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Peptidomic profile of milk of Holstein cows at peak lactation

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Abstract

Bovine milk is known to contain naturally occurring peptides, but relatively few of their sequences have been determined. Human milk contains hundreds of endogenous peptides and the ensemble has been documented for antimicrobial actions. Naturally occurring peptides from bovine milk were sequenced and compared with human milk peptides. Bovine milk samples from six cows in second stage peak lactation at 78–121 days post- partum revealed 159 peptides. Most peptides (73%) were found in all six cows sampled, demonstrating the similarity of the intra-mammary peptide degradation across these cows. One peptide sequence, ALPIIQKLEPQIA from bovine perilipin 2 was identical to another found in human milk. Most peptides derived from β-casein, α_{s1}-casein and α_{s2}- casein. No peptides derived from abundant bovine milk proteins like lactoferrin, β- lactoglobulin and secretory immunoglobulin A. The enzymatic cleavage analysis revealed that milk proteins were degraded by plasmin, cathepsins B and D and elastase in all samples.

Keywords

eptidomics; bovine milk; bioactive peptides; protease

INTRODUCTION

The peptides that are naturally occurring in bovine milk have not been completely identified, nor have they all been investigated for biological activity. In this study, breakthroughs in

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The authors declare no competing financial interest.

ASSOCIATED CONTENT

Additional information as mentioned in the text. The supporting information includes a list of all peptides identified including their sequence, modifications and peak volume in each of the bovine milk samples. This information is available free of charge via the Internet at http://pubs.acs.org.

analytical chemistry of peptides have enabled us to perform a peptidomic profiling of the endogenous peptides in bovine milk.

Human milk contains hundreds of naturally occurring peptides released by plasmin and other milk proteases within the mammary gland [1, 2]. Eighteen percent of the peptides in human milk had over 50% homology with known functional peptides [2]. Most of these peptides matched known antimicrobial peptides. Assays demonstrated that isolated ensemble of endogenous human milk peptides inhibited the growth of *Escherichia coli* and *Staphylococcus aureus*. As *S. aureus* and *E. coli* are common causative agents in human mastitis, these peptides may be a mechanism of maternal resistance to mastitis. This previous work in human milk demonstrated that naturally occurring milk peptides may influence the health of the mother and the infant.

The presence of active proteases—plasmin [3–5] (as well as tissue-type and urokinase-type plasminogen activators [6, 7]) and possibly active cathepsin B and cathepsin D [8–10]—in non-mastitic bovine milk suggested that naturally occurring peptides would also be present in bovine milk. The appearance of low molecular weight bands in gel electrophoresis experiments has suggested that naturally occurring peptides exist in healthy bovine milk: typically referred to as the proteose peptone fraction [11]. The proteose peptone fraction has been separated into components and, for some, their sequences determined, typically with gel electrophoresis and amino acid sequencing [12–14]. That research showed this fraction to be composed of casein degradation products and some whey proteins [15, 16]. A previous analysis of bovine milk peptides isolated by immobilized metal affinity chromatography and analyzed using matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF/TOF) mass spectrometry identified 15 peptides but only 2 complete sequences, both from α_{s1}-casein [17]. Eleven naturally occurring phosphorylated peptides and one non-phosphorylated peptide from bovine milk β- and α_{s2}-casein were identified with MALDI-TOF/TOF and ultra-high pressure liquid chromatography electrospray ionization mass spectrometry [18]. In another paper, 21 peptide sequences were identified in healthy bovine milk by LC-MS/MS [19].

This research analyzed naturally occurring peptides in bovine milk by a shotgun nano-liquid chromatography quadrupole time-of-flight tandem mass spectrometry approach. The cleavage sites were matched to cleavage patterns of known milk enzymes to determine which were likely responsible for protein cleavages in the mammary gland.

MATERIALS AND METHODS

Sample collection

Bovine milk samples were collected from the UC Davis Dairy Barn in the winter from six Holstein cows. All six cows were deemed mastitis-free based on the following criteria: 1) prior to milking, each teat was checked to ensure there was no abnormal milk (*e.g.* watery (serous) milk, flakes or clots) or issues in the quarter (*e.g.* swelling, redness); 2) after milking, each pre-tank filter was checked to ensure no abnormal milk entered the tank; 3) milk weights were recorded and any cows with low milk production are checked again for mastitis. None of the cows used in this study had abnormal milk, udder swelling or low milk

production. In addition, samples from the milk tank were cultured raw for total bacteria and coliform bacteria counts and then lab pasteurized for thermophilic bacterial counts. All bacterial counts were normal. All cows sampled were lactating for the second time (second lactation period). Milk samples represented a range of days post-partum: 78–121 days. Animals were kept in free stall housing, fed total mixed ration (a mixture of grain (barley and corn), alfalfa, cottonseed, almond hulls, oat hay, added minerals and salts, beet pulp and canola meal), and had access to water *ad libitum.* Cows were milked twice daily, at 4 a.m. and 4 p.m. in a milking parlor managed according to American Association for Accreditation of Laboratory Animal Care guidelines. Samples employed in this study are all from 4 p.m. milkings. Cows were milked with automated milking machines (Metatron, GEA Farm Technologies) from all four quarters at once. The resulting stream of milk was split from delivery to the bulk tank throughout the pumping process to provide samples representative of each cow's individual milk for this study. Before attaching the pumps, all four teats were washed first with water and then with an antiseptic solution (Chlorhexidine Active Mastitis Prevention) with 0.5% chlorhexidine gluconate, a chemical antiseptic, as the active ingredient. The time from initiation of suction to end of milking was 5.5–6.5 min. The cows produced from 41–52 lbs. of milk. After pumping, milk was immediately placed in a 100°C water bath for 4 min to deactivate the milk proteases. Samples were then transferred to a −20°C freezer.

