RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

RSC Advances

Modification of peptide functionality during enzymatic hydrolysis of whey proteins

Aishwarya Mohan, M. Chinonye Udechukwu, Subin R.C.K. Rajendran, Chibuike C. Udenigwe*

Food Functionality and Health Research Laboratory, Department of Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, NS, B2N 5E3, Canada

*Corresponding author

E-mail: cudenigwe@dal.ca; Phone: +1 902 843-6625; Fax: +1 902 843-1404

Abstract

Peptides derived from food proteins have shown promise as active ingredients for functional food formulation. Due to their reactivity, we evaluated the effects of conditions used for enzymatic hydrolysis of whey protein isolate (WPI) on the functionality of the resulting peptides. Free amino contents were increased when papain and Alcalase were used for WPI hydrolysis, but the proteins (especially β-lactoglobulin) were mostly resistant to pepsin activity. The release of peptides during WPI hydrolysis was associated with increase in ferric reducing capacity, but there were also notable decreases in the redox-active sulfhydryl (SH) groups in the papain and Alcalase reactions. Apparently, the reducing capacity of the hydrolysates was not dependent on their SH contents, which could have been utilised in disulfide formation. Moreover, considering that the WPI contained 1% lactose and other sugars, we observed that intermediate and advanced Maillard reaction products (MRPs) were formed during WPI hydrolysis, and this can directly impact both the reducing capacity and SH content of peptides. MRPs, such as reductones, can be highly antioxidative and possibly contributed to the reducing capacity observed for the protein hydrolysates, even with the depletion of the SH moieties. A model Maillard reaction with arginine, lactose or glucose, and reduced glutathione was used to confirm SH depletion in the presence of MRPs, and this was attributed to nucleophilic reaction with carbonyl derivatives generated during the non-enzymatic glycation reaction. Although this can be an opportunity for generating strong redox-active ingredients, it presents some challenges particularly when the native structure of peptides needs to be conserved for particular biological properties.

Keywords: Bioactive peptides; Reducing capacity; Enzymatic hydrolysis; Sulfhydryl group; Maillard reaction; Whey proteins

1. Introduction

There is a growing evidence of the link between food and health, and particularly the beneficial roles of food-derived bioactive peptides in the management of health aberrations in humans. A myriad of food proteins are known to be precursors of bioactive peptides.[1] However, the development of bioactive peptides into functional food products can be impeded by a number of factors including limited clinical evidence of efficacy in human subjects, and issues with the sensory properties (particularly bitterness), absorption, oral bioavailability and physiological stability of the peptides.[2,3] Moreover, there is a dearth of knowledge of the compatibility of peptide in different food matrices and the possibility of release of peptide derivatives that may alter their biological functions.

In order to produce bioactive peptides, food proteins are subjected to enzymatic hydrolysis at the optimum conditions of the hydrolytic proteases.[1] To date, limited consideration has been given to the possible effects of differences in protease specificity, the optimum reaction conditions (e.g. temperature, pH) and heat inactivation of proteases on the chemical functionality and bioactivity of the resulting peptides within the protein hydrolysates. Although not conducted at extreme conditions, protein hydrolyses are usually extensive and conducted at the hydrolytic optimum condition of the proteases for a prolonged duration (typically 2-5 h). This can encourage side reactions leading to changes in the structure of the generated peptides.[4] Since peptides are more reactive than intact proteins owing to their reactive nucleophilic amino, carboxylic acid and sulfhydryl groups, they are susceptible to physicochemical alterations during food processing[4], and this can alter their biological properties. For instance, we recently observed that the amount of reactive sulfhydryl (SH) group of a whey protein hydrolysate was different from that of the protein precursor (unpublished), and it was not apparent if the final hydrolysate

product contained the peptides in their natural and modified states. Therefore, the objective of this study was to evaluate the effect of three proteases that differ in their specificity and hydrolytic optimum conditions (pH, temperature) on SH functionality and ferric reducing capacity of the peptides resulting from the enzymatic hydrolysis of whey proteins. We also investigated the potential interactions occurring between reactive SH of the peptides and the complex hydrolysate matrix.

2. Materials and Methods

2.1. Whey protein hydrolysis: Bovine whey protein isolate (WPI) powder was purchased from Bulk Barn Foods Ltd. (Truro, NS Canada). A 5% (w/v) suspension of WPI was subjected to hydrolysis with papain from papaya latex (E.C. 3.4.22.2) at E/S ratio of 1:100 (w/w) at 65°C and pH 7.0 for 5 h. The pH was maintained during hydrolysis by the addition of 0.1 M NaOH. Similarly, WPI hydrolysis was conducted with Alcalase, a protease from *Bacillus licheniformis* (≥2.4 U/g), at 55°C and pH 8.3 and pepsin from porcine gastric mucosa (E.C. 3.4.23.1) at 37°C and pH 2.0. Two sets of samples were withdrawn every 30 min during hydrolysis. One sample set was heated to 95°C for 15 minutes to terminate the enzymatic activity, while the other set was placed on ice for the same duration. Thereafter, the whey protein hydrolysates (WPH) were stored at -20°C for further analysis.

2.2. Free amino group determination: Free amino content in the WPH was determined by the O-pthaldehyde (OPA) method reported by Nielsen *et al*.[5] WPH (33 µL) was added to 250 µL of the OPA reagent and absorbance was measured at 340 nm after 2 min. Serine (0.1 mg/mL) was used

as the standard and the amount of free amino group was calculated as milliequivalent serine NH_2/g protein.

