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Revised and submitted Word version of O'Loughlin et al. (2013) Food Chem, 141(3), 2334-2342. doi: 10.1016/j.foodchem.2013.05.056

Whey Protein Isolate Polydispersity Affects Enzymatic Hydrolysis Outcomes

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ABSTRACT

The effects of heat-induced denaturation of whey protein isolate (WPI) on the enzymatic breakdown of α-La, caseinomacropeptide (CMP), β-Lg A and β-Lg B were observed as hydrolysis proceeded to a 5 % degree of hydrolysis (DH) in both unheated and heat-treated (80 ºC, 10 min) WPI dispersions (100 g L^{-1}). Hydrolysis of denatured WPI favoured the generation of higher levels of free essential amino acids; lysine, phenylalanine and arginine compared to the unheated substrate. LC-MS/MS identified 23 distinct peptides which were identified in the denatured WPI hydrolysate – the majority of which were derived from β-Lg. The mapping of the detected regions in α-La, β-Lg, and CMP enabled specific cleavage points to be associated with certain serine endo-protease activities. The outcomes of the study emphasise how a combined approach of substrate heat pre-treatment and enzymology may be used to influence proteolysis with attendant opportunities for targeting unique peptide production and amino acid release.

KEYWORDS: heat-treatment; whey protein isolate; enzymatic hydrolysis; peptide analysis; essential amino acids

1. INTRODUCTION

The functional applications for whey proteins, both biological and technological, are of significant current interest. Whey as a commercial ingredient can be concentrated as whey protein isolate (WPI) which is high in branched chain amino acids, a source of health benefits (Marshall, 2004; Nilsson, 2007). The amino acid profile of whey also contains higher levels of essential amino acids compared to most other proteins (Schaafsma, 2006). The desire to find novel functional attributes to whey has led to the development of processes other than standard WPI or WPC production. One such process, enzymatic hydrolysis, has been much utilised to improve the functional characteristics of the parent protein (Gauthier & Pouliot, 2003).

The susceptibility of whey proteins to heat-induced structural changes has the potential to alter hydrolysis by exposing previously inaccessible amino acid residues to cleavage (Mullally, Mehra & FitzGerald, 1998; Reddy, Kella & Kinsella, 1988). In a mixed protein material the normal course of heat-induced denaturation is affected by intra- and inter-protein interactions (Dalgleish, Senaratne & Francois, 1997; Havea, Singh & Creamer, 2002) and, with heat-treatment, the covalent linkages which contribute to stabilise tertiary structures will be affected (Bowler, 2007). The continued application of heat can result in irreversibly aggregated protein which is prevented from returning to the native form by thermodynamic barriers (Kauzmann, 1959; Mulvihill & Donovan, 1987).

The kinetics of enzymatic hydrolysis may also benefit from improved accessibility of active sites in proteins. The use of pre-hydrolysis heat-treatments to induce structural changes in the protein substrate can influence hydrolysis reaction rates. Therefore, endo-protease activity can be affected by the degree of protein denaturation (Panyam & Kilara, 1996) where a positive influence on hydrolysis reaction rates has been demonstrated in individual protein fractions (Guo, Fox, Flynn & Kindstedt, 1995; Schmidt & van Markwijk, 1993) and

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heterogeneous concentrates (Kim et al., 2007a). Kim et al. (2007a) reported that a pre-hydrolysis heat-treatment (100 ºC for 10 min) increased peptic and tryptic hydrolysis of WPC, while heat-treatments > 70 ºC for 15 min also have a positive effect on the rate of hydrolysis of WPI that may be associated with differing aggregation phenomena (O'Loughlin, Murray, Kelly, FitzGerald & Brodkorb, 2012).

Changing the conformation of the substrate pre-hydrolysis leads to a novel hydrolysis reaction which differs from that obtained with un-treated / native protein substrate (Meisel, 1998). The folded structure of native whey proteins provides resistance to hydrolysis by digestive enzymes (Schmidt, Meijer, Slangen & Van Beresteijn, 1995) – a feature that is increasingly exploited for techno-functional and bio-activity opportunities. Previous studies have shown that controlled proteolysis of heat-denatured whey proteins can increase the techno-functionality of WPI (Mutilangi, Panyam & Kilara, 1996) and improve the bio-functionality of WPC (Kim et al., 2007b). Hydrolysis of the proteins will, in many cases, also release free amino acids as well as peptides and many of these amino acids may have important nutritional and physiological benefits. Free amino acids perform many functions including modulation of insulin release (van Loon, Kruijshoop, Menheere, Wagenmakers, Saris & Keizer, 2003) and induction of human growth hormone release (Isidori, Lo Monaco & Cappa, 1981).

The objective of this study was to determine how the combined approach of substrate heat pre-treatment and enzymology may influence proteolysis particularly with respect to the opportunity to target unique peptide production and amino acid release. In light of the effects of aggregation phenomena previously encountered (O'Loughlin et al., 2012) it was opportune to examine how heat-induced modification of WPI affected the molecular level properties of enzymatic hydrolysates obtained following incubation with Corolase® PP (E.C. 3.4.21.4). This porcine pancreatic enzyme preparation is well documented for its high proteolytic

activity in whey due to its three serine-protease activities; trypsin, chymotrypsin and elastase, as well as exo-peptidases such as carboxypeptidase (CYP) zymogens CYP A and CYP B (Mullally, O'Callaghan, FitzGerald, Donnelly & Dalton, 1994) which favour the production of non-bitter hydrolysates (Uhlig, 1998). The study was also an opportunity to determine how specific cleavage points associated with certain serine endo-protease activities present in Corolase® PP were affected by the polydisperse conditions created by heat-induced changes to WPI prior to hydrolysis.

2. MATERIALS AND METHODS

2.1 Materials

Whey Protein Isolate (Isolac®) was provided by Carbery Food Ingredients, (Ballineen, Co. Cork, Ireland). The powder contained 89.3 % protein by Kjeldahl [*N* x 6.38 (Merrill & Watt, 1973)] comprising 56.5 % β-Lactoglobulin (β-Lg) (29.3 % β-Lg A, 27.2 % β-Lg B), 14.3 % α-Lactalbumin (α-La), 10.3 % glycosylated caseinomacropeptide (CMP), 8 % non-glycosylated CMP, and 1.7 % bovine serum albumin (BSA). Denatured material amounted to 15.4% of total protein as determined by urea denaturing RP-HPLC.

