



A proteomics approach to characterizing limited hydrolysis of whey protein concentrate

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ARTICLE INFO

Keywords:

Proteomics
Whey protein hydrolysate
Hydrophobic peptides
Bioactive peptides

ABSTRACT

Whey Protein Concentrate was hydrolyzed by trypsin and Protease M, a novel endo/exopeptidase mix from *Aspergillus oryzae*. Protein peptide profiling demonstrated that Protease M preferentially hydrolyzed α -lactalbumin (α -la), while trypsin targeted β -lactoglobulin (β -lg). Peptide fractions were analyzed by liquid chromatography coupled with tandem mass-spectrometry to characterize differences in enzyme specificity, peptide hydrophobicity, and bioactivity, using bioinformatics tools. While trypsin cleaved at the C-terminal end of lysine and arginine, Protease M contributed to pepsin-like endopeptidase activity coupled with carboxyl, amino, and leucine exopeptidase activity, resulting in relatively more hydrophilic peptides compared to those released by trypsin hydrolysis. While trypsin and Protease M had varying specificity, 9 bioactive peptides were common among the hydrolysates, which was attributed to the exopeptidase activity of Protease M. The proteomics coupled with bioinformatics approach provided fundamental knowledge needed to optimize whey protein hydrolysis in a direct and efficient manner for targeted applications.

1. Introduction

Whey protein ingredients are used in many applications as nutritionally complete protein sources with a clean taste and versatile functionality. Commonly used whey protein ingredients include isolates (WPI), concentrates (WPC) and hydrolysates (WPH). Modification of whey protein by limited hydrolysis is commonly employed to improve interfacial properties, enhance water solubility, and release bioactive peptides, including those with antioxidant activity (Chobert & Bertrand-Harb, 1988; Maduereira, Tavares, Gomes, Pintado, & Malcata, 2010; Dryáková, Pihlanto, Marnila, Čurda, & Korhonen, 2010). The functionality of WPH is dependent on the hydrolysis parameters, such as enzyme type, enzyme-to-substrate ratio, hydrolysis time, temperature, and pH. These parameters can be optimized for a targeted functionality such as the release of bioactive peptides.

A major challenge when producing protein hydrolysates is the

release of hydrophobic and bitter peptides. Accordingly, protein hydrolysis is typically limited to minimize bitterness. Traditionally, degree of hydrolysis (DH), which is a measure of the percentage of peptide bonds cleaved, is used to monitor extent of protein hydrolysis. When using endopeptidases, often the aim is to maintain a DH < 8% to minimize bitterness and maximize functional properties (Adler-Nissen, 1984). Another approach to control bitterness is the use of exopeptidases, which debitter hydrolysates by cleaving terminal amino acids such as bulky basic and hydrophobic amino acids (Tan et al., 1993; Huang et al., 2015). However, when using a combination of endo- and exo-peptidases, DH becomes a poor measure for predicting bitterness and functionality of different hydrolysates.

To better characterize the impact of hydrolysis conditions and the use of different enzymes on the properties of the resulting hydrolysates, it would be best to apply a proteomics approach. A proteomics approach consists of: (1) chromatographic fractionation of proteins/peptides

Abbreviations: ACE, angiotensin converting enzyme; α -CHCA, α -cyano-4-hydroxy-cinnamic acid; α -la, α -lactalbumin; β -lg, β -lactoglobulin; DDW, double deionized water; DH, degree of hydrolysis; DPP-IV, dipeptidyl peptidase-IV; DPPH, 2,2-diphenyl-picrylhydrazyl; E:S, enzyme-to-substrate ratio; GMP, glycomacropeptide; GRAVY, grand average of hydropathy; HWPC, hydrolyzed whey protein concentrate; kDa, kilodaltons; MALDI-TOF, matrix assisted laser desorption ionization; MS, mass spectrometry; MBPDB, Milk Bioactive Protein Database; PTM, post-translational modification; PHWPC, Protease M hydrolyzed whey protein concentrate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SE-HPLC, size exclusion-high performance liquid chromatography; THWPC, trypsin hydrolyzed whey protein concentrate; WPC, whey protein concentrate; WPI, whey protein isolate; WPH, whey protein hydrolysate; UHPLC, ultra-high performance liquid chromatography.

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<https://doi.org/10.1016/j.foodchem.2021.129235>

Received 22 September 2020; Received in revised form 22 January 2021; Accepted 26 January 2021

Available online 9 February 2021

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within a hydrolysate; (2) mass spectrometry (MS) analysis of individual fractions to identify peptide sequences; and (3) bioinformatics analysis to process the data. This approach has been previously used on whey protein hydrolysates to characterize enzyme cleavage patterns (Cheison, Brand, Leeb, & Kulozik, 2011; Deng, van der Veer, Sforza, Gruppen, & Wierenga, 2018) and to identify bitter (Liu, Jiang, & Peterson, 2014) as well as bioactive peptide sequences (O'Keeffe, Conesa, & FitzGerald, 2017; Zhang, Wu, Ling, & Lu, 2013). For example, the specificity of trypsin for β -lactoglobulin at non-optimum conditions was characterized following a proteomics approach to track the order in which lysine and arginine sites were cleaved (Cheison et al., 2011). Proteomics was similarly used to predict trypsin's specific cleavage sites on β -lactoglobulin and α -lactalbumin with increasing DH (Deng et al., 2018). Proteomics has been mostly used to identify bitter and bioactive peptides in whey protein hydrolysates following peptide fractionation, measurement of bitterness and bioactivity of the fractions, and peptide sequencing of the most potent fractions using tandem MS. The identified peptides are then synthesized and tested for their bitterness and/or bioactivity (Liu et al., 2013; O'Keeffe et al., 2017; Zhang et al., 2013). Zhang et al. (2013) compared six different hydrolyzed WPC and found that alcalase hydrolyzed WPC had the highest 2,2-diphenyl-picrylhydrazyl (DPPH) free radical, superoxide radical scavenging activity, and lipid peroxidation inhibition ability. The researchers followed the protocol listed above and identified seven peptides with potent antioxidant activity.

Proteomics research led to the development of bioinformatics tools including outputs of theoretically digested proteins by various enzymes (Dallas & Nielsen, 2018) and databases of bioactive peptide sequences (Dallas & Nielsen, 2018; Murray et al., 2019; Bhattacharya et al., 2019). By coupling these bioinformatics tools to a proteomics approach, a comprehensive characterization of protein hydrolysates can be achieved. For example, sensory guided fractionation was used to identify bitter and non-bitter peptide fractions of hydrolyzed sodium caseinate. The bitter peptide fractions peptides were sequenced using ultra high performance liquid chromatography (UHPLC)-quadrupole-time-of-flight tandem MS followed by bioactive peptide database search to screen for peptides known to reduce blood glucose levels, i.e. insulinogenic peptides (Murray et al., 2018). The use of this combined approach was also utilized by Bhattacharya et al. (2019) who determined the peptide content of 10 commercial dairy products using ion-exchange HPLC, and sequenced the peptides using HPLC-orbitrap tandem MS. A bioactive peptide database was then used to identify bioactive peptides across all 10 products. A similar approach was also applied to identify bioactive peptides in commercial donkey milk (Piovesana et al., 2015) and buffalo-milk dairy products (Basilicata et al., 2018). Applying this combination of proteomics and bioinformatics to WPH production would allow the characterization of cleavage patterns of multiple enzymes, while screening generated peptides for properties such as hydrophobicity and bioactivity, irrespective of DH.