2.3. *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*: WPI and the hydrolysates withdrawn at 1, 2, 4 and 5 h were diluted 8 times (or 10 times for pepsin reaction samples) in deionized water. The diluted samples (30 µL) were mixed with 10 µL of sample buffer (containing SDS and β-mercaptoethanol) and incubated at 50°C for 10 min. Then, 20 µL was loaded on a 12% polyacrylamide SDS gel, and electrophoresis was carried out in a Mini-Protean Tetra System cell at 75 V. The gel was washed afterwards, stained with Coomassie Brilliant Blue (1%, R250) and then visualized using Bio-Rad ChemDoc™ MP Imaging system and Image Lab 5.2 software (Hercules, CA, USA).

2.4. Ferric reducing capacity: The ferric reducing antioxidative capacity of the protein hydrolysates was determined as reported by Pownall *et al.*[6], with modifications. The protein hydrolysates were diluted 12.5 times, mixed with equal volume (250 µL) of potassium ferricyanide (1% in 0.2 M phosphate buffer, pH 6.6) and incubated at 50°C for 20 min followed by the addition of 10% trichloroacetic acid. Thereafter, 250 µL of the mixture was combined with 200 µL of deionized water and 50 µL of $FeCl_3$ followed by incubation for 10 min at room temperature and measurement of absorbance at 750 nm. Reduced L-glutathione (GSH) was used to prepare the standard curve and ferric reducing capacity was expressed as mM GSH equivalent.

2.5. Sulfhydryl group (SH) determination: The SH content of the protein hydrolysates was determined as reported by Van der Plancken *et al.*[7], with modifications. Briefly, samples were

diluted 10 times with 0.1 M Tris-glycine buffer (pH 8.3) containing 5% SDS. Thereafter, 10 µL of 5,5′-dithiobis(2-nitrobenzoic acid) (4 mg/mL in the Tris-glycine buffer) was mixed with 1 mL of the diluted samples and incubated at 40°C for 30 min. The mixtures were then centrifuged at 5,000 × g for 5 min and absorbance of the supernatant measured at 412 nm. Extinction coefficient of 14,150 $M^{-1}cm^{-1}$ was used to calculate the SH content, which was expressed as µM SH/mg protein.[7]

2.6. Maillard reaction products (MRPs) determination: The presence of MRPs in the hydrolysates was determined by measuring the absorbance of 10-fold diluted sample solutions at 294 nm and 420 nm, for detecting intermediate and advanced MRPs, respectively. The intermediate products were also detected by measuring fluorescence at excitation and emission wavelengths of 347 nm and 420 nm, respectively.

2.7. Model Maillard reaction: The model reactions were set up as follows: S1 comprising of 5% Arg (w/v), 1% lactose or glucose, and 0.5 mg/mL GSH; S2 comprising of 5% Arg and 0.5 mg/mL GSH; and S3 comprising of 5% Arg and 1% lactose or glucose. Lactose+GSH, glucose+GSH and GSH only were also set up as controls. The reaction set ups were incubated at 65°C for 5 h, similar to the papain reaction condition during WPI hydrolysis. Thereafter, the SH content and intermediate MRPs were determined before and after incubation.

2.8. Statistical analysis: Assays were conducted in triplicates and expressed as mean ± standard deviation. Statistical significance of difference was analysed by one-way analysis of variance followed by Holm-Sidak multiple comparison test using SigmaPlot 12.1 (Systat Software, San

Jose, CA, USA). Relationships between the sample properties was analysed by Spearman's Rank Order Correlation (r_s) using SigmaPlot 12.1.

3. Results and Discussion

3.1. Free amino contents and protein profile during WPI hydrolysis: As shown in Figs. 1A and 1B, the amount of free amino group progressively increased with time for papain and Alcalase reactions, indicating peptide bond cleavage. This process is known to release peptides that are more bioactive than the parent proteins.[1] Alcalase catalysis was found to release slightly higher free amino content than papain, except for the heat-inactivated sample at 5 h, and this can be attributed to the broader specificity of the microbial protease.[8] However, pepsin activity did not result in substantial increase in free amino content and had a degree of hydrolysis as low as 3% (Fig. 1C). These findings are reflected in the SDS-PAGE profiles in Figs. 2A-C. The major whey proteins, β-lactoglobulin (β- LG) and α-lactalbumin (α-LA), were completely hydrolysed by both papain and Alcalase during the 5-h reaction with the appearance of lower MW peptide bands (Figs. 2A & 2B). However, β- LG can be largely resistant to peptic proteolysis[9,10] and has been found to be structurally stable at acidic pH with more internal hydrogen bonds,[10] which can contribute to the resistance to pepsin digestion. This was confirmed in this study as shown in Fig. 2C, which also shows the susceptibility of α-LA to peptic digestion. After hydrolysis, heat inactivation of the proteases at 95°C did not appear to have substantially affected the net free amino content of the resulting hydrolysates, except for the difference ($p<0.05$) observed at 3.5 h for the Alcalase reaction. However, SDS-PAGE revealed apparent differences between heat and cold inactivation especially for products derived from Alcalase and pepsin reactions. In the Alcalase reaction inactivated by heat, there were remnants of the major protein bands at 1 h, and