The digestive-enzyme complex Corolase® PP (E.C. 3.4.21.4.) was sourced from AB Enzymes GmbH, Darmstadt, Germany, and had a minimum activity of 2,500 units with haemoglobin as a substrate (UHb) and 220,000 Lohlein-Volhard units (LVU) g^{-1} at pH 8.0.

2.2 Heat treatment and characterization of WPI solutions

WPI (33.59 g) was dissolved in 300 mL dH_2O (giving a 100 g L^{-1} protein dispersion) and the solution allowed to hydrate overnight at 4 ºC. A thermostatically controlled water bath was brought to 80 ºC and allowed to pre-equilibrate for 30 min. Samples were adjusted

to pH 8 with NaOH and dispersions for heat-treatment were then heated for 10 min under stirring followed by cooling in ice-water.

The conformational state of the major proteins in WPI were characterised by high performance liquid chromatography (HPLC) and the loss in native protein quantified as previously described (O'Loughlin et al., 2012).

2.3 Enzymatic hydrolysis of WPI solutions

Degree of hydrolysis (DH) represents the number of peptide bonds cleaved (h) as a percentage of total peptide bonds (h_{tot}) and can be related to the consumption of base as hydrolysis releases protons according to Eqn. 1 (Adler-Nissen, 1986):

$$\mathrm{DH}\% = 100 \times \mathrm{B} \times N_{\mathrm{B}} \times \frac{1}{\alpha} \times \frac{1}{\mathrm{MP}} \times \frac{1}{h_{\mathrm{tot}}} \qquad \text{(Eqn. 1)}$$

Where: B is the volume of base, N_B is the normality of the base, $1/\alpha$ is the average degree of dissociation of α-NH_2 residues (= 1.13 at pH 8, 50 ºC), MP is the mass of protein and h_{tot} is the total number of peptide bonds given in meq g^{-1} protein based on Kjeldahl nitrogen (N) and a conversion factor (f_N) of 6.38. The h_{tot} for whey protein concentrates is 8.8.

For hydrolysis experiments, 300 mL solutions of WPI (100 g L^{-1} protein) were hydrolysed to a target DH of 5 % with Corolase® PP. Hydrolysis was performed at an enzyme:substrate ratio of 1:100 (w w^{-1}) on a protein equivalent basis and the hydrolysis conditions were 50 ºC and pH 8 which were controlled throughout the reaction. The pH was controlled by titration with 2 N NaOH using a Metrohm 842 Titrando dosing unit (Metrohm

Ltd., Herisau, Switzerland) and the reaction was agitated utilising an over-head stirrer at 300 rpm. The reaction was terminated by heating the enzyme at 85 ºC for 20 min. All hydrolysis experiments were conducted in triplicate.

The hydrolysates were then lyophilized prior to molecular level analysis. For analysis of the changes in protein distribution and peptide formation taking place during the course of hydrolysis, aliquots of the reaction were withdrawn at different DH points and the enzyme activity was stopped by dilution in 0.1 % (v v^{-1}) trifluoroacetic acid (TFA) instead of heat-inactivation.

2.4 Solubility and high performance liquid chromatographic analysis of hydrolysates

Solubility was determined through centrifugation and subsequent protein determination of supernatant according to the method of O'Loughlin et al. (2012). Solubility experiments were conducted in duplicate.

Reversed-phase HPLC profiles were generated utilizing a Phenomenex Jupiter C18 (4.6 mm x 250 mm, 5 µM, 300 Å, Phenomenex, Cheshire, UK) column under linear gradient elution conditions; Solvent B: from 0 % to 100 % in 30 min, 100 % for 5 min, 100 % to 0 % in 5 min, and 0 % for 5 min at a flow-rate of 1 mL min^{-1} where Solvent B is 80 % acetonitrile (MeCN) containing 0.1 % TFA (v v^{-1}). Diluted protein solutions (20 µL of 2.5 mg mL^{-1}) were loaded onto the column.

Size exclusion chromatography (SEC) was carried out on a TSK Gel G2000SW, 7.8 mm x 600 mm, (Tosoh Bioscience GmbH, Stuttgart, Germany) on an isocratic gradient of 30 % MeCN containing 0.1 % TFA (v v^{-1}) with a run-time of 60 min. The samples were diluted in 30 % MeCN containing 0.1 % TFA (v v^{-1}). 20 µL of 2.5 mg mL^{-1} Protein solutions (20 µL of 2.5 mg mL^{-1}) were also loaded onto the column. Commercial α-La, β-Lg A and B, BSA, lactoferrin, and CMP (Sigma-Aldrich, Dublin, Ireland) were used as standards with

Ribonuclease A, Cytochrome C, Aprotinin, Bacitracin, His-Pro-Arg-Tyr, Leu-Tyr-Met-Arg, Bradykinin, Leu-Phe, Tyr-Glu (Bachem AG, Bubendorf, Switzerland) used as molecular weight standards. Samples and standards were pre-filtered through 0.22 μm low protein binding membrane filters (Sartorius Stedim, Surrey, UK) prior to application to the column. All solvents were filtered under vacuum through a 0.45 μm high velocity filters (Millipore (UK) Ltd., Durham, UK).

2.5 Amino acid analysis and peptide analysis

Free amino acid analysis was performed using a Jeol JLC-500/V AminoTac^{TM} amino acid analyzer fitted with a Jeol Na^+ high-performance cation-exchange column (Joel (UK) Ltd., Garden city, Herts, UK). De-proteination was accomplished with 10 % (v v^{-1}) trichloroacetic (TCA) followed by centrifugation (Eppendorf 5417 R, Eppendorf AG, Hamburg, Germany) at 20,000 x g for 20 min. The supernatant was extracted and amino acid detection was accomplished through visible adsorption photometry after separation using ion-exchange. Ninhydrin derivatization with norvaline was used as an internal standard. For total amino acid analysis samples were heated in 6 M HCL for 23 hrs under reflux at 105 ºC before application to the JLC-500/V analyzer. The amino acids glutamine and asparagine are not determined individually and tryptophan is not determined at all.

