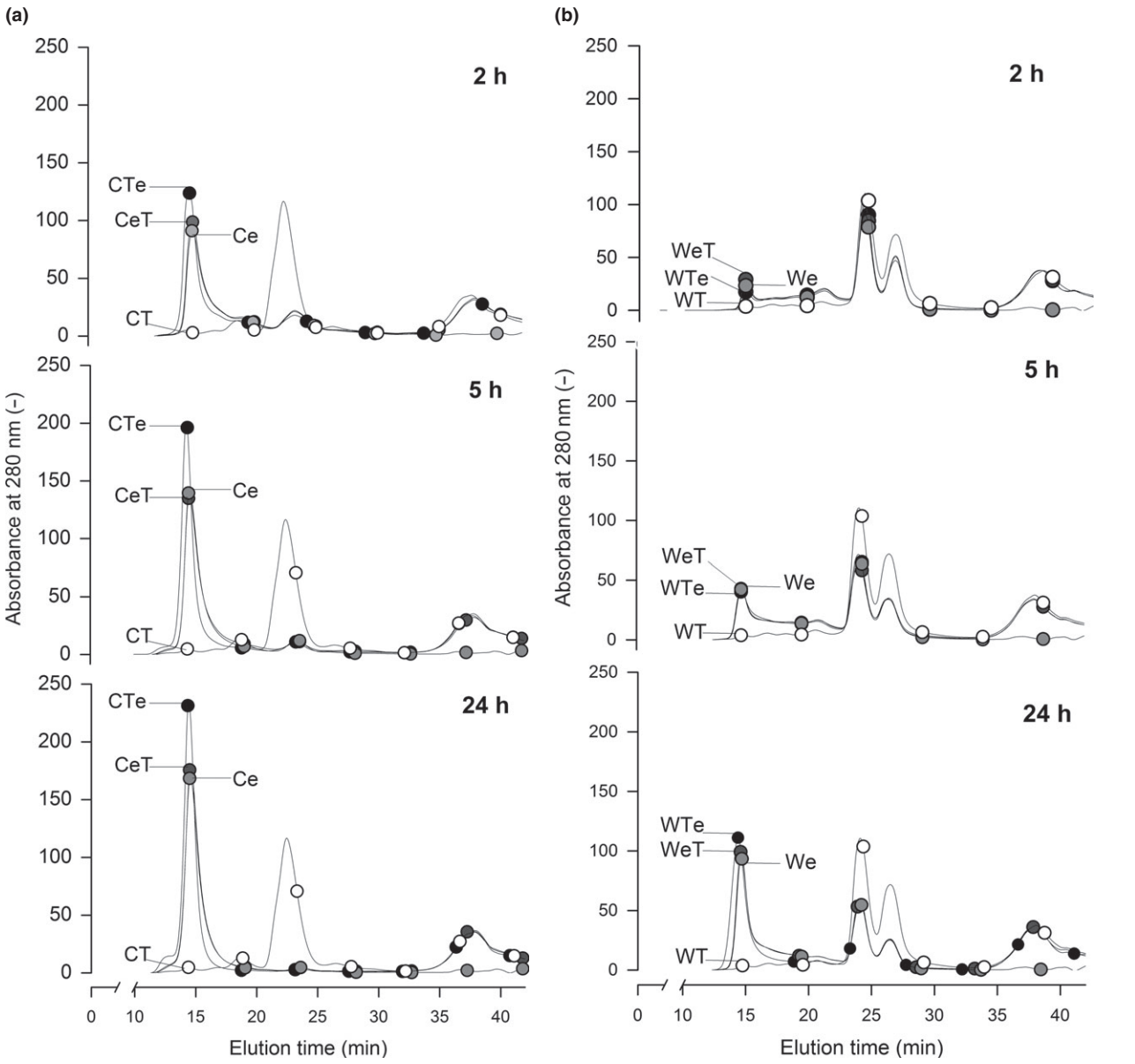

Figure 1 Size exclusion chromatograms of sodium caseinate (a) and whey protein isolates (b). CT: sodium caseinate mixed with tiger nut proteins, WT: whey protein isolate mixed with tiger nut proteins, Ce: enzymatically cross-linked sodium caseinate without tiger nut protein, We: enzymatically cross-linked whey proteins without tiger nut proteins, CeT: enzymatically cross-linked sodium caseinate before the addition of tiger nut proteins, WeT : enzymatically cross-linked whey protein isolate before the addition of tiger nut proteins, CTe: enzymatically cross-linked sodium caseinate after the addition of tiger nut proteins, WTe: enzymatically cross-linked whey protein isolate after the addition of tiger nut proteins. 2, 5 and 24 h refer to duration of enzymatic cross-linking.

gels (Raak *et al.*, 2017, 2017b). Addition of crude tiger nut proteins containing 2.8 g kg^{-1} minerals to sodium caseinate before enzymatic cross-linking (CTe) may have affected the ionic characteristics of the milieu and favoured protein association during cross-linking, contributing to a more pronounced decrease in gel stiffness because of the formation of larger polymers.