these disappeared as the reaction progressed to 5 h (Fig. 2B). Conversely, the major protein bands were not observed for cold inactivation, where a lower MW band at 1 h persisted for the hydrolysis duration. It is likely that Alcalase was not inactivated on ice thereby leading to continued reaction and complete hydrolysis of the major whey proteins. This was also observed for the cold-treated pepsin reaction products, which did not show α-LA band even at 1 h (Fig. 2C). The SDS-PAGE also showed that bovine serum albumin was hydrolysed by all the treatments whereas lactoferrin appeared to have remained in the hydrolysate after pepsin reaction. Random and spontaneous structural changes, such as N-terminal cyclisation, can result during heat treatment of peptides leading to loss of the free amino group. For instance, N-terminal glutamine residues are susceptible to heat-induced cyclisation forming pyroglutamate.[4] However, there was no apparent difference in the net amino contents of the heat-inactivated and cold-treated hydrolysates after 5 h of hydrolysis, although this does not preclude the formation of cyclized products which can be unstable and hydrolysed in the aqueous environment.[4]

3.2. Reducing capacity during WPI hydrolysis: Protein hydrolysis has been reported to increase the ferric reducing (antioxidative) capacity of the resulting protein products.[11] As shown in Figs. 3A-C, the hydrolysates showed varying capacities in reducing ferric to ferrous ions measured as the equivalent to GSH activity. Reducing capacity was found to remain mostly unchanged during WPI hydrolysis with Alcalase and pepsin, except for the slight differences observed at the mid hydrolysis stage for the pepsin reaction (Figs. 3B, 3C). Although the latter had extremely low degree of hydrolysis (Fig. 1C), its reducing capacity was close to values obtained for the more extensively hydrolysed product from the Alcalase reaction. Conversely, time-dependent increase in ferric reducing capacity was observed when papain was used for WPI hydrolysis (Fig. 3A)

with the cold-treated hydrolysates showing significantly higher ($p<0.05$) reducing capacity than the heat-inactivated sample with maximum values of 0.86±0.04 and 0.32±0.02 mM GSH equivalent, respectively after 5 h. This clearly indicates the impact of heat-induced protease inactivation, following WPI hydrolysis with papain, on the antioxidative capacity of the resulting peptide products. For the cold-treated protein hydrolysate from papain reaction, reducing capacity was found to initially decrease and later increased after 2 h, suggesting evolution of the redox-active factors with more extensive hydrolysis. Low molecular size has been thought to be an important factor in determining peptide bioactivity, including their reducing capacity.[1,11] Although the small size of peptides can enhance solvent accessibility in bioassay matrices, the more accepted mechanisms of the antioxidative capacity of peptides involve direct scavenging of free radicals and chelation of prooxidant metals.[1,12] Moreover, the reducing capacity of peptides has been linked to their redox-active SH of cysteine residues, which can readily donate electrons in the oxidative system.[12] Therefore, the SH dynamics during WPI hydrolysis is expected to offer insight on possible structural changes that led to differences in the reducing capacity of the protein hydrolysates.

3.3. SH contents during WPI hydrolysis: Protein hydrolysis is thought to result in the cleavage of disulfide linkages in native protein structures, thus, the resulting hydrolysates are expected to possess similar or higher SH content compared to the protein precursors. Primary structures derived from UniProtKB indicate that the major bovine whey proteins contain 23 disulfide linkages (two in β-LG, four in α-LA and 17 in albumin) and two free SH groups in the native structures. Thus, enzymatic proteolysis is expected to release more SH groups as recently reported for corn glutelin, which had decreased number of disulfide linkages after enzymatic

hydrolysis.[11] Conversely, we observed significant decreases ($p<0.05$) in SH contents during WPI hydrolysis with papain and Alcalase (Figs. 4A, 4B). Papain catalysis resulted in a gradual decrease in SH content with up to 50% loss at the end of hydrolysis, compared to hydrolysis with Alcalase where a rapid decrease in SH was observed to occur with a plateau at 2 h. In the latter, SH was found to decrease by up to 50% within the first 30 min and by 6 folds at the end of 5 h. However, WPI hydrolysis with pepsin did not affect the SH content, which remained the same for the hydrolysis duration (Fig. 4C). The SH content reduction was initially thought to be due to oxidation of the exposed cysteine residues leading to inter- and intramolecular disulfide linkages in the whey peptides. This was considered to be a major factor for the reduction of SH groups since disulfide linkages are quite easily formed in proteins and peptides even under mild conditions.[4] Nevertheless, since the SH content did not change when pepsin was used for protein hydrolysis, it becomes clear that SH loss was higher in samples with higher amino group content (i.e. higher degree of hydrolysis; Figs. 1A-C) and for the reactions catalysed by proteases with optimum conditions at higher temperatures (i.e. 65°C for papain and 55°C for Alcalase).

Heat inactivation of the proteases after hydrolysis did not cause notable changes in the SH content of WPH during hydrolysis. In fact, cold treatment after hydrolysis was observed to result in slight decreases in the reactive SH at mid-to-late stages of hydrolysis with Alcalase (Fig. 4B), indicating possible disulfide formation. It is also apparent from the findings that the SH content dynamics have no major bearing on the reducing potential (Fig. 3A-C), especially for the protein hydrolysate generated with papain. The SH groups are theoretically liberated from disulfides during protein hydrolysis, but found to decrease in this case (Fig. 4A) even with increase in the reducing capacity (Fig. 3A). For all the samples combined, Spearman Rank Order correlation indicated a negative relationship between the SH and reducing capacity (r_s= - 0.363,

P=0.005, n=58).This suggests that factors in addition to the SH of cysteine residues are contributing to the total reducing capacity of the protein hydrolysates.