For LC-MS/MS analysis, each sample was dissolved in 3 % MeCN, 0.1 % formic acid (Buffer A) at a concentration of 2.5 mg mL^{-1} and centrifuged at 5,000 x g for 5 min. The supernatant was purified using a C18 resin (ZipTip®, Millipore, MA, USA), dried and re-suspended in Buffer A for LC-MS/MS. Samples were analysed in triplicate on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA, USA) connected to a Dionex Ultimate 3000 RSLCnano chromatography system (Thermo Scientific, Waltham, MA, USA). MS was performed over the range 300 – 2000 m/z. MS/MS was performed on

the seven most abundant precursor ions, with a dynamic exclusion of 90 secs. Raw data from the MS/MS files were processed using Mascot version 2.3 (Matrix Science Inc., MA, USA) against an in-house bovine milk database, with no enzyme specificity, to determine the peptides present. A precursor ion tolerance was set to 20 ppm and product ion tolerance to 0.8 Da. Variable modification of methionine oxidation was employed. This in-house database contains 17 bovine protein sequences (N) and with a false discovery rate, or significance threshold, of 0.05 (P), where peptides with an ion score of -10 x log(P/N) = 25 or greater were considered present in the sample. As each sample was analysed in triplicate, only peptides present in two or more of the analyses were accepted.

Peptides detected by LC-MS/MS in each sample were mapped to their corresponding parent protein. Determination of cleavage points which could be attributed to a particular endo-protease in Corolase® PP was accomplished through the use of the Protein Digest tool of the Institute for Systems Biology (Seattle, WA, USA) and the 'generate-peptides' utility in the Crux suite of programs (Park, Klammer, Kall, MacCoss & Noble, 2008).

2.6 Statistical analysis

Statistical analysis of the results from this study was accomplished through the use of Minitab 15 software (Minitab Inc., State College, PA, USA). Analysis of variance (ANOVA) was performed followed by Tukey test to determine the statistical difference of means where the significance level was established for $P < 0.05$. For analysis between two particular sample sets a t-test was performed. Data is presented herein as mean ± SD.

3. RESULTS & DISCUSSION

3.1 Changes in the protein distribution during hydrolysis of un-heated and heat-treated WPI

A previous paper (O'Loughlin et al., 2012) highlighted how the type of aggregate formed in the course of WPI heat-treatment may influence the rate of enzymatic hydrolysis. This study addressed how these physico-chemically modified polydispersions changed the proteolytic pathway of individual proteases and altered free amino acid composition. Peptide and amino acid analysis of hydrolysates was used to map proteolytic cleavage patterns by a commercial enzyme preparation and discern altered amino acid profiles.

The initial un-heated WPI contained ~ 69 % soluble material in the molecular weight range 30 – 10 kDa – a range that represents the major native whey protein fractions β-Lg and α-La (Fig. 1 A). However, heat-induced (80 ºC, 10 min) denaturation of WPI reduced the solubility substantially from 89 % (Fig. 1 A) to 31 % (Fig. 1 B). A significant ($P < 0.01$) decrease of 83 % in soluble material between 30 – 10 kDa during hydrolysis of non-heat treated (NHT) WPI was accompanied by increases of 29 % and 17 % in the M_w < 1 kDa and > 30 kDa, respectively, while the amount of insoluble material decreased to 4 % (Fig. 1 A). In the corresponding heat-treated (HT) WPI, soluble material concentrations in the various M_w categories amounted to 11 % (30 – 10 kDa) and 39 % (< 1 kDa), with that of the 10 – 5 kDa decreasing unlike the hydrolysate of the NHT WPI (Fig. 1). The higher percentage of material > 30 kDa was attributed to aggregates not hydrolysed at the 5 % DH level. A shortcoming to utilisation of the heat-treated substrate is the relatively large amount of residual insoluble material (46 %) in the matrix at the 5 % DH point. This constrains the relative concentration of material < 1 kDa, when taken as a percentage of the total protein distribution, to 21 % compared to 27 % for the corresponding NHT WPI hydrolysate (Fig. 1). Thus, while hydrolysis of HT WPI preferentially favours the generation of small M_w (< 1 kDa) soluble material over that of the NHT WPI (as determined via SEC, where the M_w distribution of soluble material alone is shown in Supplementary Figure 1), there remains an issue with the high overall sample insolubility (Fig. 1).

The heat-inactivation (80 ºC, 20 min) of the hydrolysates (at 5 % DH) did not induce additional denaturation of the system as determined utilising chromatography and solubility (results not shown). Also, the increase from 50 to 80 ºC (away from Corolase® PP optimum temperature) of the 10 mL hydrolysate aliquot (accomplished in ~ 2.5 min) did not result in any initial increase in hydrolysis rate.

During the course of experimentation, the individual protein components of WPI possessed different susceptibilities to proteolysis depending on prior exposure to heat (Fig. 2 A and B). Prior heat-treatment of the substrate reduced the native protein content of β-Lg A, β-Lg B and α-La by approximately 80 %, 55 % and 58 %, respectively as determined by RP-HPLC (Fig. 2 A and B). Only native-like protein fractions were determined via this methodology. From Figure 2 B, CMP was largely unaffected by heat, given the limited reduction, approximately 6 %, in its native structure. This reduced thermal lability of CMP compared to the other protein components of whey has been noted previously under similar conditions (O'Loughlin et al., 2012). The order of hydrolysis at 1 % DH of the individual fractions from the NHT WPI reaction was: β-Lg A (39 %) > β-Lg B (21 %) > α-La (9 %) (Fig. 2 A). Reducing SDS-PAGE also demonstrated this increased susceptibility to hydrolysis by Corolase® PP of β-Lg in comparison to α-La (Supplementary Figure 2). This hydrolytic susceptibility to Corolase® PP is roughly in agreement with previous work utilising trypsin and chymotrypsin (van Willige & FitzGerald, 1995).

Native CMP was completely broken-down on reaching a DH of 1 % in both NHT and HT WPI hydrolysis samples – a factor that owes its origins to the 'macro-peptide' nature of CMP where little secondary (2^o) structure presents a more accessible cleavage substrate than β-Lg or α-La. This finding concurs with previous work showing that CMP is readily hydrolysed by trypsin and chymotrypsin (Shammet, Brown & McMahon, 1992). On reaching 5 % DH enzymatic activity had degraded 98.8 % and 95.2 % of the native β-Lg and α-La

respectively. Relative to the NHT WPI, the overall effect of heat denaturation was to increase the rate of hydrolysis of both β-Lg variants, while decreasing that of α-La (Fig 2 B).

Differences in the peptide profiles of the NHT and HT hydrolysates were discerned to a greater extent by RP-HPLC than by electrophoresis. Three distinct peaks were evident in both samples (P1, P2, and P3) under gradient elution although in varying intensities (Fig. 2 insets). Two peaks (P4 and P5) with large intensities (at 214 nm) appear only to be present in the hydrolysate of HT WPI (Fig. 2 B inset) and not in the NHT hydrolysate (Fig. 2 A inset).