Sample preparation

Samples were removed from the freezer and allowed to thaw at room temperature. Then, they were vortexed for 3–4 min each. Peptides were extracted according the procedure of Dallas et al. [2] but adapted to a 96-well plate format for increased sample preparation speed, reproducibility and throughput. Briefly, 50 μl of each milk sample were added to the 96-well plate with 150 μl of nanopure water followed by pipette mixing. The 96-well plates were centrifuged at 2,800×g for 30 min at 4°C, and 160 μl of the infranate (skim milk) was removed from the upper lipid layer by multi- channel pipette and added to a second 96-well plate. Proteins were precipitated with addition of 160 μl of 200 g/L trichloroacetic acid (TCA). A plate cap was added and the samples were vortexed for 5 min. After centrifugation at 2,800×g for 30 min at 4°C, the peptide supernatant was collected. The peptide supernatant was cleaned up on a C18 solid phase extraction column plate to remove salts, sugars and TCA. Columns were prepared with 99.9% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) (v/v), followed by 1% ACN, 0.1% TFA (v/v). The peptide samples were then added. Three column volumes (200 μl) of 1% ACN, 0.1% TFA (v/v) were added to remove sugars, salts and TCA. Then, three column volumes of 80% ACN, 0.1% TFA (v/v) were used to elute peptides. Solvents were passed through the columns by centrifugation for 30s at 2,800×g without heating. Peptides were dried down in the plate by centrifugal evaporation. Peptides were resolubilized in 20 μl of nanopure water with 5 min sonication and 30 min of plate vortexing. Samples were then centrifuged at 16,000×g for 30 min to pull down any remaining solids. Supernatants were transferred to sampling vials for Q-TOF analysis.

Mass spectrometry

Peptides were analyzed by mass spectrometry according to the procedure of Dallas et al. [2]. Samples were injected on the Agilent 6520 (Santa Clara, CA) nano-LC-chip-Q-TOF

MS/MS (Chip-Q-TOF) with an Agilent chip C18 column. Changes to the procedure in Dallas et al. [2] include using a drying gas flow rate of 3 L/min and a spectral acquisition rate of 1 spectra/s. The gradient elution solvents were (A) 3% ACN/0.1% FA (v/v) and (B) 90% ACN/0.1% FA (v/v). The gradient employed was ramped from 0–8% B from 0–5 min, 8–26.5% B from 5–24 min, 26.5–100% B from 24–48 min, followed by 100% B for 2 min and 100% A for 10 min (to re-equilibrate the column). Ion polarity was set to positive. The peak collection thresholds were set at 200 ion counts or 0.01% relative intensity for MS spectra and 5 ion counts or 0.01% relative intensity for MS/MS. Automated precursor selection based on abundance was employed to select peaks for tandem fragmentation with an exclusion list consisting of all peptides identified in previous analyses in this study. The acquisition rate employed was .63 spectra/s for both MS and MS/MS modes. The isolation width for tandem analysis was 1.3 m/z. The collision energy was set by the formula (Slope)*(m/z)/100+Offset, with slope = 3.6 and offset = −4.8. Five tandem spectra were collected after each MS spectrum, with active exclusion after 2 MS/MS for 2 min. Precursor ions were only selected if they had at least 1000 ion counts or 0.01% of the relative intensity of the spectra.

Spectral analysis

Spectra were analyzed by database search according to the procedure of Dallas *et al*. [2]. Briefly, data was exported from Agilent MassHunter in .mgf format and imported into the offline search engine X!Tandem [20]. X!Tandem searches were against a bovine milk library compiled from previous bovine milk proteome studies [21–23]. Peptides were accepted if e-values were less than or equal to 0.01, corresponding to a 99% confidence level. Masses were allowed 20 ppm error. No complete (required) modifications were included but up to four potential modifications were allowed on each peptide. Potential modifications allowed were phosphorylation of serine, threonine and tyrosine; oxidation of methionine and tryptophan; and deamidation of asparagine and glutamine. A non-specific cleavage ([X]|[X]) (where ‘X’ is any amino acid) was used to search against the protein sequences. Because the instrument did not always select the monoisotopic ion for tandem fragmentation, isotope errors were allowed (allowing up to one C13). No model refinement was employed in X! Tandem.