Therefore, the objectives of this study were to: (1) examine differences in the cleavage patterns of trypsin and Protease M and the subsequent effect on the peptide composition; and (2) characterize differences in hydrophobicity and bioactivity of the generated peptides. Trypsin was chosen as a common endopeptidase with high specificity, commonly used to produce WPH (Deng et al., 2018; Gauthier & Pouliot, 2003). Whereas, Protease M, from *Aspergillus oryzae*, was chosen as it exhibits both exo- and endo- peptidase activity that would mostly generate hydrophilic peptides, some of which could be bioactive (de Castro & Sato, 2014; Zanutto-Elgui et al., 2019). The understanding developed from proteomics coupled with bioinformatics approach will allow the optimization of whey protein hydrolysis for targeted applications in a direct and efficient way compared to traditional approaches.

2. Materials and methods

2.1. Materials

WPC 80 (77.5% protein) and Protease M SD (~40,000 U/g protein) were kindly donated by Agropur (Eden Prairie, MN) and Amano Enzyme Inc. (Elgin, IL), respectively. Bovine Trypsin (~10,000 BAEE units/mg protein, EC 3.4.21.4) was purchased from Millipore Sigma (St. Louis, MO). Criterion™ TGX™ 8 – 16% precast gels, Precision Plus™ all blue molecular weight (MW) marker, Laemmli sample buffer, and 10x tris/glycine/sodium dodecyl sulfate (SDS) running buffer were purchased from BioRad Laboratories, Inc. (Hercules, CA), while the Imperial Protein™ Coomassie blue stain was purchased from Thermo Fisher Scientific (Waltham, MA). Molecular weight calibration kits were purchased from GE Healthcare (Solingen, Germany). C18 ZipTips and Protein Calibration Standard I and II were purchased from Bruker Daltronics (Billerica, MA) while Empore™ C18 extraction disks were purchased from 3 M (Maplewood, MN). Magic™ C18_{aq} resin was purchased from Michrom Bioresources (Auburn, CA). All other analytical grade reagents were purchased from Millipore Sigma or Thermo Fisher.

2.2. Preparation of hydrolyzed WPC 80 (HWPC)

WPC 80 was subjected to enzymatic hydrolysis, in triplicate, using either trypsin or Protease “M” SD, following the pH-stat approach described by Adler-Nissen (1984), and using the method optimized by Boyle, Hansen, Hinnenkamp, and Ismail (2018). Preliminary trials were carried out to determine hydrolysis parameters needed to produce a Protease M (PHWPC) and a trypsin hydrolysate (THWPC) with DH $\leq$ 8% and hydrolysis times < 35 min. Since exopeptidase activity can reduce hydrolysate bitterness, a second Protease M hydrolysate at a higher DH ($\leq$ 14%) was also produced. Dispersions (5% protein) were prepared using double deionized water (DDW) pre-heated to 45 °C, stirred for 30 min, and adjusted to pH 8 for trypsin and pH 6 for Protease M hydrolysis with 2 N NaOH or HCl. For the production of PHWPC, pH adjusted dispersions were then heated to 40 °C, and Protease M was added at an enzyme to substrate ratio (E:S) of 0.67 g enzyme/ 100 g protein to result in DH $\leq$ 8%, and 1.5 g enzyme/ 100 g protein to result in DH $\leq$ 14%. Dispersions were then incubated for 35 min and 10 min for the lower and higher DH hydrolysates, respectively. pH 6 was maintained throughout the incubation period by titrating 0.2 N NaOH at 5 min intervals. After incubation, PHWPC samples were neutralized and heated at 65 °C for 12 min to inactivate the enzyme (stability is reported as being lost at pH > 7 and temperatures > 60 °C). For the production of THWPC, pH adjusted dispersions were heated to 37 °C, and trypsin was added at an E:S of 0.32 g enzyme/ 100 g protein. The dispersions were incubated for 15 min. pH 8 was maintained throughout the incubation by titrating 0.2 N NaOH at 5 min intervals. Trypsin was then inactivated by neutralizing the pH followed by boiling for 1 min. All hydrolysates were lyophilized, and the protein content of lyophilized samples was determined using a FP 828 nitrogen analyzer (Leco Corp., St. Joseph, MO), following the Dumas method (AOAC 990.30), and using 6.38 as the nitrogen conversion factor. The DH was calculated following the formula reported by Adler-Nissen (1984) and updated by Boyle et al. (2018) using an h_{tot} of 8.8 for whey protein. The three hydrolysates produced were PHWPC 8% with an exact DH of $7.5 \pm 0.7\%$ (78.6% protein); PHWPC 12% with an exact DH of $12.5 \pm 0.3\%$ (77.3% protein), and THWPC 5% with an exact DH of $4.8 \pm 0.1\%$ (78.9% protein). Lyophilized samples were kept at –20 °C until further analysis.

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

Protein profiling of WPC and the three hydrolysates was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions as described by Laemmli (1970)

and reported by Boyle et al. (2018). Briefly, samples (5 μ L; containing $\sim$ 25 μ g protein) and Precision Plus™ MW standard (10 μ L) were loaded onto an 8 – 16% gradient gel. The gel was electrophoresed, stained/desained, and imaged as outlined by Boyle et al. (2018).

2.4. Size exclusion – High performance liquid chromatography (SE-HPLC)

WPC and hydrolysates were subjected to size exclusion HPLC (SE-HPLC) using ÄKTAbasic™ 10 system (Amersham Biosciences, Uppsala, Sweden), consisting of a P-900 pump, UV-900 detector, UV-flow cell (10 mm), INV-907 injection valve, M-925 mixer, and a FR-904 flow restrictor, and equipped with a Superdex 200 Increase 10/300 GL column (30 cm $\times$ 10 mm, 13 μ m avg. particle size; GE healthcare). Data was acquired using UNICORN™ software version 5.01 (Amersham Biosciences). The analysis was performed at room temperature following the method of Brückner-Gühman, Heiden-Hecht, Sözer, and Drusch (2018), with modifications. Samples were dissolved (0.1% w/v) in the mobile phase (0.05 M NaCl; 0.1 M sodium phosphate buffer, pH 7) and stirred for 90 min. Samples were then passed through a 0.45 μ m filter, manually injected (100 μ L) and separated isocratically using a mobile phase flow rate of 0.5 mL min⁻¹ for a total run time of 72 min. UV-detection and analysis was performed at 280 nm. Molecular weights were calculated using gel filtration calibration kits with low and high molecular weight standards: ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease (13.7 kDa), and aprotinin (6.5 kDa). Peak identities were assigned based on reported molecular weights (Andrews, Taylor, & Owen, 1985). Relative peak areas—the ratio of the area of a single peak to total peak area for a sample—were used to monitor changes in intact protein. Three fractions from the peptide region of each hydrolysate were collected at 18 – 20.5 mL, 21 – 24 mL and 24 – 26 mL of eluate, for proteomics analysis.