3.2 Amino acid analysis of hydrolysates produced from NHT and HT WPI

Total amino acid analysis of both the NHT and HT hydrolysates of WPI (Supplementary Table 1) revealed high average concentrations of glutamate (~150 g kg^{-1}), aspartate (~93 g kg^{-1}), leucine (~87 g kg^{-1}), and lysine (~83 g kg^{-1}). The hydrolysates had relatively low concentrations of the amino acids glycine (~15 g kg^{-1}) and histidine (~15 g kg^{-1}).

The respective leucine concentrations of 1668 ± 3 mg kg^{-1} and 1690 ± 6 mg kg^{-1} at 5 % DH in the NHT and HT hydrolysates were statistically different ($P < 0.05$) (Table 1), while noting that it was most abundant only in the case of NHT. The concentration of free phenylalanine, the most abundant free amino acid in the HT hydrolysate (2906 ± 2 mg kg^{-1}), increased by a factor of 2.1 compared to the concentration in the NHT (1394 ± 7 mg kg^{-1}) at the same % DH (Table 1). The greatest disparity between the two hydrolysates was seen in the concentration of free lysine – 274 ± 5 mg kg^{-1} and 1545 ± 2 mg kg^{-1} for the NHT and HT hydrolysates respectively at 5 % DH. This represents a 5.6 fold increase in free lysine as a result of the pre-hydrolysis heat-treatment.

As enzymatic hydrolysis continues, amino acids are liberated through endo-protease and exo-peptidase activities. The change in concentration as hydrolysis proceeded is shown

in Fig. 3 for four of the most abundant free amino acids in the NHT and HT hydrolysates (arginine, leucine, lysine and phenylalanine). The reaction hydrolysis curves show the reduced reaction time to arrive at equivalent DH points in the HT reactions (Fig. 3). Leucine is released relatively quickly compared with phenylalanine which begins to increase from 2.5 % DH onwards, and eventually surpasses free leucine in the HT hydrolysate (Fig. 3 B). Porcine elastase preferentially cleaves after small non-polar amino acid residues such as alanine, leucine and valine (Powers, Gupton, Harley, Nishino & Whitley, 1977). A large increase in free lysine concentration is observed from 2.5 % DH onwards in the HT reaction (Fig. 3 B) after approximately 20 min. However, no such increase is seen in the NHT reaction at the equivalent DH after approx. 40 min hydrolysis time (Fig. 3 A).

Free amino acid analysis was also utilised to illustrate which proteolytic activities benefitted from the molecular changes to the substrate as a result of heat-treatment. A high proportion of aromatic and hydrophobic aliphatic free amino acids were present in both sets of hydrolysates (Table 1) possibly due to secondary cleavages associated with the primary activities in Corolase® PP (elastase, chymotrypsin, and to a lesser extent, trypsin). Chymotrypsin primarily cleaves after tryptophan, tyrosine and phenylalanine with secondary activity after leucine and methionine. Corolase® PP is reported to have a high chymotrypsin to trypsin activity ratio (Mullally et al., 1994).

Hydrolysates from HT WPI possessed increased non-polar free amino acids, which could be due to the presence of a greater proportion of soluble smaller peptides being available for carboxypeptidase (CYP) cleavage. CYP B acts on basic amino acids such as arginine and lysine, and CYP A acts on aromatic (CYP A2) and aliphatic side-chains on the C-terminus of peptides. The higher proportion of free aromatic amino acids, such as phenylalanine, in the HT hydrolysates (Fig. 3 B) is indicative of increased CYP A2 activity.

3.3 Peptide analysis of hydrolysates produced from NHT and HT WPI

Peptide analysis was carried for the NHT and HT hydrolysates within the size region 300 – 2,000 Da at varying degrees of hydrolysis (1, 2.5 and 5 % DH). Therefore, peptides outside this threshold are not presented in this study. Peptides are included with an ion score >25 which is based on a 5 % false discovery rate. Using these criteria it is possible that a small number of peptides, that may have been present, were excluded. However, the authors employed these strict criteria in order to have the greatest confidence that the peptides reported are present in each sample. β-Casein-associated peptides (possible proteose peptone components) in the un-hydrolysed control samples (results not shown) were excluded as they were not the result of enzymatic hydrolysis with Corolase® PP.

The total number of peptides detected using LC-MS/MS in the size range 300 – 2,000 Da were 34 (1 %), 36 (2.5 %) and 38 (5 %) for the respective WPI fractions β-Lg, CMP and α-La (according to % DH in parenthesis) while the corresponding numbers for the HT hydrolysate were 36, 32 and 38, respectively. Heat-treatment prior to hydrolysis resulted in a decrease in the detected cleavage of peptides corresponding to β-Lg and an increase in the number of peptides derived from α-La (Supplementary Table 2). However, assertions made regarding increases / decreases in assigned peptides, e.g., the reduced detection in the HT hydrolysate at 2.5 % DH, are made carefully with regard to the low number of peptides detected using the stringent criteria (5 % false discovery) for peptide analysis as laid out in Section 2.5.

Unique peptides in the NHT hydrolysate included five peptides derived from α-La, 17 from β-Lg, and four from CMP which are not present in the HT hydrolysate (Table 2). The corresponding unique peptides detected in the HT hydrolysate, not present in the NHT hydrolysate, were; 10 from α-La, 12 from β-Lg and one from CMP.

The unique peptides obtained are postulated to be the result of different cleavages in the parent proteins arising from structural alterations due to heat pre-treatment of the substrate. Denaturation of proteins reduces the tertiary structures internalising and protecting certain residues from proteolytic attack (Benyon & Oliver, 1996) and therefore hydrolytic cleavage is theoretically increased with denaturation. The nature of this denaturation and the characteristics of any resulting aggregates will dictate the alteration of the reaction. For example, the pH, mineral content and heterogeneity of the substrate during heat-treatment will determine the size of any resulting aggregates and whether that matrix restricts or facilitates enzymatic cleavage (Leeb, Kulozik & Cheison, 2011; O'Loughlin et al., 2012).