3.4. MRPs formation during enzymatic WPI hydrolysis: Maillard reaction can occur when heat is applied to a mixture of reducing sugars and amino acids, peptides or proteins. The non-enzymatic glycation reaction can also occur under a wide range of conditions, even at room temperature, albeit at slower rates.[13] Heat treatment of whey protein has been reported to lead to lactosylation of β-LG.[14] Considering that the WPI used in this study contains 1% (w/w) lactose and other sugars, we evaluated MRPs formation during protein hydrolysis by absorbance and fluorescence measurements. As shown in Figs. 5A-C, absorbance values of the hydrolysates at 294 nm increased over the 5-h duration for papain and Alcalase reactions, and not for the pepsin reaction, suggesting the formation of intermediate MRPs. This was then confirmed by the observation of a similar trend in the production of fluorescence-active factors, with papain catalysis resulting in the highest amounts of the MRPs (Figs. 5D-F). The increase in intermediate MRPs was slightly ($p<0.05$) affected by heat inactivation of proteases at various time points during hydrolysis, and this was also observed for the pepsin-catalysed reaction. It is noteworthy that the evolution of intermediate MRPs is inversely related to the SH group contents of the protein hydrolysates derived from papain. As expected under the acidic condition (pH 2.0), WPI hydrolysis with pepsin did not show detectable changes in the absorbance and fluorescence values over time, suggesting limited MRPs formation. A recent study reported that the presence of glucose, similar in amount to the sugar content of the WPI, resulted in the loss of SH groups of serum albumin due to oxidation after 1-week incubation at 37°C.[15] In addition, the MRPs formation dynamics during WPI hydrolysis appear consistent with the observed pattern in ferric

reducing capacity (Figs. 3A-C). It is possible that the MRPs contribute to the redox activity of the hydrolysates. Previous studies have reported enhanced reducing capacity with the formation of MRPs from protein hydrolysates and peptides.[16,17,18,19] However, correlation analysis revealed no significant relationship ($P>0.05$) between reducing capacity and each of the three MRPs measurements in this study, except for the pepsin-catalysed fluorescence active MRPs, which showed a strong positive relationship (r_s=0.805, P=0.000, n=60) even with limited evidence of progressive MRPs formation in the treatment. In a previous study, MRPs produced from whey proteins at lower pH were reported to possess weak ferric reducing capacity.[18] However, we could not explain the low reducing capacity that was observed for the heat-inactivated hydrolysate from papain reaction (Fig. 3A) based on reaction pH. The reducing capacity of MRPs derived from whey proteins has been mostly attributed to a group of redox-active reductones, although much is still unknown about the identity of the compounds.[18]

Advanced MRPs contribute to the brown colour of thermally produced food. As shown in Fig. 5G, absorbance measurement at 420 nm showed a time-dependent increase in the advanced MRPs when papain was used for WPI hydrolysis. However, the Alcalase and pepsin reactions did not show a significant increase in absorbance (Figs. 5H, 5I). This was unexpected particularly for the Alcalase reaction considering that it was conducted at a more alkaline pH, which is known to promote Maillard reaction, compared to the papain and pepsin reactions. Wang *et al.* reported that MRPs from WPI produced at alkaline pH had higher antioxidant capacities than MRPs generated at neutral or slightly acidic pH.[18] At the early stage of Maillard reaction, the pathway for the Amadori products at pH>7 results in the formation of redox-active reductones, whereas neutral to acidic conditions support the formation of fission products and Schiff's bases that have lower reducing capacity compared to the reductones.[20] Although pH is

an important factor that determines the occurrence and progression of Maillard reaction, temperature can also play an important role.[20] The higher temperature for papain reaction in this study appears to have facilitated Maillard reaction even at neutral pH during enzymatic hydrolysis of WPI.

3.5. Depletion of SH during MRPs formation: A model MRP system containing arginine (source of the amino group), lactose or glucose (reducing sugar) and GSH (for the SH group) was used to evaluate the plausible interactions occurring during the papain catalysed hydrolysis of WPI, particularly the relationship between MRP formation and the SH contents.

3.5.1. SH contents: Among the seven set ups, S3 (arginine+lactose) or (arginine+glucose) had no SH group and was used as the negative control (Fig. 6A). The SH group in S1 (arginine+lactose+GSH) or (arginine+glucose+GSH) was found to be depleted by 5 and 11 folds, respectively; the latter was found to decrease to the baseline value observed for S3 (no GSH) after the 5-h incubation. Notably, the SH content of S2 (arginine+GSH, no MRPs) did not change after the reaction. The findings clearly demonstrate the existence of a possible interaction of the peptide SH group with components of the Maillard reaction system, in addition to possible disulfide linkage formation. Furthermore, the reactive SH content remained unchanged after the reaction for lactose+GSH and glucose+GSH, indicating the absence of direct SH interaction with the reducing sugar under the experimental conditions.