Library search

X!Tandem peptide results were used to construct a peptide library from all samples. Duplicates with the same sequence, protein, and modifications were removed. In addition, all peptides that were the same except for the position of the modification were reduced to a single entry for each. The library information consisted of neutral mass, retention time and a peptide ID. Compounds were identified from MS-only runs of all 6 samples by the “Find by Molecular Feature” function in Agilent MassHunter Qualitative Analysis version 6. The target data type was small molecule (chromatographic). Retention time was restricted from 3.5–36 min (the range of library peptides). Only peaks with at least 1,000 ion counts were selected. The selected charge carrier was “protons”. The isotope model was “peptides” and the maximum assigned charge was 7. After compounds were extracted, they were matched to the peptide library by mass and retention time to identify peptides. The database match employed a mass error tolerance of 40 ppm and a retention time tolerance of 1 min. Each

exact peptide mass in the library was converted to an m/z for all charge states from 1–7 for the search. All amino acid sequence ranges expressed throughout this article will include the signal sequence.

Enzyme analysis

A custom program written in Python was used to estimate the activity of selected enzymatic systems. This program works with the output from the peptide library from an individual trial. Each peptide is assigned a location within a protein based on the unique sequence in order to determine the sequence context of the peptide. After assigning context, both termini of each peptide are compared to a selected set of proteolytic enzyme rules. As a measure of simplification, rules were assumed to only act on P1 and P1'. P1 is the amino acid directly before the cleavage site and P1' is the amino acid directly after the cleavage site, where the protein *N*-terminus is on the left and the *C*- terminus is on the right. Enzymatic cleavage rules were derived from a list published on ExPASy [24]. The enzyme specificity patterns used in the algorithm for evaluating cleavages are shown in Table 1. Peptides having termini that pass a comparison to an enzymatic rule have their mass spectral intensity (peak volume) added to the sum of the respective enzyme. In the case that a peptide matches multiple rules simultaneously, the full intensity of that peptide are added to both enzymatic sums, thus the intensity value output represents potential activity rather than uniquely specific activity. Peptides failing all enzymatic comparisons have their intensity added to a list of remainders whose purpose is to assist in the identification of new enzymatic systems.

Homology searches for comparison of bovine and human milk peptides

Sequences identified in the bovine milk samples were searched for homology against endogenous peptides previously identified in human milk [2]. This search was performed with protein- protein BLAST. For each query, matches were retained only if peptides shared at least 50% identity (at least 50% of the amino acids were the same and in the same positions).

RESULTS AND DISCUSSION

The base peak chromatograms from all 6 samples (shown in Figure 1) have a substantial number of overlapping peaks. The large degree of overlap is interpreted to indicate that release of peptides in the mammary gland is thus similar across the six cows measured. The annotated tandem spectrum in Figure 2 demonstrates how peptide identifications were verified in an automated process in X!Tandem; in this case, confirmation of the presence of RGSKASADESLALGKPGKEPR from bovine fibroblast growth factor-binding protein 1 in all six samples from 8.3–8.8 min retention time.

The Nano-LC Q-TOF and database searching methodologies developed for peptidomic analysis identified 159 endogenous peptides in the six bovine milk samples. All 159 peptide sequences are shown in Supplementary Table 1. Previous research on endogenous bovine milk peptides revealed fewer peptides. One study employing MALDI-TOF/TOF identified 2 peptides sequences, both from α_{s1}-casein [17]. Another study employed MALDI-TOF and LC-MS to identify 11 phosphopeptides and 1 non- phosphorylated peptide [18]. Mansor *et al.*

identified 21 peptide sequences from healthy bovine milk by LC-MS/MS [19]. The increased number of peptides identified in the present research is likely due to improved identification methods.

Six of the peptides found in Mansor *et al.* were identified in the present research (identified in Supplementary Table 1). Most of the other peptides in the work of Mansor *et al.* matched to segments of the proteins identified here but were not identical sequence matches. The 2 bovine milk peptides found in Meltretter *et al.* were not found here but overlapping peptides were identified in the present data [17]. Of the 12 peptides found in Baum *et al.* [18] 3 were identical to those found here (2 phosphorylated, 1 non-phosphorylated) (see Supplementary Table 1), 4 overlapped but were incomplete matches, and 5 matched by sequence but had a lower degree of phosphorylation in the present paper.

The peptides identified ranged in charge states from 1 to 6. Peptide sequence length ranged from 8 to 45 amino acids. The average number of amino acids in a sequence was 16.7. Peptide mass ranged from 784.4 to 4416.2 Daltons. The average peptide mass was 1938.8 Da.

The majority of identified peptide sequences (73%) were present in all 6 samples. 16% were found in 5 of the 6 samples, thus 89% of peptides identified were found in at least 5 of the samples. This dataset was interpreted to indicate that the degradation processes within the mammary gland are relatively similar across these six healthy Holstein cows in second stage peak lactation. Analysis of more cows would be necessary to extend this interpretation to healthy Holstein cows in general, as the cows examined had similar feed and climate.

The parent proteins for all peptides identified are present in healthy bovine milk as protein fragments and as intact proteins. Gel electrophoresis implied the presence of these intact milk proteins [25]. In the present research, 15 proteins gave rise to endogenous milk peptides, including α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, glycosylation- dependent cell adhesion molecule 1, polymeric immunoglobulin receptor, fibroblast growth-factor-binding protein 1, lactoperoxidase, perilipin-2, butyrophilin and osteopontin. Previous research by Meltretter *et al.* identified only α_{s1}-casein-derived peptides [17]. The work of Baum et al. identified peptides only from α_{s2}-casein and β-casein [18]. Mansor *et al.* identified peptides from most of the proteins identified in the present research, but did not identify peptides from polymeric immunoglobulin receptor, perilipin-2 and butyrophilin [19]. The increased number of protein precursors identified in the present research compared to previous research is likely due to more exhaustive tandem fragmentation of peptide precursors and searching against the large in-house library of milk proteins with X!Tandem.