The fact that the majority of these unique peptides come from β-Lg is not surprising considering β-Lg constitutes 56.5 % (w w^{-1}) of the protein content in the WPI. However, while the same number of peptides (38) within the 300 – 2000 Da size-range were detected in the hydrolysates of the NHT and HT WPI at 5 % DH, the probability exists that a sizeable proportion of the material < 1 kDa in the hydrolysates of the HT WPI was below the detectable range of the LC-MS/MS.

3.4 Proteolytic mapping of peptides found within the hydrolysates of NHT and HT WPI

Peptide analysis of the produced hydrolysates allowed for the mapping of the cleavage sites in α-La, β-Lg and CMP; these proteins constituting 89 % (w w^{-1}) of the total protein in the WPI starting material. As the analysis is limited to peptides within a certain size range, there were gaps in the protein sequences where no peptides could be detected by LC-MS/MS. All peptides detected in the 5 % DH NHT and 5 % DH HT hydrolysates were mapped to the primary (1^{o}) structures of α-La (Fig. 4), β-Lg (Supplementary Fig. 3) and CMP (Supplementary Fig. 4) in order to visualise the coverage of the respective proteins resulting from LC-MS/MS analysis. In Fig. 4 the cleavage points detected for α-La are shown for both

NHT (Fig. 4 A) and HT hydrolysates (Fig. 4 B). Utilising known information about the preferential cleavage sites for trypsin, chymotrypsin and elastase (the three major endo-protease constituents of Corolase® PP) the authors employed protein digest software to determine which cleavage points could be attributed to a particular enzymatic activity. The number of cleavages in the α-La sequence attributed to chymotrypsin activity is five in both hydrolysate sets. Three cleavage points were assigned to elastase in the NHT and two in the HT hydrolysate. For trypsin, it was two in the NHT hydrolysate and four in the HT samples from a theoretical 13.

The cleavage points of β-Lg were also deduced and are presented in Supplementary Fig. 3. Noteworthy is the detection of peptides from the A variant of β-Lg in the HT hydrolysate only and the presence of a large section in the parent β-Lg sequence, corresponding to amino acids 105 to 121, from which no peptides were detected in the NHT hydrolysate. In relation to the non-detection of *f*(105-121) of β-Lg, Cheison et al. (2010) demonstrated the non-ionisability of β-Lg *f*(102-124) which could be a possible reason for the non-detection (Cheison, Schmitt, Leeb, Letzel & Kulozik, 2010). Also, while a greater percentage of the sequence of β-Lg is accounted for by the HT hydrolysate (98.8 %) compared to the NHT hydrolysate (89.5 %), there is a reduction in the assigned cleavages for elastase in the HT hydrolysate from 12 to five and two more cleavage sites attributed to trypsin activity in the HT hydrolysate (10) than the NHT hydrolysate (8) (Supplementary Fig. 3).

For CMP, greater percentage coverage of the 65-mer macro-peptide is accounted for in the NHT (90.8 %) than the hydrolysate of the HT WPI (86.1 %) and no cleavage points were assigned to chymotrypsin by the software due to the lack of tyrosine, tryptophan and phenylalanine residues in the 1^{o} structure sequence of CMP (Supplementary Fig. 4). Only the main protease activities were considered and the cleavages as a result of exo- or di-peptidase

activities known to be present in Corolase® PP (Mullally et al., 1994) were not investigated in this study.

4. CONCLUSION

It is concluded that alteration of substrate molecular conformation can change the protein hydrolysis patterns attributed to the serine endo-protease activities that comprise Corolase® PP. Substrate heat-treatment appeared to affect hydrolytic susceptibility of the different WPI fractions, e.g., a reduction in the number of β-Lg peptides cleaved during hydrolysis of heated WPI contrasted with an increased peptide release from α-La due to trypsin activity. In the case of β-Lg, it was notable that peptides were detected from the A variant of β-Lg in the HT hydrolysate only while none were released from a large section, *f*(105-121), of the parent β-Lg in the NHT hydrolysate. It would appear that heat-treatment of WPI appeared to adversely affect associated β-Lg cleavage by elastase. Hydrolysis of pre-heated WPI favored the release of phenylalanine and lysine by factors of 2.0 and 5.6, respectively, compared to un-heated substrate. Both lysine and, to a lesser extent, leucine release were evident during the early stages of hydrolysis – the latter being more abundant in the case of non-heated whey protein.

Overall, heat-treatment creates an opportunity to achieve greater prediction and targeting of the outcomes of whey protein hydrolysis, however consideration needs to be given to the utilization of the higher levels of insoluble material present, even at 5 % DH, in the prior heat-treated substrate.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the help and assistance gratefully provided by Paula O'Connor of Teagasc Food Research Centre, Moorepark.

SUPPORTING INFORMATION

Supporting Information Available: Total amino acid concentrations of WPI and hydrolysates (Supplementary Table 1). Number of peptides released from α-La, β-Lg and CMP at different degrees of hydrolysis (Supplementary Table 2). Molecular weight distribution of soluble protein material by SEC (Supplementary Figure 1). Reducing SDS-PAGE of WPI and hydrolysates (Supplementary Figure 2). LC-MS/MS peptide alignment patterns for β-Lg and CMP (Supplementary Figures 3 and 4).

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FINANCIAL SUPPORT

The work herein was funded by Enterprise Ireland (EI) as part of the Food for Health Ireland project. I. B. O'Loughlin is a Teagasc Walsh Fellow supported by EI grant number CC/2008/0001/A.

Figure 1. Changes in the proportion of insoluble and soluble protein material at pH 2 during Corolase® PP hydrolysis (0–5% DH) of protein dispersions (100 g L^{-1} protein): (A) un-heated control whey protein isolate (WPI), and (B) heat-treated (80 ºC for 10 min) WPI.