2.5. Mass spectrometry (MS) and proteomics analysis of peptide fractions

Proteins are typically digested by trypsin or chymotrypsin prior to HPLC-MS/MS analysis to reduce them to a suitable molecular weight. To maintain the integrity of the peptides produced during the Protease M and trypsin hydrolyses and avoid additional digestion, peptide fractions from SEC-HPLC fractionation were first analyzed by matrix assisted laser desorption and ionization – time of flight mass spectrometry (MALDI-TOF MS) to ensure the peptides were under 8,500 Da. Prior to MALDI-TOF MS analysis, the samples were desalted using C18 ZipTips. The manufacturer's standard protocol for peptide cleanup was followed. After elution from the ZipTip, 1 μ L of each sample was spotted on a MALDI-TOF 384 spot stainless steel target (Bruker Daltonics, Billerica, MA) and mixed with 1 μ L of α -cyano-4-hydroxy-cinnamic acid (α -CHCA) matrix (15 mg mL⁻¹ α -CHCA in 50% acetonitrile). Each spot was allowed to dry at room temperature. The target was placed in a Bruker Autoflex Speed MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a Nd-YAG 355 nm pulsed laser. To check for peptides from $\sim$ 350–4,500 Da, the data was collected in reflectron mode, positive polarity, with an accelerating potential of 19 kV. Each spectrum was the accumulation of 4,000 laser shots with complete spot raster of the laser during data collection. External calibration was performed using 0.5 μ L α -CHCA and 0.5 μ L of Peptide Calibration Standard I. To check for large polypeptides from $\sim$ 1,000–8,500 Da, the data was collected in linear mode, positive polarity, with an accelerating potential of 19.5 kV. Each spectrum was the accumulation of 4,000 laser shots with complete spot raster of the laser during data collection. External calibration was performed using 1.0 μ L sinapinic acid and 1.0 μ L of Peptide Calibration Standard II. All of the Bruker data files were converted to an mzXML file using MSConvertGUI tool in ProteoWizard open-source software. The mzXML files were viewed and analyzed with mMass v5.5 open-source software.

MALDI-TOF results showed that all peptide fractions met size requirements for UHPLC-MS/MS analysis (data not shown). Peptide fractions were desalted using Empore™ C18 extraction disks according to the “Stage Tip” procedure of Rappsilber, Ishihama, and Mann (2003) and analyzed by HPLC-MS/MS using the parameters established by Lin-Moshier et al. (2013) with the exception that a locked mass was not used during MS data acquisition. Briefly, samples (<1 μ g) were separated using a fused silica, pulled-tip capillary column (12 cm $\times$ 75 μ m internal diameter; New Objective, Woburn, MA) packed in-house with Magic™ C18_{aq} resin (5 μ m, 200 Å pore size;) in-line with a Velos Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA) for MS/MS analysis, with data dependent acquisition. Peaks Studio 10 (Bioinformatics Solutions, Inc, Waterloo, CA) was used for database searching using the following parameters: bovine (taxonomy ID 9913) protein sequence database from UniProt (<http://www.uniprot.org/>, downloaded 2017–02-14) concatenated with the common lab contaminant database from <http://www.thegpm.org/crap/> (downloaded 2019–03-13); precursor mass error tolerance of 50.0 ppm; fragment mass error tolerance of 0.1 Da; and a monoisotopic precursor mass search. The following amino acid modifications were also included: methionine oxidation, cysteine carbamidomethylation, pyroglutamic acid, protein N-terminal acetylation, and deamidation of asparagine and glutamine. The Peaks post-translational modification (PTM) results were used selecting a False Discovery Rate (FDR) of 0.5% for peptides, $-10\text{lgP} \geq 20$ for protein score, and minimum of 1 unique peptide. The Peaks software defined unique peptides as peptide sequences associated with only one protein group in bovine (*Bos taurus*).

2.6. Bioinformatics analysis

Peptide coverage maps were generated by Peaks PTM based on the search parameters previously detailed. Theoretical trypsin cleavage sites along with the cleavage probability of each site were determined using the ExPASy PeptideCutter tool (https://web.expasy.org/peptide_cutter/, accessed on 2019–11-15). Grand average of hydropathicity (GRAVY) scores were calculated using the amino acid values determined by Kyte and Doolittle (1982), where hydrophobic amino acids were assigned scores >0 and hydrophilic amino acids were assigned scores <0. To compare the effect each hydrolysis condition had on generating hydrophobic peptides, the GRAVY score distributions for specific proteins where visualized using box whisker plots generated using SigmaPlot (version 14). Finally, the Milk Bioactive Peptide Database (MBPDB; <http://mbpdb.nws.oregonstate.edu>; accessed on 2019–10-15) was used to identify bioactive peptides (Nielsen, Beverly, Qu, & Dallas, 2017). The database was searched to identify peptide sequences with a 100% similarity threshold to the database sequences, thus peptides with post-translational modifications were not considered.

3. Results and discussion

3.1. Effect of hydrolysis on protein profile

Protein/peptide profiles of PHWPC 8%, PHWPC 12%, and THWPC 5% (Fig. 1, lanes 4–6) demonstrated that Protease M and trypsin hydrolyzed α -lactalbumin (α -la) and β -lactoglobulin (β -lg) to varying degrees. Protease M preferentially hydrolyzed α -la as evidenced by a slight reduction in its band intensity in PHWPC 8% sample (lane 3), and a much reduced intensity in PHWPC 12% sample (lane 4). Protease M, to a lesser extent, hydrolyzed β -lg as noted by the slight reduction in its band intensity in both PHWPC samples. On the other hand, trypsin preferentially hydrolyzed β -lg as evidenced by the marked reduction in its band intensity, compared to only a slight reduction in α -la band intensity (lane 5). A similar observation of trypsin selectivity was reported by Pintado and Malcata (2000). Although the DH of THWPC is lower than that of both PHWPC samples, a higher smearing intensity below 10 kDa was noted for the THWPC 5% sample (compare lane 5 to lanes 3–4),

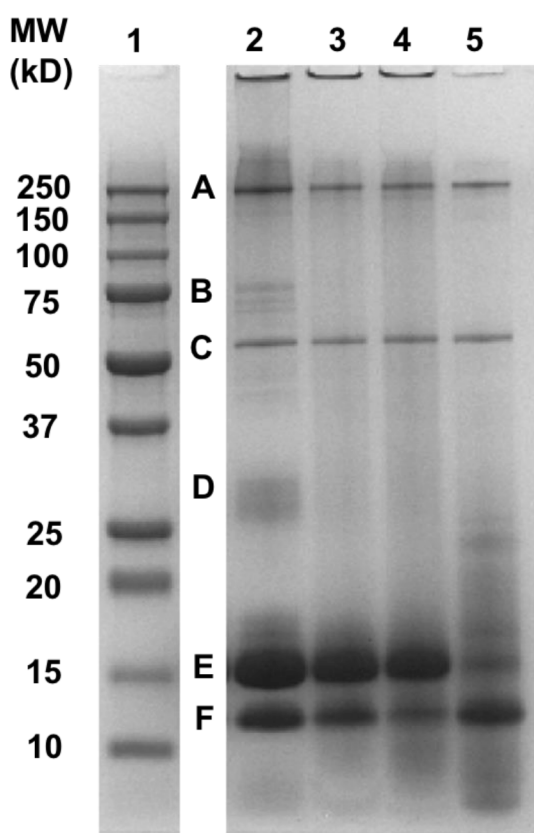


Fig. 1. SDS-PAGE profile of whey protein samples under non-reducing conditions. Lane 1: Molecular weight marker (MW); Lane 2: WPC; Lane 3: PHWPC 8%; Lane 4: PHWPC 12%; Lane 5: THWPC 5%; A: Immunoglobulins; B: Lactoferrin; C: Bovine Serum Albumin; D: Glycomacropeptide; E: β -lactoglobulin; F: α -lactalbumin.

confirming trypsin's endopeptidase dominant activity compared to Protease M.