However, other high abundance milk proteins including lactoferrin, β-lactoglobulin and secretory IgA did not produce peptides. These proteins were also not present as peptides in human milk [2]. One peptide from β-lactoglobulin in healthy bovine milk in Mansor *et al.*, but, like the present research, they detected no peptides from lactoferrin or secretory IgA [19]. Likewise, these peptides were not identified in milk in the previous works of Meltretter *et al.* [17] and Baum *et al.* [18].

This variation in which proteins are degraded in the mammary gland could be due to protein structural differences. Milk enzymes may more easily degrade the looser three- dimensional structure of the caseins [2] than the more rigid, compact, globular structures of lactoferrin, β-lactoglobulin and sIgA [2]. The type and position of glycan post-translational modifications on lactoferrin and sIgA may also influence resistance to protein degradation.

Forty percent of all peptide sequences were derived from α_{s1}-casein. The number of peptides found for each protein is shown in Figure 3. In human milk, the majority of peptides identified in mothers studied were derived from β-casein [2], whereas α_{s1}-casein peptides were present but less abundant. The number of peptides identified from each protein were similar across the 6 bovine milk samples (Figure 3). This data is interpreted to indicate that protein degradation in the cow mammary gland is well regulated and controlled across cows. However, evaluation of more milks is necessary to confirm this interpretation, as these cows were exposed to identical feeding and climate conditions. This similarity of peptides released across bovine milk samples was mirrored in the results showing that human milk peptide profiles are highly similar between mothers [2].

Each sample had between 35–37 phosphorylated peptides. The total number of phosphorylated peptides for all 6 samples combined was 38 (24% of all peptides identified by count). The number of phosphopeptides identified here is higher than the 11 identified in Baum *et al.* [18]. Research by Meltretter *et al.* and Mansor *et al.* did not identify any phosphopeptides [17, 19]. The high number of phosphorylated peptides detected in the present research is not surprising as most of the phosphorylated peptides come from the four casein proteins, which are all known to be multiply phosphorylated [18]. Some of these phosphopeptides contained multiple phosphorylations–2 had 3 phosphorylations and 4 had 2 phosphorylations. This is less phosphorylation than found in the work of Baum *et al.*: they identified 9 peptides with 4 phosphorylations [18]. The presence of phosphorylations on caseins is known to allow formation of organophosphate salts for minerals, especially calcium [26]. Peptides containing multiple phosphorylation sites may bind calcium and aid in calcium delivery [27]. Other modifications found include methionine oxidation, deamidation of glutamine, *N*-terminal acetylation and pyroglutamination of glutamine. No glycosylated peptides were searched for with the methodology employed, therefore, no glycopeptides were identified.

Only one bovine milk peptide sequence matched exactly to a known endogenous peptide in human milk: ALPIIQKLEPQIA from human and bovine perilipin 2 (amino acids 65–77 in both species, including the signal sequence). This peptide was not identified in milk peptide profiles in Mansor et al. [19], Meltretter *et al.* [17] and Baum *et al.* [18]. No function has been ascribed to this peptide sequence. Though many bovine milk peptides derived from the same proteins as peptides found in human milk [2], the amino acids within these sequences differed, presumably due to evolutionary changes.

All 6 cows released peptides from the same locations within each protein (α_{s1}-, α_{s2}-, β-, κ-casein, osteopontin and glycosylation-dependent cell adhesion molecule 1), but with varying levels. None of the protein fragments were derived from the protein signal sequence of any of the proteins.

Enzyme cleavage

Plasmin (EC 3.4.21.7) was interpreted by computation to be a major agent of cleavage in the bovine mammary gland (see Figure 4). Plasmin is a serine proteases that cleaves on the *C*-terminal side of arginine or lysine [28]. Cleavage after arginine or lysine accounts for the majority of released peptides by count. Plasmin and trypsin cannot be differentiated with these methods, because both cleave after arginine or lysine; however, no evidence for trypsin in bovine milk exists [29]. Plasmin is known to be present and active in bovine milk [30]. Plasmin is thought to enter milk from the blood [30] and this interpretation is supported by the lack of mRNA for plasminogen in bovine mammary epithelial cells [31]. The remaining cleavage sites are interpreted to be created by elastase and cathepsins B and D (Figure 4) based on their match to the cleavage specificity of the remainder of cleavages. More cleavages were attributed to cathepsin D than cathepsin B for all milks. A separate column shows that many peptide cleavage sites could not be differentiated between cathepsins B and D. Likewise, several peptide cleavages were assigned to elastase in each milk sample and a substantial number of cleavages could not be differentiated between the cathepsins and elastase. Cathepsins D (3.4.23.5), cathepsin B (EC 3.4.22.1) and elastase (EC 3.4.21.36) have been proven to exist in milk via either sequencing or antibody recognition [32]. Other cathepsins (*i.e.* cathepsins L (EC 3.4.22.15) and G (EC 3.4.21.20)) may also be present in milk, but their presence has not been confirmed [32]. Therefore, in this analysis, only plasmin, cathepsin B, cathepsin D and elastase were assessed. Not all cleavage sites were matched to these enzymes: on average, 237.6 cleavage sites for each milk sample were assigned to plasmin, cathepsin B, cathepsin D or elastase. However, on average, another 108.3 cleavage sites could not be assigned to these enzymes. This finding suggests that other enzymes not assessed here (nor proven to exist in bovine milk) are active in the bovine mammary gland. Further research to determine which enzymes are responsible is warranted.

