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Peptidomic analysis of healthy and subclinically mastitic bovine milk

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ABSTRACT

A variety of proteases release hundreds of endogenous peptide fragments from intact bovine milk proteins. Mass spectrometry-based peptidomics allows for high throughput sequence assignment of a large number of these peptides. Mastitis is known to result in increased protease activity in the mammary gland. Therefore, we hypothesized that subclinically mastitic milks would contain higher concentrations of released peptides. In this work, milks were sampled from three cows and, for each, one healthy and one subclinically mastitic teat were sampled for milk. Peptides were analyzed by nano-liquid chromatography quadrupole time of flight tandem mass spectrometry and identified with database searching. In total, 682 peptides were identified. The total number of released peptides increased 146% from healthy to subclinically mastitic milks ($p < 0.05$), and the total abundance of released peptides also increased significantly ($p < 0.05$). Bioinformatic analysis of enzyme cleavage revealed increases in activity of cathepsin D and elastase ($p < 0.05$) with subclinical mastitis.

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1. Introduction

Mastitis, an inflammation of the mammary gland, is one of the most prevalent diseases in dairy cattle, ranging from 10.4 to 64% of cows in their first lactation (Nickerson, Owens, & Boddie, 1995). The disease is particularly problematic for the dairy industry because it results in significant reductions in milk and milk-fat yield as well as poor quality milk (Daniel, Barnum, & Leslie, 1986). Mastitis is caused by bacterial infections and risk increases with physical injuries to the gland (Radostits & Done, 2007). Bacteria associated with mastitis include a variety of *Staphylococcus* and *Streptococcus* strains, *Escherichia coli*, *Corynebacterium pyogenes*, *Micrococcus* spp. (Daniel et al., 1986). These bacteria are thought to be acquired from the cow's environment (soil, manure, bedding materials, etc.; Hogan & Smith, 2003). Clinical mastitis signs include heat, swelling and redness of the mammary gland. Mastitic milk can be identified by presence of clots, discoloration of the gland or milk and high leukocyte counts (Radostits & Done, 2007). Even without visible abnormalities, subclinical mastitic inflammation can be present, which can be detected by the California test.

Milk proteases including plasmin, cathepsins B and D and elastase are known to be up-regulated in mastitic milks (Kelly, O'Flaherty, & Fox, 2006; Kirschke, Barrett, Glaumann, & Ballard, 1987; Saeman, Verdi, Galton, & Barbano, 1988). Overall proteolysis occurs at higher rates in mastitic milk than healthy milks (de Rham & Andrews, 1982). The increased activity of these enzymes suggests that mastitic milk will have higher concentrations of released peptides. Urokinase-type plasminogen activator (u-PA), which activates plasminogen to plasmin, is up-regulated in the inflammatory response during bovine mastitis (Heegaard et al., 1994). Procathepsin D, which, after conversion to cathepsin D, can also hydrolyze caseins, is 250-fold higher in mastitic milk than normal healthy milk with low somatic cell count (SCC) (procathepsin D increased linearly with log SCC; Larsen, Rasmussen, Bjerring, & Nielsen, 2004). This increase in procathepsin D did not, however, translate to an increase in the active form of the enzyme, cathepsin D (Larsen et al., 2006). In contrast with the

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plasmin system, an increased activation of procathepsin D does not occur at elevated SCC.

Invading bacteria present in mastitis often release exogenous enzymes into the milk, including elastase (Fleminger, Heftsi, Uzi, Nissim, & Gabriel, 2011). The increased levels of these bacterial proteases may also contribute to milk protein degradation.

Neutrophils enter the mammary gland as part of the inflammatory process to eliminate the bacterial infection (Hogan & Smith, 2003). These neutrophils release enzymes like elastase (Kelly et al., 2006). The release of these enzymes likely also increases proteolysis in the mastitic mammary gland.

The milk proteome changes with mastitis: mastitic milks contain lower concentrations of caseins, α-lactalbumin, and β-lactoglobulin (Hogarth et al., 2004) due to increased protease activity resulting in less intact protein. Additionally, mastitis is problematic for the dairy industry as it impairs the stability and texture of fermented products, like yogurt and cheese (Auldist et al., 1996; Kelly et al., 2006) and reduces product shelf-life due to flavor and textural changes (Datta & Deeth, 2003).

Previous work using capillary electrophoresis with MS demonstrated that several peptides derived from α_{S1}- and β-casein increased in milk from cows with clinical mastitis in comparison with non-mastitic cow milk (Mansor et al., 2013). Non-mastitic samples were confirmed to be non-mastitic by having SCC <100,000 cells mL^{-1}. In that study, 48 peptides were significantly different between the milks of healthy and mastitic cows. In a recent paper, we performed an extensive peptidomic profile for healthy bovine milk (Dallas et al., 2013b). In the present research, we apply these techniques to understand differences between healthy and subclinically mastitic milks.

2. Materials and methods

2.1. Sample collection and preparation

Milk collection from Holstein cows was carried out at the UC Davis dairy facility following a protocol approved by the UC Davis Institutional Animal Care and Use Committee (IACUC). Milk somatic cells are known to release proteases (Kelly et al., 2006); to maximize the amount of peptides in milk, samples were collected 3 h after the morning milking to obtain the highest percentage of viable somatic cells (Wickramasinghe, Rincon, Islas-Trejo, & Medrano, 2012). Using examination gloves, the cow's teat was cleaned with a gauze wetted in 70% isopropanol, and milk was collected by hand, milking directly into sterile 50 mL tubes (BD Biosciences, San Jose, CA, USA) using a 3-cm diameter plastic cannula (Genesis Industries Inc., Elmwood, WI, USA) to collect samples from the inside of the teat canal and to reduce possible external contamination (Canovas et al., 2014). Milk was collected from three cows having subclinical mastitis in one quarter. Samples were collected from a healthy and a subclinically mastitic quarter from each of these three cows. Milk was kept on ice until processing and stored at −20 °C directly thereafter. The California mastitis test was used to detect subclinical mastitis, i.e., the early presence of mastitic inflammation without visible abnormalities in the milk. Peptides were extracted from the samples according to the procedure previously described (Dallas et al., 2013a).