Molecular weight distribution and hydrolysis patterns were confirmed using SE-HPLC (Fig. 2, Table 1). No major differences in soluble aggregates, immunoglobulins, and bovine serum albumins were noted among the hydrolysate samples (peaks 1–3). The significant increase in soluble aggregates in the hydrolysates compared to WPC, could be attributed to relative changes in the monomers (Table 1), and the possibility of some aggregation upon hydrolysis that may have involved intact proteins and potentially some peptides. However, in the presence of SDS, hydrophobic interactions would be disrupted, yet some disulfide linkages may have occurred during enzyme inactivation contributing to the observed increase in the soluble aggregates.

Because β -lg forms a dimer at close to neutral pH, it migrated through the column as a dimer in the absence of SDS in the sample buffer (McKenzie & Sawyer, 1967). The molecular weight of the β -lg dimer was not computed in THWPC sample due the absence of its respective peak, confirming the SDS-PAGE observation. The absence of the β -lg peak in the THWPC sample was coupled with a significantly higher peak area % of the peptide region in comparison to that of the PHWPC samples (Table 1). On the other hand, the β -lg dimer shifted to lower molecular weight in the PHWPC samples, potentially due to exopeptidase activity. No major change in β -lg peak area % was observed, confirming limited hydrolysis of β -lg by Protease M. Although the relative peak area % of α -la is significantly higher in THWPC compared to WPC, the reduction in peak height (Fig. 2) indicated that trypsin did partially hydrolyze α -la. The relative peak area % distribution shifted due to lack of β -lg peak. The selectivity of Protease M to α -la was more pronounced as evidenced by the significant reduction in α -la peak area % in PHWPC samples

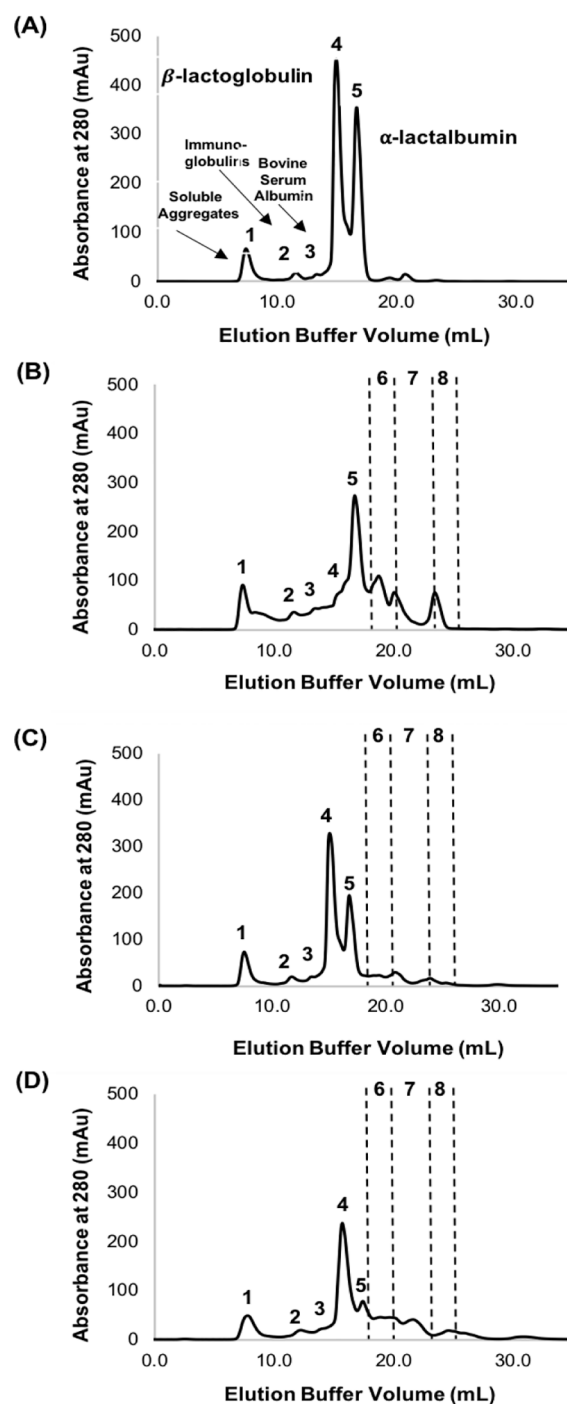


Fig. 2. SE-HPLC chromatograms for (A) WPC; (B) THWPC 5%; (C) PHWPC 8%; (D) PHWPC 12%; Peptide region is denoted by Peaks 6 – 8 with dashed lines indicating boundaries of the fractions collected for LC-MS/MS analysis.

compared to WPC. The increase in peak area % of the peptide region, however, was significant for PHWPC 12% and not for PHWPC 8%. Additionally, there was a significant reduction in the molecular weight of α -la in the PHWPC 12% sample. Both observations indicated that at a higher DH, endopeptidase activity of Protease M was evident in addition to exopeptidase activity. Exopeptidase activity results in the release of terminal amino acids, dipeptides, and some tripeptides, leaving behind polypeptides closer in molecular weight to the intact protein, which may explain the shifts in molecular weights observed. Singular amino acids, dipeptides, and tripeptides would elute past the final collected fractions (peaks 6–8), and may not contain aromatic amino acids, hence will not

Table 1

Molecular weight and peak relative % area of the different protein components in WPC and HWPC samples.

Sample	Soluble Aggregates		Immunoglobulins		Bovine Serum Albumin		β -lactoglobulin dimer		α -lactalbumin		Peptide Region ¹ Fractions 6–8 PeakArea (%)
	Peak 1 Mwt (kDa)	PeakArea (%) ²	Peak 2 Mwt (kDa)	PeakArea (%)	Peak 3 Mwt (kDa)	PeakArea (%)	Peak 4 Mwt (kDa)	PeakArea (%)	Peak 5 Mwt (kDa)	PeakArea (%)	
WPC	1650 a	7.8c	210 a	1.4 a	87.7 a	0.92b	39.1 a	51.9c	16.9 a	35.8b	2.3c
THWPC 5%	1660 a	15.7 a	202 a	1.6 a	84.0 a	1.8 a	Hydrolyzed		16.0 a	50.6 a	28.9 a
PHWPC 8%	1590 a	11.8b	202 a	1.2 a	84.0 a	0.54b	33.8 ab	55.3b	16.2 a	26.5c	4.7c
PHWPC 12%	1390 a	15.9 a	131b	1.2 a	66.3b	0.02c	26.7b	60.1 a	11.8b	10.5 d	12.7b