α_{s1}-casein

Isracidin, a peptide derived from α_{s1}-casein (RPKHPIKHQGLPQEVLNENLLRF, 16–38) was identified in the bovine milks. Isracidin is known to be active against *Staphylococcus aureus* and the yeast *Candida albicans* [33]. The signal sequence of α_{s1}-casein is amino acids 1–15, so in the terminology of this paper, the position would be 16–38 (which includes the signal sequence). This paper is the first to identify isracidin as a naturally occurring peptide in bovine milk. Previously, isracidin was identified after digestion with chymosin [33].

α_{s1}-casokinin-5 (FFVAP, 38–42) a known angiotensin converting enzyme inhibitory peptide [34] is found within the sequences of three identified peptides (38–49 **FFVAP**FPEVFGK, 38–51 **FFVAP**FPEVFGKEK, 38–54 **FFVAP**FPEVFGKEKVNE). Whether the FFVAP sequence will exhibit identical actions within a longer sequence is unknown. A closely overlapping peptide with these, α_{s1}-casein (39–52), was previously found in Mansor et al. [19]

α-casein exorphin (RYLGYLE, 105–111), a known opioid agonist peptide [35] from α_{s1}-casein is found within three sequences found (95–115 HIQKEDVPSE**RYLGYLE**QLLR, 95–117 HIQKEDVPSE**RYLGYLE**QLLRLK, 95–119 HIQKEDVPSE**RYLGYLE**QLLRLKKY). It is not known whether these longer sequences

will retain the opioid inhibitory function. RYLGYLE has naloxone-inhibitable opioid-like properties [36]. RYLGYLE is a δ-selective opioid receptor ligand [35]. Opioid receptors are found in the digestive tract and the brain.

α_{s1}-immunocasokinin-6, TTMPLW (208–214) from α_{s1}-casein which has angiotensin-converting enzyme inhibitory [36] and immunomodulatory function [37] is present within two sequences (187–214, 191–214). α_{s1}-immunocasokinin-6 is known to decrease proliferation of lymphocytes [38], decrease proliferation of human peripheral blood mononuclear cells [39], increase antibody formation and macrophage phagocytosis [40], and decrease *Klebsiella pneumoniae* infection in mice [41, 42].

Caseicin B (EVFGKEKVN, 45–53) from α_{s1}-casein inhibits the growth of *E. sakazakii* of α_{s1}-casein and is found within the sequence identified 40–54. Whether this longer sequence has antimicrobial function is unknown.

β-casein—Peptide 16–39 from β-casein (RELEELNVPGEIVESLSSSEESIT) is a known caseinophosphopeptide which binds to calcium with its phosphorylation sites [37]. This binding of peptides to calcium hinders calcium phosphate precipitation in the milk, increasing the bioavailability of this mineral [43]. This peptide is present within the sequences of 5 peptide sequences found in this dataset (16–40, 16–43, ranging from 1–3 phosphorylations).

Casoparan (41–45, INKKI) [44] is present within one larger sequence detected in β- casein: **INKKI**EKFQSEEQQQTEDELQDK (41–63). Casoparan activates macrophages to increase their phagocytic activity and peroxide release. Casoparan also acts as a bradykinin-potentiating peptide (i.e. increase the duration or magnitude or bradykinin's vasodilatory effects) [44]. Whether casoparan is functional within this larger sequence is unknown.

Casohypotensin (129–136, YPVEPFTE) [45], another bradykinin-potentiating peptide and a competitive inhibitor of angiotensin converting enzyme is present within longer sequences in the identified β-casein protein fragment: HKEMPFPK**YPVEPFTE**SQSL (121–140). Whether casohypotensin is functional within this larger sequence is unknown.

β-casokinin-7, an ACE inhibitor [46] from β-casein AVPYPQR 192–198 is present in several larger peptides in bovine milk but missing the initial Alanine as 193–201 **VPYPQR**DMP and 193–204 **VPYPQR**DMPIQA. Whether the missing Alanine is important to function as an ACE inhibitor is unknown. In addition, β-casein (193–209) in previous literature has been shown to have bitter taste perception [47]. β-casein (193–204) found here may also have bitter taste.

Casecidin-15 YQEPVLGPVRGPFPI (208–222) from β-casein has been shown to be antimicrobial towards *Escherichia coli* [48] and is present in the identified peptide sequences but lacking two amino acids: β-casein (210–222). A similar peptide, casecidin- 17 YQEPVLGPVRGPFPIIV (208–224) that is also antimicrobial toward *E. coli* [48] is also present but missing two amino acids: β-casein (210–224).