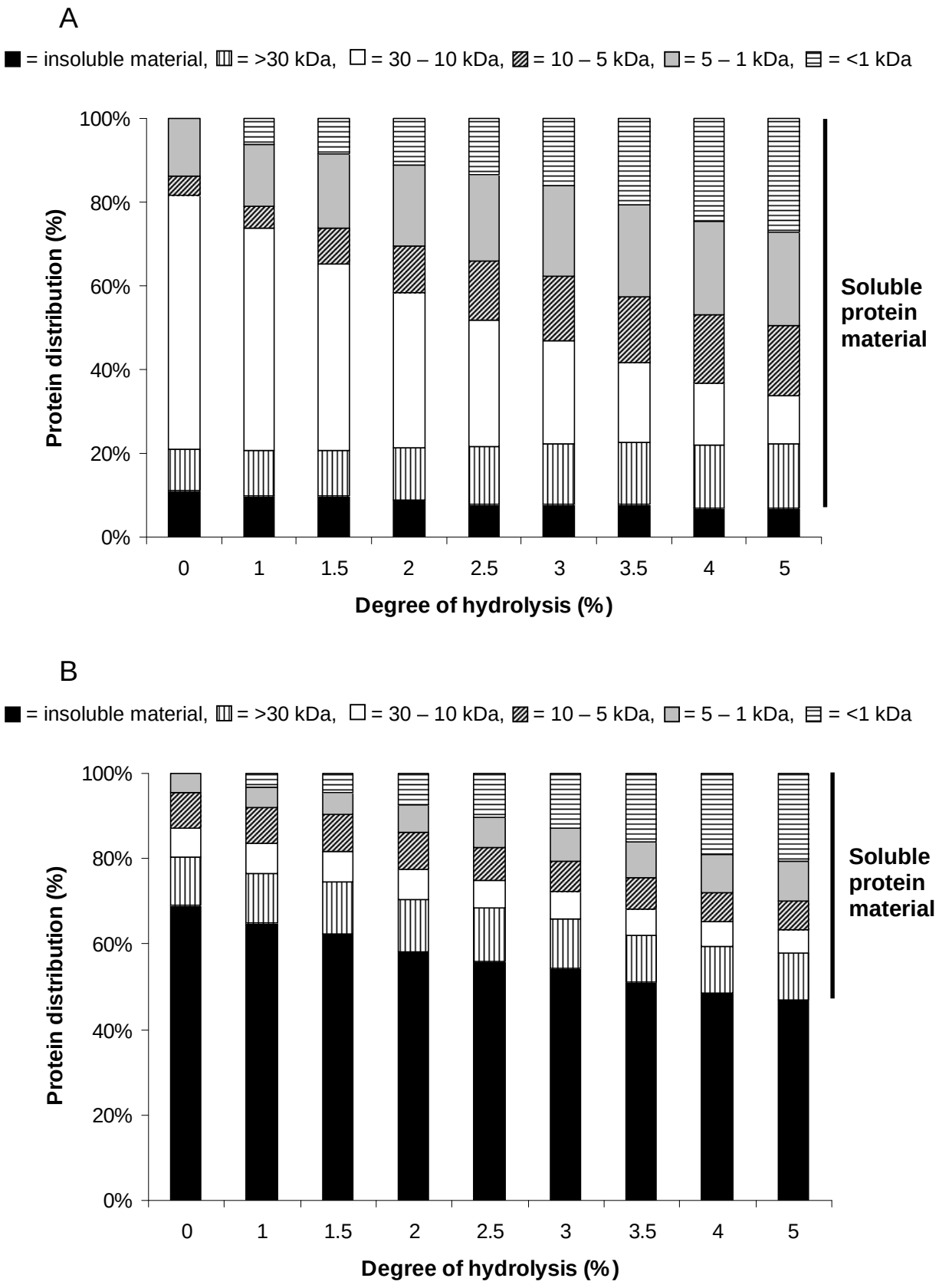
Figure 2. Changes to native protein as determined by RP-HPLC during Corolase® PP hydrolysis (0–5% DH) of whey protein isolate (WPI) dispersions (100 g L^{-1} protein) of (A) control WPI and (B) heat-treated (80 ºC for 10 min) WPI. Insets show C18 RP-HPLC chromatographs of hydrolysates of WPI at 5% DH with dashed lines showing predetermined retention times for α-Lactalbumin and β-Lactoglobulin.

Figure 3. Changes in the concentration of selected free amino acids during Corolase® PP hydrolysis (0–5% DH) of whey protein isolate (WPI) dispersions (100 g L^{-1} protein): (A) un-heated control WPI and (B) heat-treated (80 ºC for 10 min) WPI.

Figure 4. Alignment of sequences detected by LC–MS/MS during Corolase® PP - induced cleavage (5% DH) of the α-Lactalbumin fraction in whey protein isolate (WPI) dispersions

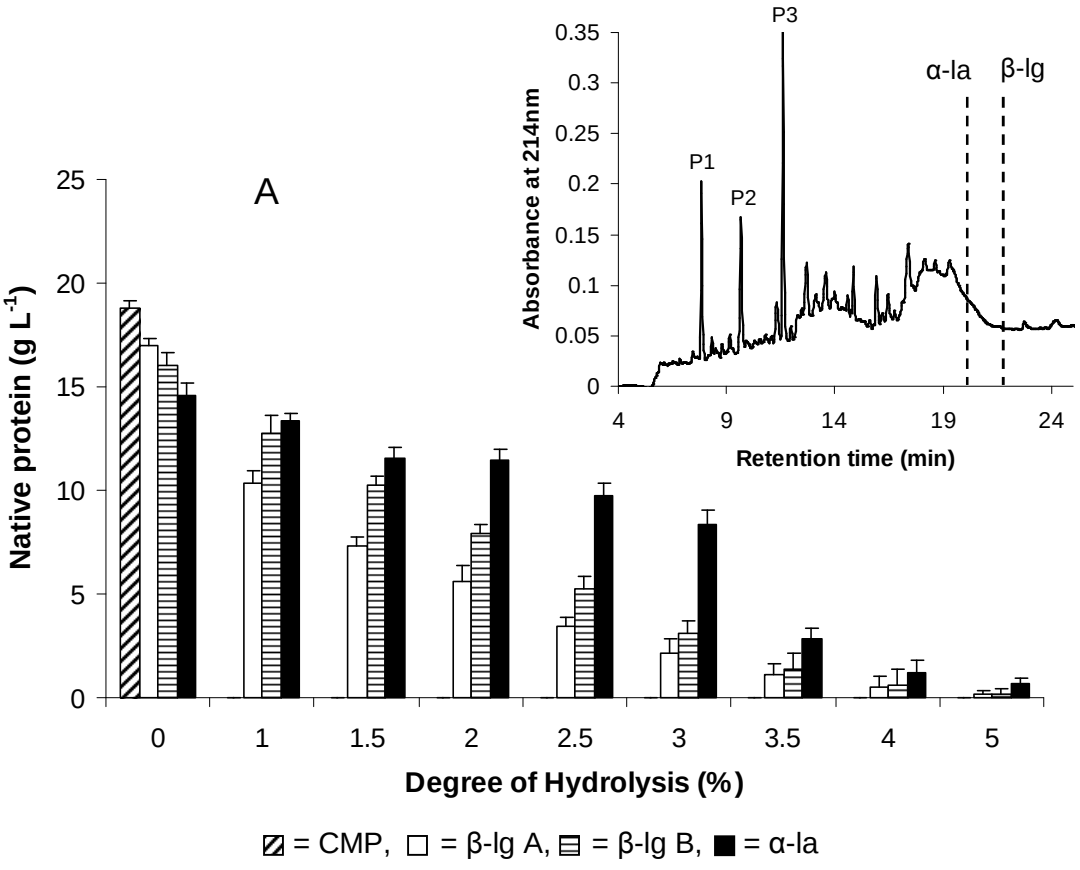
(100 g L^{-1} protein): (A) un-heated control, and (B) heat-treated (80 ºC for 10 min) WPI. Sequences detected by LC–MS/MS are listed underneath the parent protein. Cleavage points for endo-protease activities are assigned using ISB protein digest tool specifying no missed cleavages.