¹ A single molecular weight was not calculated for the peptide region because it comprises a range of molecular weights where fraction 6 has a range of 8.5–2 kDa, fraction 7: 2–0.5 kDa, and fraction 8: <0.5 kDa; ²Peak relative area (%) is the area of a specific peak divided by the total peak area for that sample; ³Lowercase letters in each column indicate significant differences among samples according to the Tukey-Kramer HSD means comparison test ($P \leq 0.05$).

be detected at 280 nm. Endopeptidases, on the other hand, cleaves at multiple sites resulting in a wide range of smaller peptides in comparison to the intact protein. Such smaller peptides were represented in peaks 6–8. When both activities are present, the exopeptidase may also further hydrolyze peptides generated by endopeptidases. Shifts in molecular weights and relative peak areas coupled with SDS-PAGE observations confirmed differential selectivity, with trypsin having endopeptidase activity targeting mostly β -lg, and Protease M having exopeptidase activity predominantly with some endopeptidase activity targeting mostly α -la. Previous research showed that trypsin preferentially hydrolyzed β -lg over α -la (Deng et al., 2018; Gauthier & Pouliot, 2003). Protease M, on the other hand, was shown to preferentially hydrolyze α -la over β -lg, resulting in 13% and 50% residual intact protein, respectively (Lisak Jakopović, Cheison, Kulozik, & Božanić, 2019). Trypsin cleavage is influenced by the primary structure, specifically the number of Lys and Arg residues, accessibility to the binding sites, their abundance in one substrate compared to another, and the concentration of each substrate, especially when both are present during the hydrolysis fixed time. Its preference for β -lg over α -la can, therefore, can mostly be attributed to β -lg having more potential cleavage sites compared to α -la, 19 vs. 13 Lys and Arg residues, respectively. The amino acids on either side of Lys or Arg and the amino acid with which either formed the scissile bond (i.e. the peptide bond cleaved) also affects the likelihood and rate of trypsin hydrolysis. α -la has a number of cleavage sites with charged amino acids positioned adjacent to the scissile bond (i.e. P2 and P2'), which is known to slow trypsin activity (Deng et al., 2018). In the case of Protease M, the hydrolysis was performed at pH 6, where β -lg would have been present as a dimer while α -la would have been a monomer. Thus, Protease M may have had better access to cleavage sites on α -la compared to β -lg.

3.2. Enzyme specificity as determined by proteomics

Proteomics analysis of fractions 6, 7, and 8 of all three hydrolysates revealed the presence of peptides originating from several major and minor protein groups (Table 2). The complete list of identified proteins and peptides can be found in [supplementary information](#) in the MS results excel file. This observation confirmed that both enzymes targeted a wide range of proteins. Hydrolysates contained peptides originating from all of the major whey proteins (α -la, β -lg, immunoglobulins, bovine serum albumin, and lactotransferrin) and the four caseins (α -S1, α -S2, β , and K). Presence of casein peptides is not surprising. According to the manufacturer, WPC 80 contains an appreciable amount of glycomacropeptide (GMP), approximately 23–25% of the total protein. The presence of GMP in WPC was apparent in the SDS-PAGE gel (Fig. 1, lane 2) at around 25 kDa (Fariás, Martinez, Pilosof, 2010). WPC may also contain α -S1, α -S2, and β -casein proteins/peptides as a result of processing conditions (van Hekken & Holsinger, 2000) and general proteolysis during cheese making (Hayes & Nielsen, 2000). Other identified peptides originated from minor components such as endogenous enzymes (lactoperoxidase, xanthine oxidoreductase, catalase, amine oxidase, and fructose biphosphate aldolase) and minor proteins (B₂-microglobulin, osteopontin, and Vitamin D-binding protein).

In comparison to hydrolysis by Protease M, trypsin hydrolysis resulted in peptides originating from more protein groups and generated the most unique peptides, i.e. peptides associated with only one protein group, in each of the fractions (Table 2), despite the lower DH. However, the LC-MS/MS method detected mostly peptides with ≥ 7 amino acids. Accordingly, the exopeptidase activity of Protease M, which contributed to higher DH, was not reflected by the number of protein groups and peptides identified. Comparing PHWPC 8% to PHWPC 12%, the higher DH in this case contributed to the generation of a higher number of

Table 2Number of protein groups and unique peptides in fractions 6–8 of each hydrolysate along with the number of unique peptides and coverage (%) of α -lactalbumin and β -lactoglobulin as identified by LC-MS/MS.

Sample	Fraction ¹	Overall Proteins		α -lactalbumin ² P00711 LALBA_BOVIN ⁴		β -lactoglobulin ² G5E5H7 G5E5H7_BOVIN ⁴		# Unique Peptides
		# Protein Groups ²	# Unique Peptides ³	–10lgP ⁵	Coverage (%) ⁶	–10lgP	Coverage (%)	
THWPC 5%	6	88	486	74.46	20	3	294.81	62
	7	59	158	120.22	8	4	229.69	30
	8	13	32	67.81	7	3	114.86	14
PHWPC 8%	6	26	243	110.89	25	11	208.05	72
	7	15	71	64.49	20	4	160.16	28
	8	7	20	33.02	13	2	119.78	9
PHWPC 12%	6	36	415	169.16	47	26	287	149
	7	38	355	146.07	32	21	243.32	103
	8	11	32	N/A	N/A		117.32	15

¹ SE-HPLC peptide fraction that was collected and analyzed by LC-MS/MS; ² Includes the different variants of a protein as single, homologous group; ³ Number of unique peptide sequences associated with only one protein group in bovine (*Bos taurus*); ⁴ Gene identifiers and accession numbers for identification of proteins in the NCBI (G) and Swiss Prot (sp) databases; ⁵ Probability of a false hit calculated by Peaks, a peptide false discovery rate of 0.5% was selected; ⁶ Percentage of the intact protein sequence identified based on the detected peptide sequences

detected peptides originating from higher number of protein groups. While the presented LC-MS/MS data is qualitative (i.e. providing information on how many different proteins were hydrolyzed rather than how much), this data demonstrated that DH alone is not a good indicator of enzyme activity or specificity.

While both enzymes hydrolyzed a wide range of proteins, analyzing enzyme specificity will be focused on the major WPC components, α -la and β -lg. Proteomics data indicating specificity toward casein proteins can be found in [supplementary information \(Table S1\)](#), while that indicating enzyme specificity toward α -la and β -lg is presented in [Table 2](#). Based on coverage % and number of unique peptides from α -la in all fractions of THWPC 5% and PHWPC 8%, and fractions 6 and 7 of PHWPC 12% ([Table 2](#)), it is apparent that Protease M had greater affinity than trypsin to α -la, confirming SDS-PAGE ([Fig. 1](#)) and SE-HPLC results ([Fig. 2](#), [Table 1](#)). There were no unique peptides from α -la detected in fraction 8 of PHWPC 12%. This observation indicated that any potential α -la peptides initially generated by Protease M may have been further cleaved by its exopeptidase activity as hydrolysis progressed. When comparing coverage % and number of unique peptides from β -lg to those from α -la in all fractions of THWPC 5%, it seemed that trypsin had more affinity toward β -lg than α -la. The same assumption could be made for Protease M, especially that fractions 6 and 7 in both PHWPC samples had greater coverage % and number of unique peptides

from β -lg compared to their counterparts in the THWPC sample. These results appear to contradict the observations of SDS-PAGE and SE-HPLC, which indicated that β -lg was hydrolyzed to a greater extent by trypsin. Proteomics data, however, was not quantitative, in fact it revealed that the combined endo-/exopeptidase activity of Protease M resulted in a higher number (not quantity) of unique peptides from β -lg and a corresponding higher coverage %. For trypsin, which is an endopeptidase, assessing affinity is more straightforward using number of unique peptides and coverage %.