α_{2S}-casein—A known antihypertensive peptide from α_{s2}-casein (TKVIP, 213–217) was present within an identified peptide sequence α_{s2}-casein (**TKVIP**YVRYL, 213–222). Whether this longer sequence will have antihypertensive function is unknown.

As endogenous milk peptides could be released by a variety of proteases, a non- specific cleavage pattern was necessary for the X!Tandem searches. Searching X!Tandem with a non-specific cleavage (rather than the typical tryptic cleavage employed for proteomic studies) against the full bovine proteome with modifications allowed resulted in a failed search because the search space was too large for the program. Therefore, to reduce the search space, a smaller list of proteins compiled all proteins identified in proteomic studies of bovine milk was used. This restriction of the protein library used may have affected the results. Improvements in database searching speed would allow more thorough analysis of non-specifically cleaved peptides.

Some endogenous milk peptides may be glycosylated. However, peptides with unknown glycosylation cannot be identified currently with X!Tandem. In addition, identification of glycopeptides with our approach is difficult as CID leads to mostly glycosidic cleavages rather than peptide chain cleavages [49]. This pattern allows identification of the glycan but not the peptide. The use of an alternate fragmentation strategy, such as electron transfer detection, which fragments both the glycan and peptide moieties [49] could resolve this problem. In addition, glycopeptides ionize poorly in comparison with peptides [49], thus making them difficult to detect without prior fractionation to increase their abundance. Identification of non-specifically cleaved naturally occurring glycopeptides from a complex biological mixture remains a major challenge in glycobiology [50].

The results of these healthy cow's milk are likely to differ from milks of cows with mastitis, an inflammation of the mammary gland. As somatic cell counts were not measured in these milks, we cannot rule out the possibility that some of these cows had subclinical mastitis, which could affect the results. Milks from cows with mastitis are known to have up-regulated plasmin, cathepsin and elastase [32, 51, 52] as well as urokinase-type plasminogen activator [7]. In addition, invading bacteria present in mastitis are also known to release exogenous enzymes into the milk, including elastase [53]. Previous work has shown that mastitic milks, compared to healthy milks, significantly differ in relative abundance of 33 identified peptide sequences [19]. All six of the peptides identified in this work that matched those identified in Mansor et al. were found in both healthy and mastitis cows but at different levels. As absolute quantification is employed for neither the work of Mansor et al. nor the present research, abundance of peptides between these cows and the mastitic cows in the previous work are not comparable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

MALDI-TOF/TOF	matrix-assisted laser desorption/ionization tandem time-of-flight
ACN	acetonitrile
TFA	trifluoroacetic acid
FA	formic acid
Q-TOF	quadrupole time-of-flight
Da	Dalton
GlyCAM-1	glycosylation-dependent cell adhesion molecule 1.

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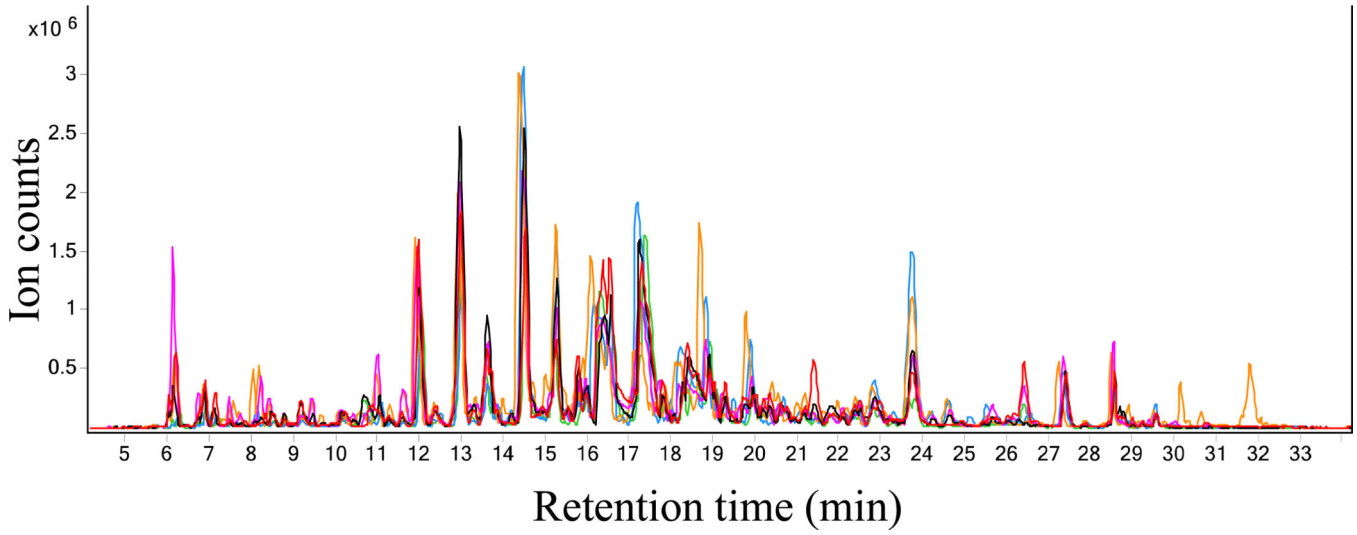

Figure 1.
Base peak chromatograms of all 6 bovine milk samples overlapped.