Prolonging the enzymatic cross-linking of whey protein isolate (We) resulted in increase in gel stiffness to a limited extent, whilst that of whey proteins enriched in tiger nut proteins showed differences in gel stiffness depending on whether cross-linking occurred before or after tiger nut proteins addition. Figure 3 shows that the gel stiffness of whey proteins

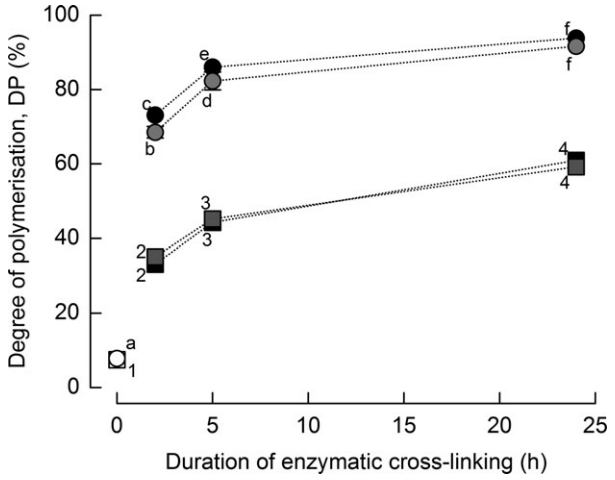

Figure 2 Degree of polymerisation, DP (%) of dairy proteins mixed with tiger nut proteins. CT(circle): non-cross-linked sodium caseinate mixed with tiger nut proteins, WT (square): non-cross-linked whey proteins mixed with tiger nut proteins, CeT(grey circle): enzymatically cross-linked sodium caseinate before the addition of tiger nut proteins, WeT(grey square): enzymatically cross-linked whey protein isolate before the addition of tiger nut proteins, CTe (black circle): enzymatically cross-linked sodium caseinate after the addition of tiger nut proteins, WTe (black square): enzymatically cross-linked whey protein isolate after the addition of tiger nut proteins. Arithmetic means with different letters or figures are significantly different.

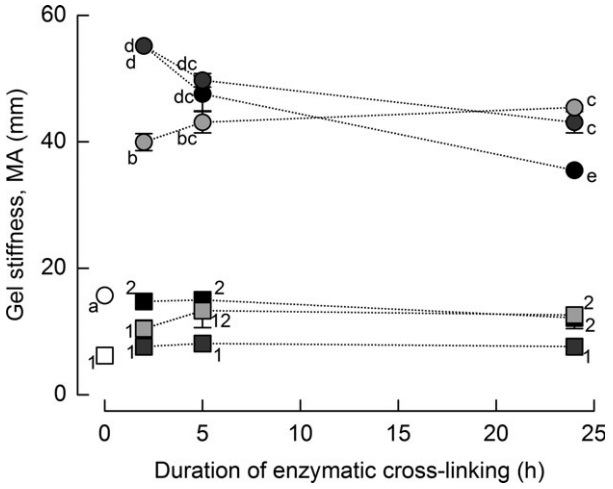

Figure 3 Gel stiffness, MA (mm) of dairy proteins mixed with tiger nut proteins. CT (circle): non-cross-linked sodium caseinate mixed with tiger nut proteins, WT (square): non-cross-linked whey proteins mixed with tiger nut proteins, Ce (light grey circle): enzymatically cross-linked sodium caseinate without tiger nut proteins, We (light grey square): enzymatically cross-linked whey proteins without tiger nut proteins. CeT (deep grey circle): enzymatically cross-linked sodium caseinate before the addition of tiger nut proteins, WeT (deep grey square): enzymatically cross-linked whey protein isolate before the addition of tiger nut proteins, CTe (black circle): enzymatically cross-linked sodium caseinate after the addition of tiger nut proteins, WTe (black square): enzymatically cross-linked whey protein isolate after the addition of tiger nut proteins. Arithmetic means with different letters or figures are significantly different.

after 2 h incubation was approx. 50% higher when cross-linking occurred in the presence of tiger nut proteins (WTe, 14.75 ± 0.64 mm) than that in its absence (WeT, 7.60 ± 0.20 mm). This means that the crude tiger nut proteins extract contributed to increase gel stiffness, which might be relevant for enhancing the textural characteristics of lactic acid fermented TNM enriched in whey proteins (Kizzie-Hayford *et al*., 2017). Further experiments are needed to elucidate the molecular basis for the effects of tiger nut proteins on enzymatically cross-linked dairy proteins.

In this study, it was observed that DP and gel stiffness were not always correlated: on one hand, even though CTe showed a higher DP than CeT systems after 2 h incubation, no significant difference in stiffness of the resulting gels was observed. On the other hand, WTe showed higher gel stiffness than WeT although there was no significant difference in their DPs (WTe, 33.12 ± 0.10%; WeT, 35.09 ± 0.74%). Polymer size and isopeptide content, that is the amount of intermolecular and intramolecular cross-links, are known to affect the molecular flexibility and compactness of polymers, thereby influencing gel stiffness of enzymatically cross-linked dairy proteins (Raak *et al*., 2017).

Conclusion

In this study, enzymatic cross-linking of dairy proteins in the presence of crude tiger nut proteins caused the formation of larger casein polymers and increased the degree of polymerisation. Gel stiffness of acidified products containing whey proteins was higher when cross-linking occurred in the presence of crude tiger nut proteins. The results imply that enrichment of tiger nut aqueous extracts (tiger nut milk) in dairy proteins prior to enzymatic cross-linking and subsequent acidification is promising for improving the textural properties of lactic acid fermented tiger nut milk. Further experiments are needed to establish the molecular basis of the influence of crude or pure tiger nut proteins on the textural properties of enzymatically cross-linked dairy proteins.

Acknowledgments

The authors acknowledge the joint scholarship support received by Nazir Kizzie-Hayford (Grant number A/12/9748) via the Government of Ghana-Ministry of

Education (GOG-MoE) and the German Academic Exchange Services (DAAD). The authors also thank Ajinomoto Foods Deutschland GmBH, Hamburg, Germany, for the generous supply of enzymes used in this study.

Conflict of interest

The authors declare that there is no conflict of interest.

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