These differences in trypsin and Protease M specificity can be visualized using peptide coverage maps with identified cleavage sites ([Fig. 3](#) and [supplementary Figures S1 - S6](#)). Trypsin is known to selectively cleave at the C-terminal end of lysine and arginine residues ([Deng et al., 2018](#)). The selectivity of Protease M is not clear due to the presence of multiple enzymes from *Aspergillus oryzae*. To determine the possible enzymes present in Protease M, the reported optimal activity (pH 6 and 45 °C) and enzyme stability parameters (pH 3 – 6 and temperature < 65 °C) was cross-referenced with the stability and activity of the *Aspergillus oryzae* enzymes listed in the BRENDA comprehensive enzyme database (<https://www.brenda-enzymes.org>; accessed on 2020-02-04). Accordingly, the endopeptidase activity could be due to aspartic acid protease (Aspergillopepsin I; EC 3.4.23.18), and to a lesser extent neutral protease II (deutrolysin; EC 3.4.23.18). The exopeptidase activity could be

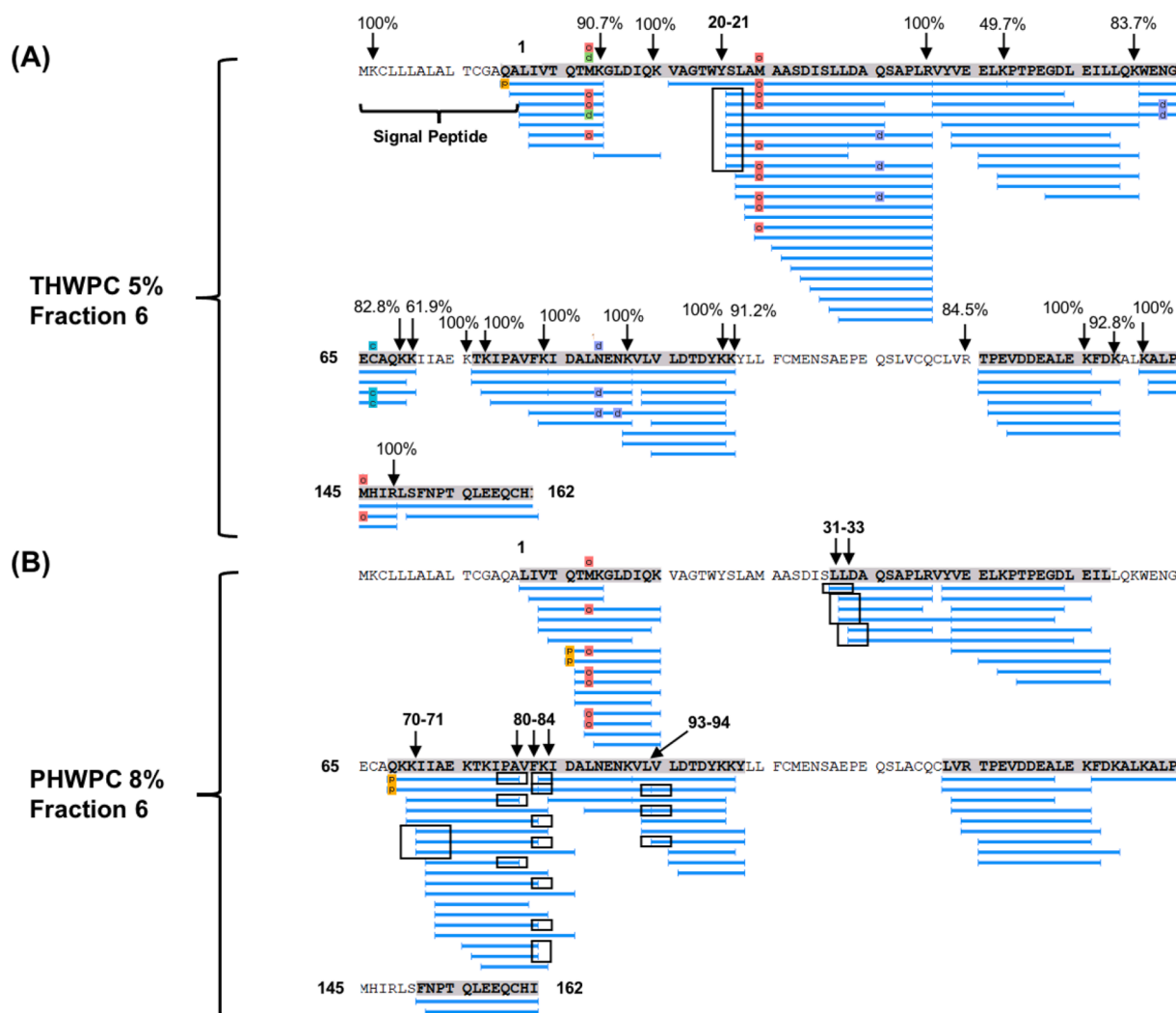


Fig. 3. Peptide coverage maps of β -lactoglobulin in fraction 6 of (A) THWPC 5% and (B) PHWPC 8%. Arrows designate possible trypsin cleavage sites, with percentages indicating the theoretical probability of cleavage. A boxed letter in the peptide sequence line denotes an amino acid that underwent a post-translational modification (each peptide sequence and its respective PTM can be found in the MS results excel file).

due to serine-type carboxypeptidase (EC 3.4.16.6), leucyl aminopeptidase (EC 3.4.11.1), and aspartyl aminopeptidase (EC 3.4.11.21). Aspartic acid protease, similar to pepsin, has a broad specificity favoring hydrophobic residues on either side of the scissile bond (P1 and P1' positions), with Leu-Val, Tyr-Thr, and Lys-Ile as targeted cleavage sites (Ichishima, 2013; Vishwanatha, Appu Rao, & Singh, 2009). Neutral protease II is a zinc metallopeptidase that also prefers hydrophobic residues and targets Asn-Gln and Gly-Ser cleavage sites (Sekine 1972). Serine-type carboxypeptidase cleaves C-terminal arginine and lysine, leucyl aminopeptidase preferably cleaves N-terminal leucine or proline, and aspartyl aminopeptidase cleaves N-terminal aspartate or glutamate (BRENDA, 2020).