3.5.2. Intermediate MRPs: The presence of free amino group of arginine and the carbonyl group of lactose or glucose at 65°C apparently initiated Maillard reaction in both S1 and S3 considering their higher fluorescence values compared to S2 (arginine+GSH), where Maillard reaction did not occur due to the absence of the reducing sugar (Fig. 6B). Although both S1 and

S3 had arginine and the reducing sugars, the amount of fluorescence-active intermediate MRPs was significantly higher ($p<0.05$) in S3 compared to S1. Furthermore, S1 with arginine+lactose+GSH had higher MRPs compared to the reaction with glucose, indicating possible differences in their MRPs and their reactivity with SH. As expected, there were low levels of fluorescence active compounds in the control (lactose+GSH, glucose+GSH and GSH only) model experiments. This further supports the possible direct interaction between the MRPs and GSH (SH in particular) leading to the depletion of both.

3.5.3. Interaction between the SH and MRPs: Taken together, the decreases in the SH group and intermediate MRPs in S1 can be due to nucleophilic reaction between the free SH group of GSH and the carbonyl group of early MRPs (Amadori products) forming thiohemiacetals and thioacetals.[21] Consequently, a portion of the carbonyl groups of the early MRPs in S1 would have become unavailable for Maillard reaction progression leading to the decrease in fluorescence active MRPs formation compared to S3 (Fig. 6B). Moreover, the depletion of the SH content of S1 indicates other plausible interactions involving other electrophilic compounds in the matrix such as intermediate MRPs, which can also readily react with the SH group.[21]

3.6. Is Maillard reaction contributing to SH depletion during enzymatic hydrolysis of whey proteins? Findings from the Maillard reaction model can provide insight on the structural changes that occurred during enzymatic WPI hydrolysis with the proteases. The gradual and consistent SH decrease observed with papain could be due to heat-induced formation of disulfide linkages and also interaction of the SH group with early, intermediate and advanced MRPs produced during hydrolysis as shown in Figs. 5A, 5D and 5G. WPI hydrolysis with papain was

conducted at neutral pH and this is expected to favour 1,2-enolisation of N-glycosylamine.[20] This pathway would be directed to the formation of 2-furaldehydes, which also have dicarbonyl intermediates, the 3-deoxyosones. The repertoire of carbonyl compounds are highly susceptible to reacting with the nucleophilic SH groups of (poly)peptides during whey protein hydrolysis. Moreover, it is apparent that direct nucleophilic attack of the SH moiety on the sugar carbonyl group is less likely as both intermediate and advanced MRPs were constantly detected throughout the papain reaction, and as demonstrated by the model reaction (Fig. 6A). Otherwise, Maillard reaction would have been arrested or not progressed at the observed rate during hydrolysis as earlier reported..[21] Despite the higher nucleophilicity of SH compared to the amino group, theoretically favouring the reaction of carbonyls with the former, Maillard reaction still progressed and this can be attributed to the relatively high amounts of the amino group (compared to SH) released during enzymatic WPI hydrolysis. However, the alkaline pH is expected to enable reducing sugars to exist in the open chain form.[20] Consequently, for the papain reaction, the SH group of the peptides can interact with the reactive carbonyl of the early and intermediate MRPs to form thio(hemi)ketals or thio(hemi)acetals as earlier proposed.[21] The sharp decrease in SH content observed during the initial 30 min of Alcalase reaction (Fig. 4B) can be due to the rapid formation of carbonyl-SH adducts that would not proceed to Maillard reaction. This can possibly explain the lower amounts of intermediate (Figs. 5B, 5E) and advanced MRPs (Fig. 5H) formed in the Alcalase reaction.

4. Conclusion

Findings from this study showed that the enzymatic hydrolysis process used to obtain peptides from WPI can lead to peptide derivatization particularly resulting in the loss of their SH groups.

During WPI hydrolysis, the SH group was found to vary over the 5-h hydrolysis duration mostly in samples that were subjected to different temperatures, pH and proteases. The complex and highly reactive hydrolysate matrix makes it likely to have inter- and intramolecular interactions of the released peptides, and also between the peptides and other components of the protein hydrolysates. Contrary to the accepted antioxidative mechanism, the ferric reducing capacity of the protein hydrolysates could not be attributed to the contents of their redox-active SH groups and, in fact, an inverse relationship was observed especially when papain was used for WPI hydrolysis. Maillard reaction was observed to have occurred during enzymatic hydrolysis of the whey proteins, and a model system confirmed the loss of the SH group possibly due to interactions with reactive carbonyl derivatives. Some MRPs (especially reductones) are highly redox-active and most likely contributed to the observed ferric reducing capacity of the whey protein hydrolysates, although positive correlation was only observed between the two properties for pepsin reaction products. The structural changes are more attributable to the differences in protease specificities in releasing amino groups and long-term exposure to processing conditions (temperature, pH) during hydrolysis. Moreover, there are marked differences in electrophoretic profiles due to the short-term heat or cold treatments used to inactive proteolytic activities, which can also contribute to differences in the hydrolysate properties. However, we could not explain what led to the low reducing capacity of hydrolysates from the heat-inactivated papain reaction. To our knowledge, this is the first report of SH group depletion (loss of functionality) and MRPs formation (gain of reducing capacity), and to establish a connection between the two, during enzymatic hydrolysis of food proteins for the purpose of bioactive peptide production. Due to their sensitive structures and reactive functionalities, thermal processing of peptides for food

applications is challenging and needs to be optimized for successful translation of bioactive peptides into functional foods with human health benefits.