Figure 1.



Revised and submitted Word version of O'Loughlin et al. (2013) Food Chem, 141(3), 2334-2342. doi: 10.1016/j.foodchem.2013.05.056

Figure 2.


A
Native protein (g L-1)
25
20
15
10
5
0
0
1
1.5
2
2.5
3
3.5
4
5
Degree of Hydrolysis (%)
Absorbance at 214nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
4
9
14
19
24
Retention time (min)
P1
P2
P3
α-la
β-lg
▨ = CMP, □ = β-lg A, ▤ = β-lg B, ■ = α-la

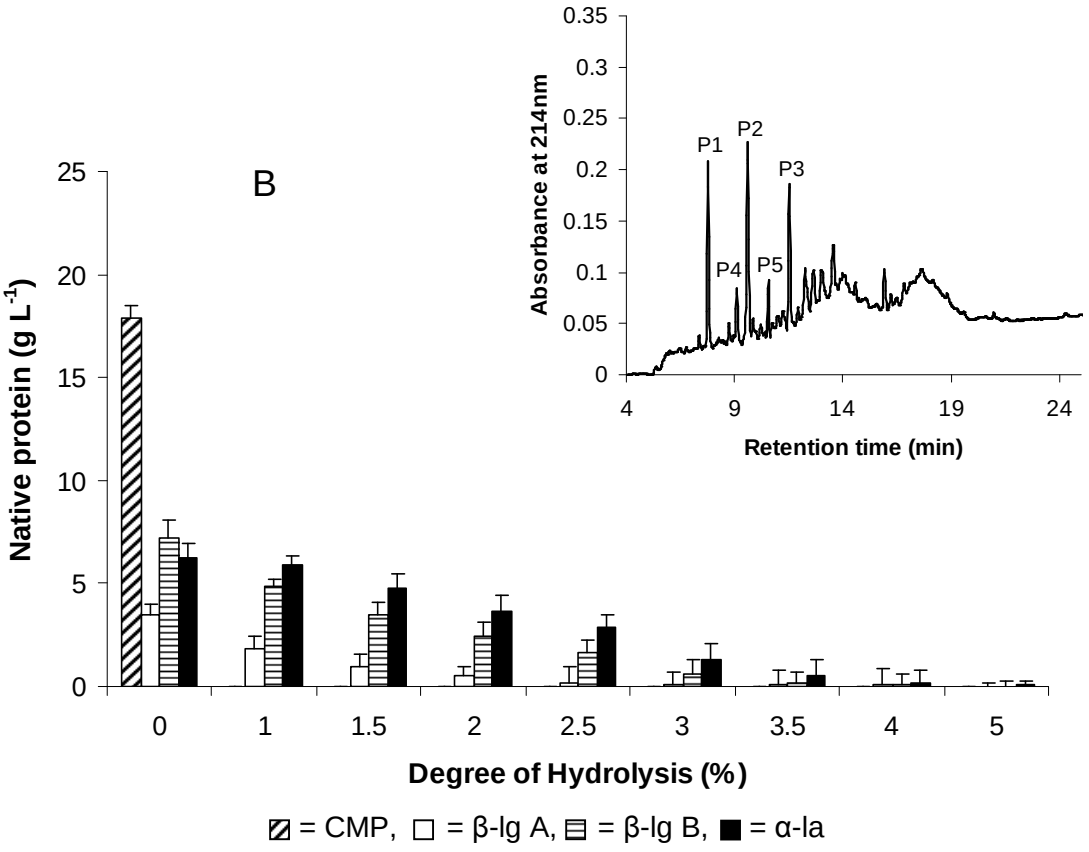
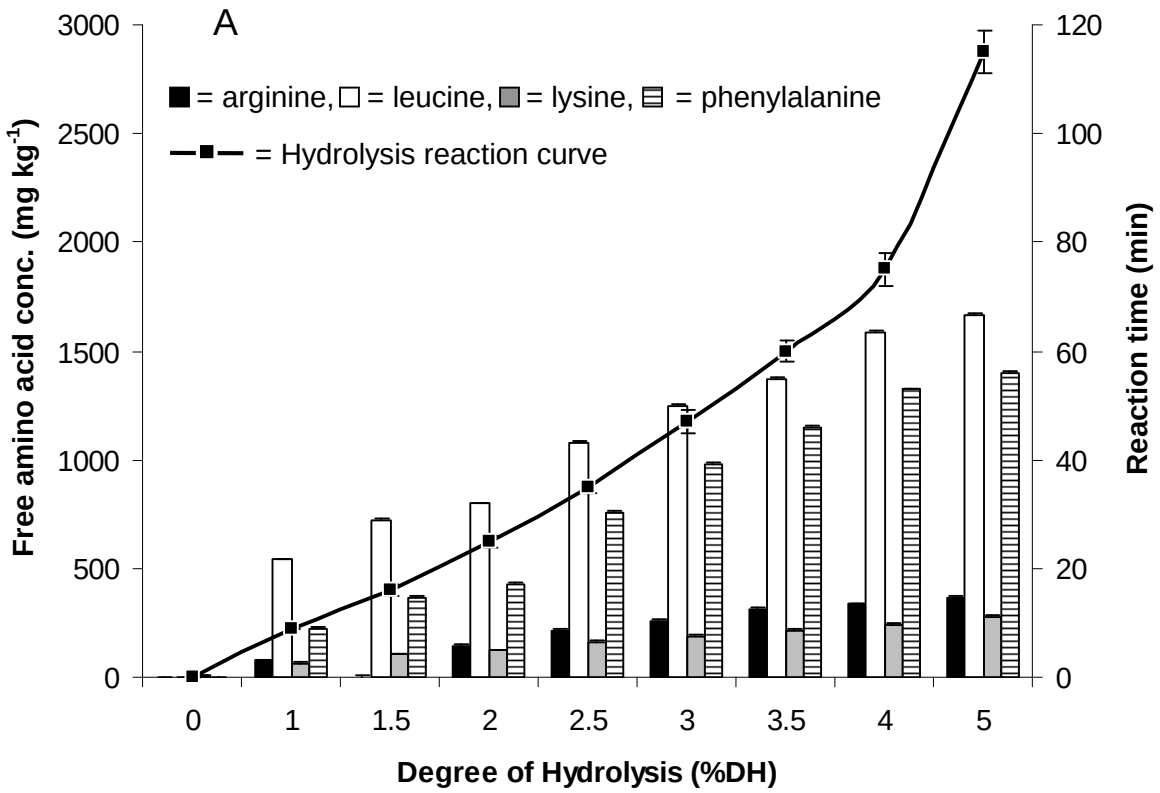