Peptide coverage maps for β -lg (Fig. 3 A&B) were used to illustrate differences in specificity of trypsin and Protease M, utilizing fraction 6 from THWPC 5% and PHWPC 8%. The β -lg peptide coverage map for PHWPC 12% had similar cleavage sites to those shown for PHWPC 8% and can be found in [supplementary information \(Figure S1\)](#). The peptide coverage maps for α -la can also be found in [supplementary information \(Figures S5 and S6\)](#), and illustrate similar specificities for trypsin and Protease M. The peptide coverage maps include the signal peptide that is cleaved from the N-terminal end of the mature protein. Thus, the β -lg sequence shown in Fig. 3 has 178 amino acids but was renumbered to reflect that the mature β -lg protein has 162 amino acids. The peptide coverage map for THWPC 5% confirmed that trypsin cleaved at the C-terminal end of lysine and arginine residues, with at least one peptide generated from each theoretical trypsin cleavage site, except the site in the signal peptide region (Fig. 3A). The β -lg peptide coverage map shows 11 sites with 100% probability of trypsin cleavage, while α -la only has 5 (Figures S5 and S6), further supporting the observed preference of trypsin for β -lg. An unpredicted cleavage site observed at Tyr₂₀ – Ser₂₁ might have been the result of chymosin activity on β -lg during the cheese making process. Upon using chymosin to hydrolyze β -lg, [Dalsgaard and Larsen \(2009\)](#), identified a peptide with Tyr₂₀ at the C-terminal end (L₁₄KVAGTWY₂₀).

The endo-/exopeptidase activity of Protease M was also evaluated using the peptide coverage map of β -lg (Fig. 3B). One peptide was cleaved at the N-terminal end of Leu₃₁, three peptides cleaved at the N-terminal end of Leu₃₂, and two peptides cleaved at the N-terminal end of Asp₃₃, supporting the assumption that Protease M contains both leucyl and aspartyl aminopeptidases. The presence of both endo- and exopeptidase activity, coupled with the inability to detect tri-/di-peptides and single amino acid residues, made it difficult to differentiate cleavage sites that could have been attributed to initial endopeptidase activity. Therefore, cleavage sites of peptides identified from both sides of the scissile bond were examined for endopeptidase activity. Three peptides were cleaved at Lys₇₀ – Ile₇₁ and 3 peptides were cleaved at Leu₉₃ – Val₉₄. These bonds have been reported to be cleavage sites of aspartic acid protease, which is an endopeptidase (Ichishima, 2013; Vishwanatha et al., 2009). The endopeptidase activity of Neutral Protease II was not clearly detected since neither β -lg or α -la have any Asn-Gln and Gly-Ser bonds. However, there were three peptides cleaved at the highly hydrophobic Ala₈₀ – Val₈₁ bond, indicating the possible presence of Neutral Protease II. The presence of eight peptides cleaved at Phe₈₂ – Lys₈₃ indicated the combined effect of endo-/exo-peptidase activity. At least 6 of these peptides might have resulted from the exopeptidase activity of carboxypeptidase, which may have occurred post potential cleavage at Lys₈₃ – Ile₈₄ by aspartic acid peptidase.

3.3. Release of hydrophobic peptides

The differential effect of trypsin and Protease M hydrolysis on the hydrophobicity of the released peptides across fractions was demonstrated for β -lg using GRAVY score distributions (Fig. 4). The GRAVY scores of α -la peptides are found in [supplementary information](#) in the MS results excel file. The median GRAVY scores for β -lg peptides in all THWPC 5% fractions were close to zero, indicating an even distribution

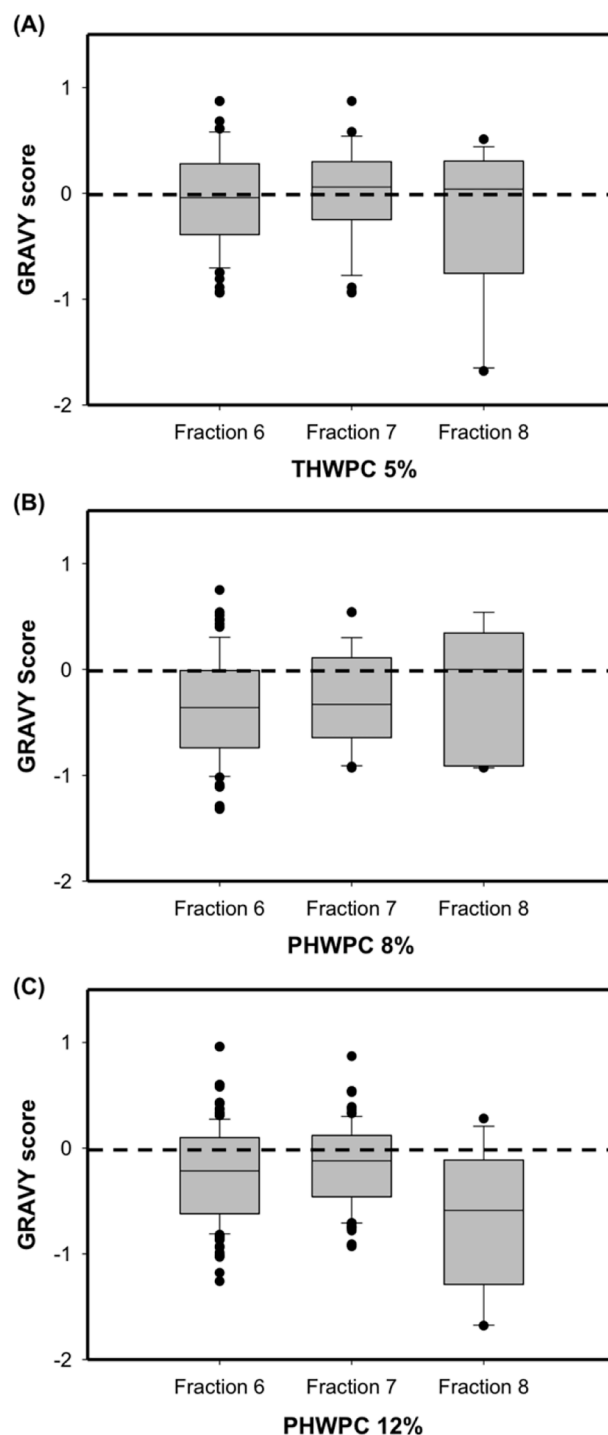


Fig. 4. Box plots showing the GRAVY (grand average hydropathicity) score distribution for peptides generated from β -lactoglobulin across all three peptide fractions for (A) THWPC 5%, (B) PHWPC 8%, (C) PHWPC 12%. Each dot represents a peptide outlier. Peptides with Scores > 0 are hydrophobic.

between hydrophobic and hydrophilic peptides (Fig. 4A). On the other hand, the median GRAVY scores for β -lg peptides in all fractions from both PHWPC samples were less than zero (Fig. 4 B&C), indicating a greater proportion of hydrophilic peptides released upon hydrolysis by Protease M. Peptides in fractions 6 and 7 of PHWPC 8% were particularly hydrophilic, with 75% of the scores falling below zero. Fraction 8 across all three hydrolysates had the widest distribution of GRAVY scores, which is mostly attributed to having the fewest peptides.