Acknowledgement. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a Discovery Grant, and Canada Foundation for Innovation (CFI) and Nova Scotia Research and Innovation Trust Fund (NSRIT) through an Infrastructure Grant. A.M. and S.R.C.K.R are recipients of the Nova Scotia Research and Innovation Graduate Scholarship.

References

1. C. C. Udenigwe and R. E. Aluko. *J. Food Sci.*, 2012, 77, R11.
2. D. Bouglé, and S. Bouhallab. *Crit. Rev. Food Sci. Nutr.*, 2015, doi: 10.1080/10408398.2013.873766.
3. C. C. Udenigwe. *Trends Food Sci. Technol.*, 2014, 36, 137
4. F. Van Lancker, A. Adams, and N. De Kimpe. *Chem. Rev.*, 2011, 111, 7876.
5. P. M. Nielsen, D. Petersen, and C. Dambmann. *J. Food Sci.*, 2001, 66, 642.
6. T. L. Pownall, C. C. Udenigwe, and R. E. Aluko. *J. Agric. Food Chem.*, 2010, 58, 4712.
7. I. Van der Plancken, A. Van Loey, and M. E. Hendrickx. *J. Agric. Food Chem.*, 2005, 53, 5726.
8. N. S. Oh, H. A. Lee, J. Y. Lee, J. Y. Joung, K. B. Lee, Y. Kim, and S. H. Kim. *J. Dairy Sci.*, 2013, 96, 4899.
9. D. G. Schmidt, R. J. G. M. Meijer, C. J. Slangen, and E. C. H. Beresteijn. *Clin. Exp. Allergy*, 1995, 25, 1007.

10. I. M. Reddy, N. K. Kella, and J. E. Kinsella. *J. Agric. Food Chem.*, 1988, 36, 737.

11. X. Q. Zheng, J. T. Wang, X. L. Liu, Y. Sun, Y. J. Zheng, X. J. Wang, and Y. Liu. *Food Chem.*, 2015, 172, 407.

12. B. Hernández-Ledesma, A. Dávalos, B. Bartolomé, and L. Amigo. *J. Agric. Food Chem.*, 2005, 53, 588.

13. Belitz, H. D., Grosch, W., & Schieberle, P. Food Chemistry, 4th Revised and Extended Edition. Springer-Verlag Berlin Heidelberg, Germany, 2009, 1070 pages.

14. J. Leonil, D. Molle, J. Fauquant, J.L. Maubois, R.J. Pearce, and S. Bouhallab, S. *J. Dairy Sci.*, 1997, 80, 2270.

15. Neelofar, J. Ahmad and K. Alam. *RSC Adv*., 2015, 5, 63605.

16. Y. Joubran, A. Moscovici, and U. Lesmes. *Food Funct.,* 2015, 6, 1229.

17. Q. Liu, B. Kong, J. Han, C. Sun, and P. Li. *Food Struct.*, 2014, 1, 145.

18. W. Q. Wang, Y. H. Bao, and Y. Chen. *Food Chem.*, 2013, 139, 355.

19. D. Stanic-Vucinic, I. Prodic, D. Apostolovic, M. Nikolic, and T. C. Velickovic. *Food Chem.*, 2013, 138, 590.

20. S. I. Martins, W. M. Jongen, and M. A. Van Boekel. *Trends Food Sci. Technol.*, 2000, 11, 364.

21. M. Friedman and I. Molnar-Perl. *J. Agric. Food Chem.*, 1990, 38, 1642.

List of Figures

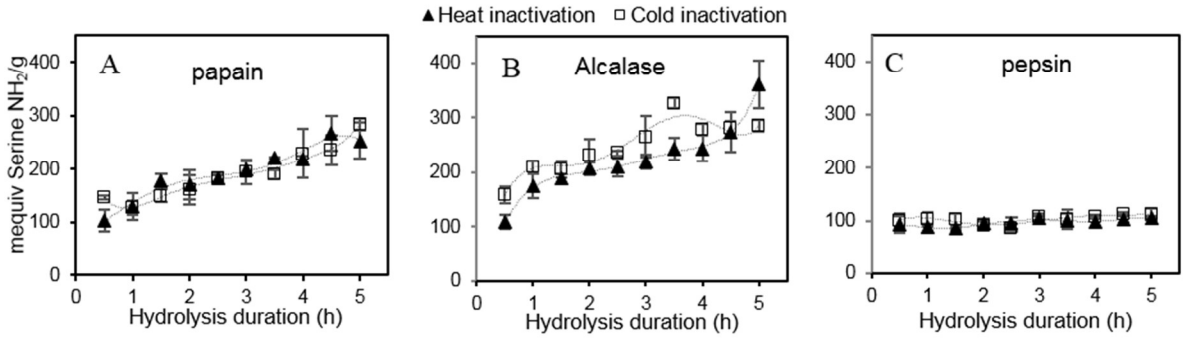


Fig. 1.