B
Native protein (g L-1)
25
20
15
10
5
0
0
1
1.5
2
2.5
3
3.5
4
5
Degree of Hydrolysis (%)
▨ = CMP, □ = β-lg A, ▤ = β-lg B, ■ = α-la
Absorbance at 214nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
P1
P2
P3
P4
P5
4
9
14
19
24
Retention time (min)

Figure 3.


A
■ = arginine, □ = leucine, ■ = lysine, ▤ = phenylalanine
—■— = Hydrolysis reaction curve
Free amino acid conc. (mg kg-1)
Reaction time (min)
Degree of Hydrolysis (%DH)
0
500
1000
1500
2000
2500
3000
0
20
40
60
80
100
120
0
1
1.5
2
2.5
3
3.5
4
5

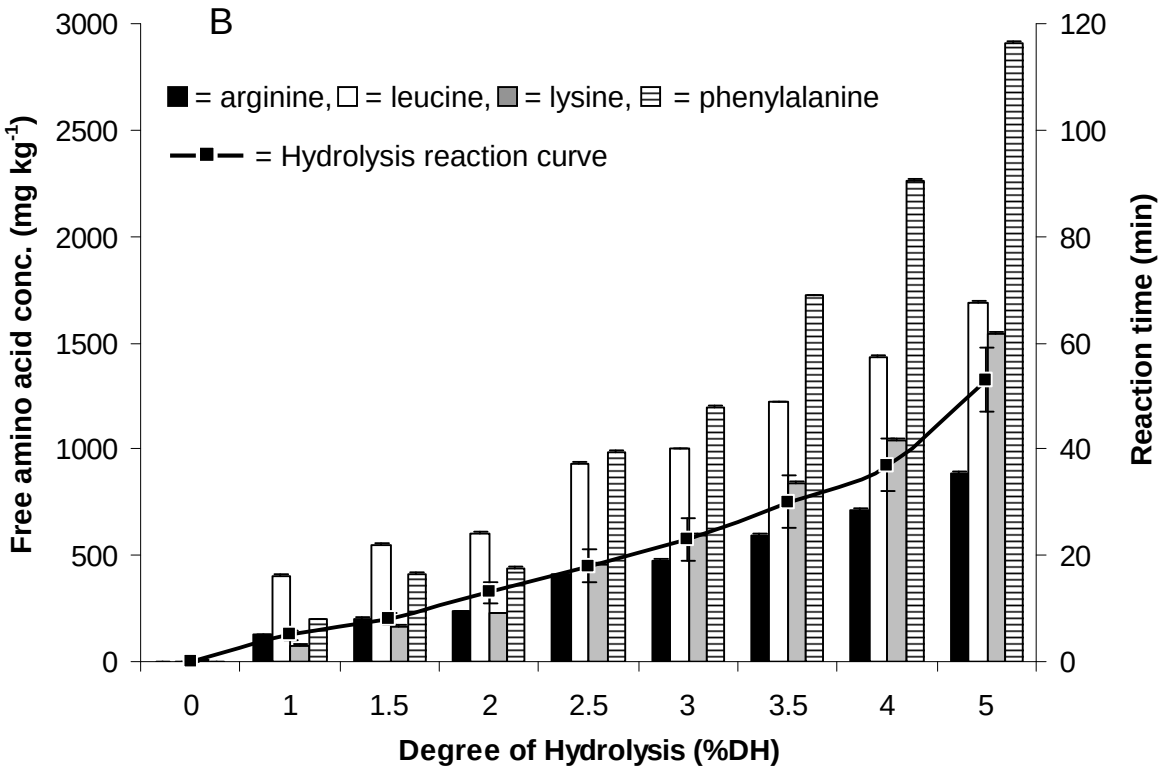

B
■ = arginine, □ = leucine, ▩ = lysine, ▤ = phenylalanine
—■— = Hydrolysis reaction curve
Free amino acid conc. (mg kg-1)
Reaction time (min)
Degree of Hydrolysis (%DH)
3000
2500
2000
1500
1000
500
0
120
100
80
60
40
20
0
0
1
1.5
2
2.5
3
3.5
4
5

Figure 4.


(A)
EQLTKCEVFRELKDLKGYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNKIWCKDDQNPHSSNICNISCDKFLDDDLTDDIM
----------ELKDLKGYGGVSLPEWVCTT----------GLFQINNKIWCKDD----------
----------TFHTSGYDTQAIV----------QINNKIWCKDD----------
----------FHTSGYDTQA----------
----------HTSGYDTQAI----------
----------TSGYDTQAIVQNNDSTE----------
----------DTQAIVQNNDSTEYG----------
(B)
EQLTKCEVFRELKDLKGYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNKIWCKDDQNPHSSNICNISCDKFLDDDLTDDIM
----------GYGGVSLPEWVCTTFHTSG----------GLFQINNKIWCKDD----------LDDDLTDDIM
----------GYGGVSLPEWVCTTFH----------GLFQINN-------QNPHSSNICNISCDK----------
----------FHTSGYDTQA----------
----------HTSGYDTQAI----------
----------DTQAIVQNNDSTEYG----------
----------VQNNDSTEYG----------
----------VQNNDSTEY----------
E = Elastase cleavage
C = Chymotrypsin cleavage
T = Trypsin cleavage

1