A comparison of the hydrophobicity and bitterness of whey protein

hydrolysates produced by trypsin and Protease M, has not been reported. Peptides with bulky, basic amino acids at the *N*-terminus are highly correlated with peptide bitterness (Kim & Li-Chan, 2009). Therefore, the bitterness of peptides released upon trypsin hydrolysis could be attributed to its preference for cleaving at the C-terminal end of lysine and arginine (Kim & Li-Chan, 2009). Additionally, the position and size of hydrophobic amino acids can influence peptide bitterness. For example, hydrophobic amino acids located at the ends of a peptide chain tends to be mildly bitter, except when proline or other bulky hydrophobic amino acids, like phenylalanine, are located at the *N*-terminus (Kim & Li-Chan, 2006; Ishibashi et al., 1988). Aspartic acid protease, a suspected endopeptidase in Protease M, preferentially cleaves at hydrophobic amino acids sites, resulting in the release of less hydrophobic and less bitter peptides, unless the released peptides have a bulky, hydrophobic amino acid at the *N*-terminus. The exopeptidase activity of Protease M specifically that of leucyl aminopeptidase, which cleaves hydrophobic *N*-terminus amino acids, could have resulted in the release of less hydrophobic peptides. The effectiveness of similar exopeptidases in reducing the bitterness of trypsin hydrolysates was demonstrated by Tan et al. (1993). Similarly, leucine aminopeptidase from *Aspergillus sojae* reduced the bitterness score of hydrolysates produced by the endopeptidase Neutral Protease I, despite increasing the DH from 7.7% to 18.1% (Huang et al., 2015). The observed decrease in bitterness was attributed to the efficient cleavage of Leu, Met, and Phe at the *N*-terminus by leucine aminopeptidase. Therefore, despite the higher DH of the PHWPC samples compared to THWPC, the presence of exopeptidase in Protease M contributed to the release of less hydrophobic peptides that would potentially be less bitter.

3.4. Bioactivity of the released peptides

Thirty-three bioactive peptides were identified with antimicrobial, antidiabetic, antithrombic, hypotensive, immunomodulatory, and opioid bioactive properties. Of those 33 peptides, 15 bioactive peptides originated from β lg and α -la, as illustrated in [supplementary information \(Tables S2 and S3\)](#). Nine peptides were found in fractions from both Protease M and trypsin hydrolysates (Table S2), while 6 peptides were unique to either Protease M or trypsin hydrolysate (Table S3). Two of the β -lg bioactive peptides, TPEVDDEALEK and VLVDLDYK, were found in all nine fractions of the three hydrolysates. The remaining bioactive peptides, originating from K-casein, β -casein, α -S1-casein, and α -S2-casein, can also be found in [supplementary information \(Tables S4 and S5\)](#).

Of the 9 bioactive peptides found in both Protease M and trypsin hydrolysates, 8 originated from β -lg (Table S2), which is not surprising since trypsin preferentially targeted β -lg. All of the 9 bioactive peptides had a lysine or arginine residue at the C-terminus. The proposed serine-type carboxypeptidase activity of Protease M resulted mostly in peptides with lysine and arginine at the C-terminus, which potentially resulted in Protease M and trypsin hydrolysates having shared bioactive peptide sequences. These results, however, were not quantitative, thus it was not possible to determine which enzyme contributed to higher quantities of the common bioactive peptides. Of the 6 peptides identified in either Protease M or trypsin hydrolysate (Table S3), only 1 peptide, VYVEELKPTPEGDLLEILLQK, was unique to THWPC 5%, while 4 peptides were unique to PHWPC 12%, with three peptides, KGYGGVSLPEW, LKGYGGVSLPE, and DKVGINY, originating from α -la. The increased hydrolysis of α -la by Protease M with the higher DH resulted in PHWPC 12% having the most unique bioactive peptides.

While peptides from different protein sources may have the same function, they could differ in potency. Angiotensin converting enzyme (ACE) inhibition was the most common bioactive function with 3 ACE inhibitory peptides, LDAQSAPLR, VLDTDYK, and VGINYWLAHK, found in both Protease M and trypsin hydrolysates (Table S2) and 3 peptides, DAQSAPLRVY, KGYGGVSLPEW, and DKVGINY, unique to Protease M hydrolysates (Table S3). The activity of ACE inhibitory peptides

generally involves the three terminal C-residues, with potency influenced by the composition of these residues (López-Fandiño, Otte, & van Camp, 2006). Proline as one of the three terminal residues has been shown to be important. Only two of the ACE inhibitory peptides, LDAQSAPLR found in all three hydrolysates and KGYGGVSLPEW in PHWPC 12%, had proline as one of the three C-terminal residues. Aromatic amino acids, leucine, and the positively charged lysine and arginine residues at the C-terminus also appear to contribute to potency (López-Fandiño, Otte, & van Camp, 2006). The C-terminus of the three ACE inhibitory peptides found in trypsin and Protease M hydrolysates had lysine or arginine, while the three unique peptides in Protease M hydrolysates had aromatic tyrosine and tryptophan at the C-terminus. Cleaving at the C-terminal end of hydrophobic amino acids by aspartic acid protease and neutral protease II present in Protease M, potentially increased the potency of the ACE inhibitory peptides.

The second most prevalent bioactive function was inhibition of DPP-IV (dipeptidyl peptidase-IV), which helps regulate blood glucose levels. The increased level of Protease M hydrolysis again resulted in PHWPC 12% having unique peptide sequences, LKPTPEGDL and LKGYGGVSLPE, with DPP-IV inhibitory action (Table S3). Dipeptides such as His-Leu, Val-Ala, and Tyr-Val and single amino acids such as Try, Met, and Leu were found to be potent DPP-IV inhibitors (Nongonierna & FitzGerald, 2013; Nongonierna, Mooney, Shields, & FitzGerald, 2013). However, the single amino acids and dipeptides, released by the exopeptidase activity such as that of leucine aminopeptidase in Protease M, were undetected by the LC-MS/MS method employed, but could have further contributed to the potency of the Protease M hydrolysates. Measuring the bioactivity of the trypsin and Protease M hydrolysates would be needed to determine the actual potency of the present peptides. These results, nevertheless, are a benchmark that demonstrates the usefulness of proteomics as a tool for targeted bioactive peptide production.

4. Conclusions

Following a proteomics approach, this work successfully demonstrated the differences between trypsin and Protease M in their hydrolysis pattern and preference toward β -lg and α -la. Results highlighted the potential benefits of using a mixture of endo-/exopeptidases. While this study did not investigate the functionality, bitterness, and bioactivity of the hydrolysates, it provided fundamental knowledge needed to predict and understand their performance in future work. The demonstrated approach will allow a variety of hydrolysate conditions to be pre-screened for potential bitterness by calculating GRAVY score distributions, reducing the cost and minimizing the challenges (i.e. food grade production, large quantities) of sensory analysis. This approach can also be used to screen hydrolysates for a specific bioactivity, such as ACE-inhibitory activity, to produce a hydrolysate for use as a nutraceutical. Accordingly, the proteomics approach employed will allow the optimization of whey protein hydrolysis for targeted applications in a direct and efficient way compared to traditional approaches.

CRedit authorship contribution statement

Chelsey Hinnenkamp: Data curation, Methodology, Formal analysis, Writing - original draft. **Baraem P Ismail:** Conceptualization, Funding acquisition, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This project was generously funded by Midwest Dairy Association™. The authors acknowledge LeeAnn Higgins and Todd Markowski or their assistance with MALDI-TOF MS and HPLC-MS/MS analyses, and Professor Stephan Drusch at TU Berlin for assistance with the SE-HPLC method.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.129235>.

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