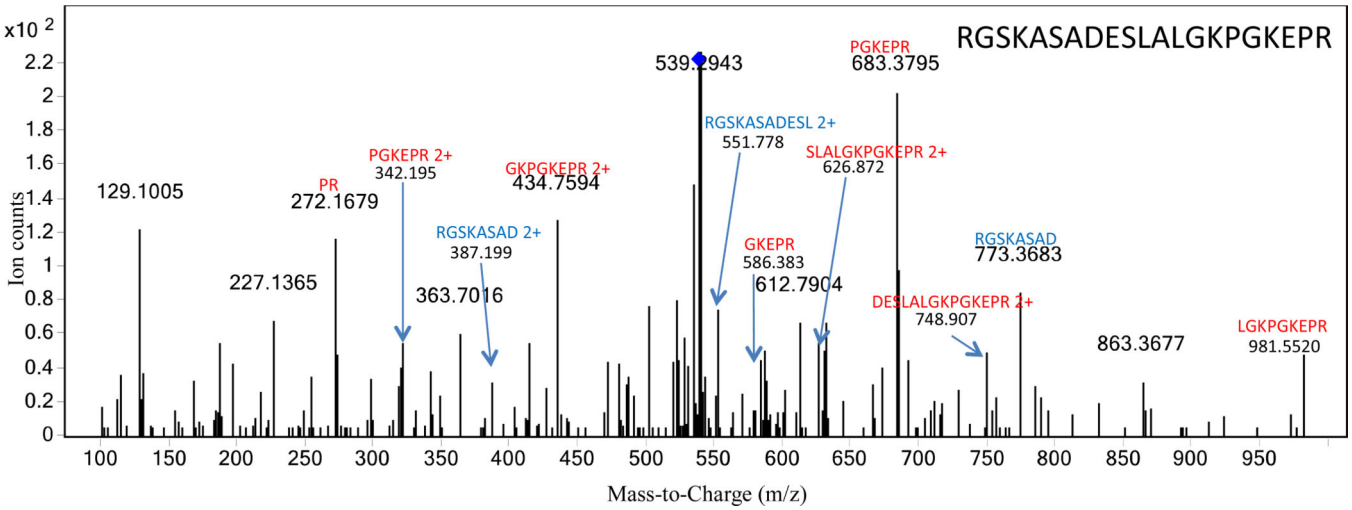

Figure 2.

Tandem fragmentation of 539.29 m/z (z=4) at one time point within its 8.3–8.8 minutes retention time with y-type ions in red, b-type ions in blue and the precursor ion denoted by a blue diamond. The tandem fragmentation analysis confirms the presence of peptide RGSKASADESLALGKPGKEPR from fibroblast growth factor-binding protein 1.

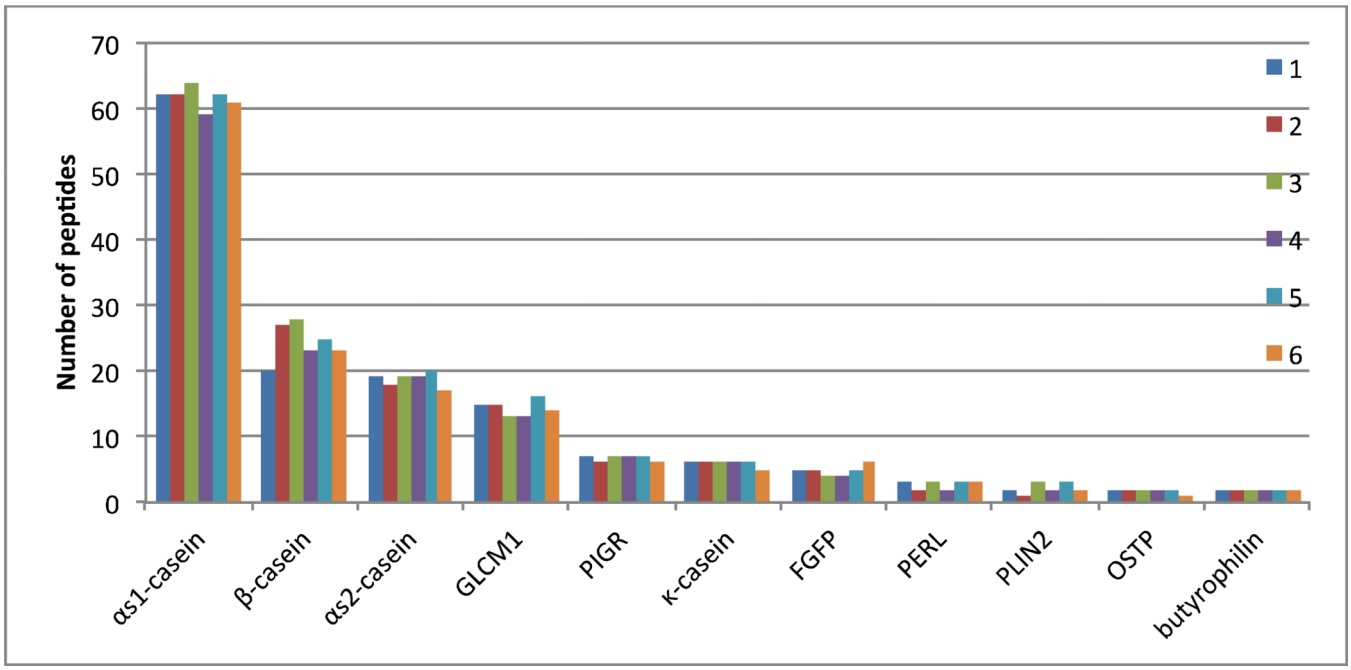

Figure 3.