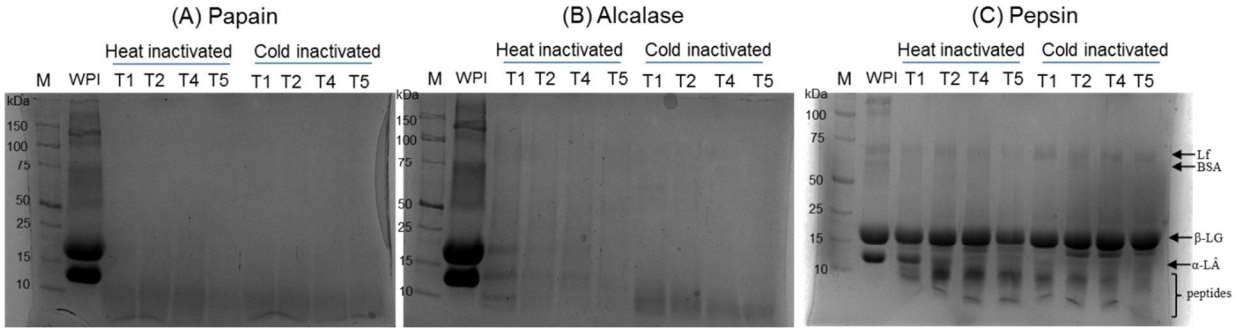


Fig. 2.

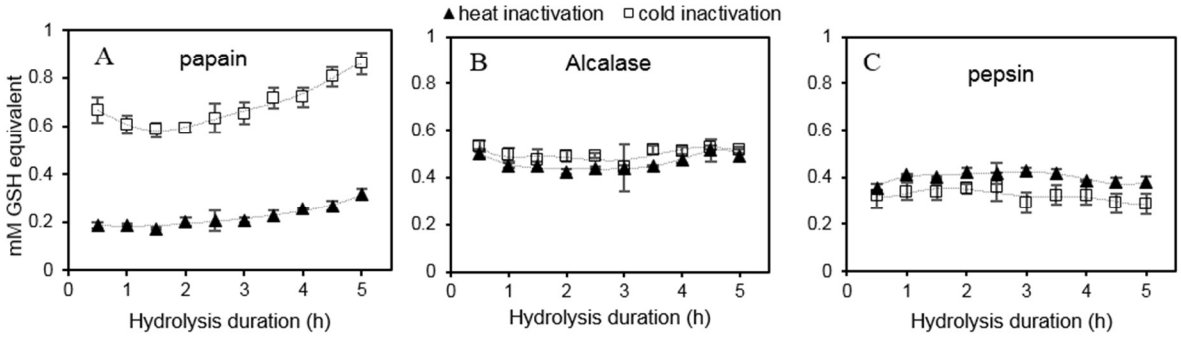


Fig. 3.

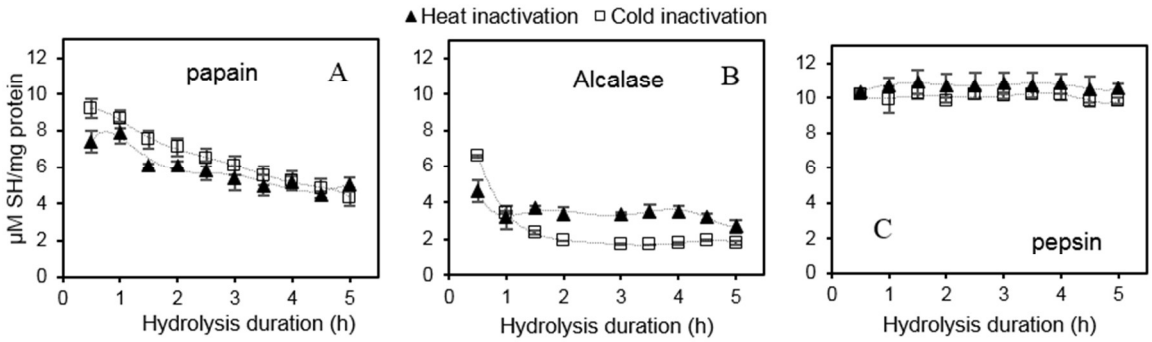
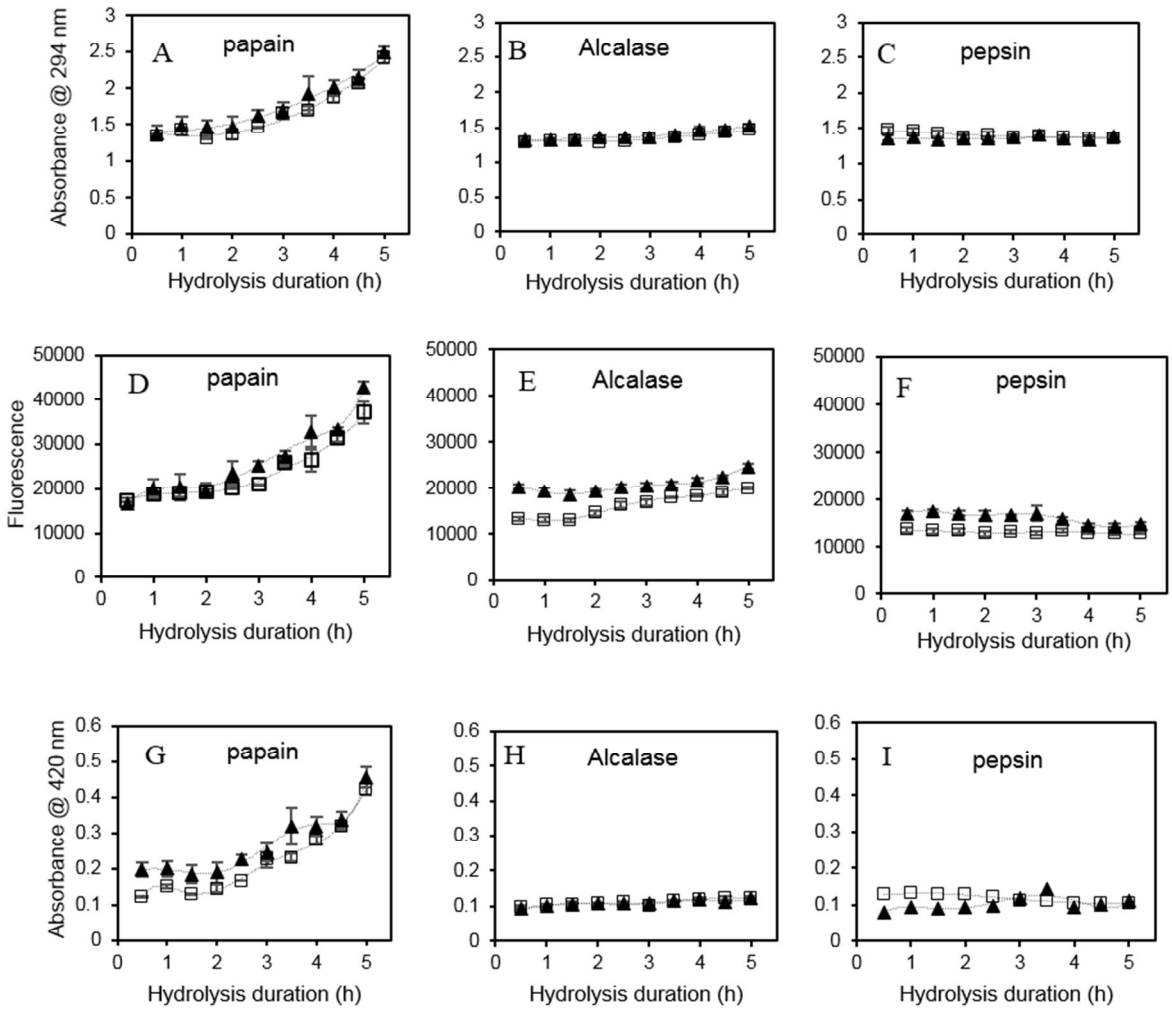


Fig. 4.

512



513

514

515 Fig. 5.

516

517

518

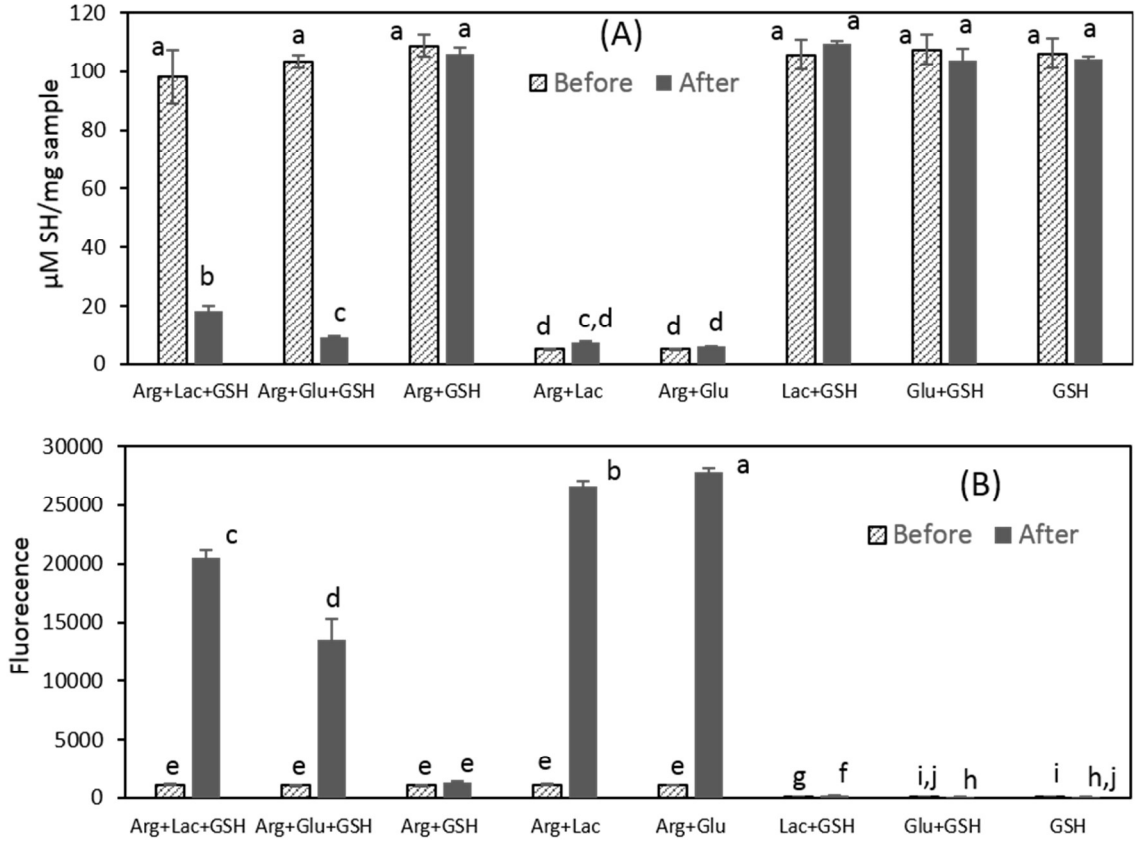
519

520

521

522

523



524

525

526 Fig. 6.

527

528