Number of peptides identified for each protein by each sample. GLCM1, glycosylation-dependent cell adhesion molecule 1; PIGR, polymeric immunoglobulin receptor; FGFP, Fibroblast growth factor-binding protein 1; PLIN2, perilipin-2; PERL, lactoperoxidase; OSTP, osteopontin.

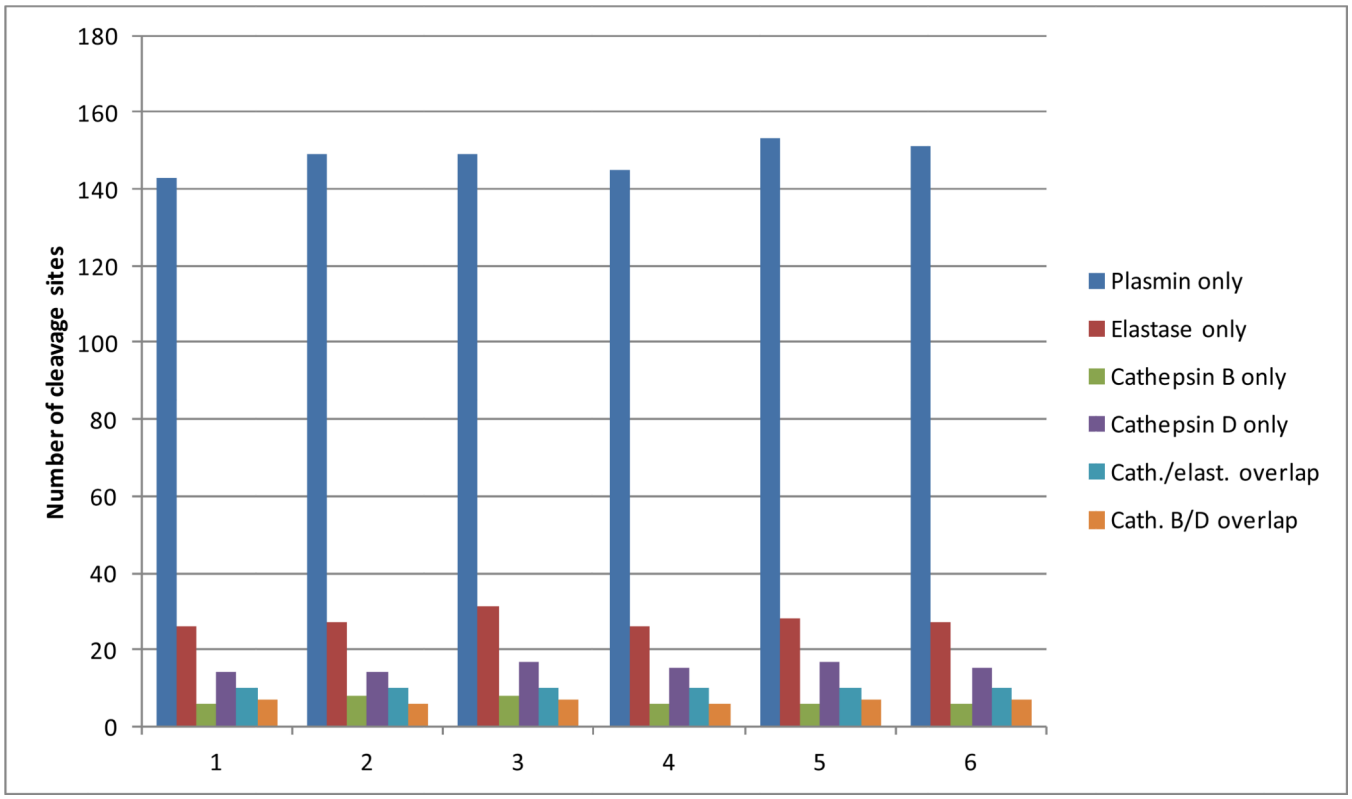

Figure 4.
Cumulative intensities for all assigned cleavage sites for each bovine milk sample.

Table 1

Enzyme cleavage specificity rules used for enzyme analysis. Specificity rules were derived from PeptideCutter as described in [24].

Enzyme	P1	P1’
Plasmin	R, K	Any
Cathepsin B	G, A, M, Q, T	F, G, I, V, L
Cathepsin D	A, V, L, I, P, M, F, W	A, V, L, I, P, M, F
Elastase	A, V, L, I, G, R	G, P, A, L, F