2.2. Mass spectrometry identification

Samples were randomized and analyzed in positive mode on an nano-LC-chip-Q-TOF MS/MS (Agilent Tech.,Santa Clara, CA, USA) both in MS and MS/MS mode using the exclusion list methodology previously described (Guerrero et al., 2014).

Data files were exported as MGF files using MassHunter Workstation Software B.05.00 (Agilent Tech.,Santa Clara, CA, USA). Peptide identification was accomplished using the database searcher X!Tandem included on GMP Manager 2.2.1 (Craig & Beavis, 2004) against a bovine milk library compiled from previous bovine milk proteome studies (Reinhardt & Lippolis, 2006, 2008; Wilson et al., 2008) using search parameters described before (Dallas et al., 2013a).

2.3. Library search

The results from X!Tandem were included in a library that contains retention times, peptide sequence, neutral mass, empirical formula, protein of origin as well as the number and nature of modifications that the peptide contains. Duplicate peptide entries were removed and their corresponding retention times were averaged. The library was used to identify peptides in each sample and for relative quantification by ion counting. MS experiments were used for this purpose. Quasi-molecular ion signals corresponding to different charge states of the same compound were grouped and searched against the library using retention time, mass and isotopic distribution. The intensity of each signal matching an entry from the library was calculated as the area under the curve of its elution time.

2.4. Data analysis

A custom script written in Python (PepEx, 2014) was used to visualize the proteolysis[2]. PepEx uses a list of peptide entries and their corresponding abundances as input. The program localizes the position of each peptide in their respective proteins and plots their abundance over the sequence.

A custom script written in Python (PEnTab, 2014) was used to estimate the activity of selected enzymatic systems. A description of the program has been previously published (Guerrero et al., 2014).

Sequences identified in the bovine milk samples were searched against a library of known functional peptides from literature. The in-house library of functional peptides contains 66 entries. This search was performed with protein–protein BLAST in the Geneious program (Biomatters Ltd., Auckland, NZ). Parameters used included max e-value: 1×10^{-1}; search matrix: BLOSUM62; gap cost: 11:1; word size: 3; no low complexity filter; maximum hits: 5000. For each query, matches were retained only if peptides shared at least 80% identity (at least 80% of the amino acids were the same and in the same positions).

3. Results and discussion

3.1. Nano-liquid chromatography-mass spectrometry analysis of endogenous milk peptides

Peptides from each sample were identified by MS/MS and the results were compiled in a library composed of 682 peptides from 69 different proteins. The identified peptides ranged in length from 6 to 47 amino acids and the average length was 19. The mass of peptides ranged from 578.3 Da to 5226.9 Da and the average mass was 2113.2 Da. The exclusion list approach proved its capability to identify low abundant peptides, increasing the size of the library four times compared with previous results (Dallas et al., 2013b). The peptide library generated in this work is shown at the supplementary material (Table S1).

Both instrumental and sample preparation variation were examined by comparison of a single milk sample extracted separately and analyzed on the mass spectrometer at the beginning,

middle and end of the experimental run. The chromatographic areas of the peptides identified in these three experiments were extracted, grouped by protein of origin and compared. The relative standard deviation based on the triplicate experiments was estimated to be ≈1% (Table S2). These results demonstrate the approach has high sample preparation reproducibility and low instrumental variation over time. Therefore, for simplicity, this variation has been neglected for the rest of the study. Potential ion-source fragmentation was examined by comparing the experimental peptide retention times against those obtained with the Normalized Elution Time Prediction Utility (Fig. S1) (NET, 2014; Petritis et al., 2003). Overall, experimental and theoretical retention time values correlated and only a few marginal points could be indicative of in-source fragmentation.

For all samples, more than 80% of the total number of peptides and more than 90% in terms of signal intensity are coming from only four milk proteins: β-casein (CASB), α_{S1}-casein (CASA1), α_{S2}-casein (CASA2) and lactophorin (also called glycosylation-dependent cell adhesion molecule 1 (GLCM1)) (Fig. 1).

Other identified peptides derive from proteins like osteopontin (OSTP), polymeric immunoglobulin receptor (PIGR), perilipin-2 (PLIN2), κ-casein (CASK), butyrophilin subfamily 1 member A1 (BT1A1) and lactoperoxidase (PERL). Nevertheless, the number of peptides from these proteins and their corresponding abundances are significantly lower, and they were not found in all the samples. Like in our previous work on healthy bovine milk, many high abundance proteins (including lactoferrin, β-lactoglobulin and secretory IgA) did not produce peptides. The fact that peptides from these abundant milk proteins were also absent in the subclinical mastitis samples suggests that the mechanisms involved in the protease selectivity for certain protein substrates is not affected by mastitis.

In peptidomics, a non-specific enzyme digestion is often required during the database search. As a consequence, the search space, computational time and number of false positives identifications greatly increase. To compensate for these problems, the search was performed against a milk protein library instead of using the whole bovine proteome. Unfortunately, this approach will miss peptides from proteins not expressed in the mammary gland. To test this possibility, a single database search was performed for one of the samples against the entire bovine proteome. Peptides from non-milk proteins were not identified, and the number of identifications decreased (results not shown) supporting the validity of the reduced library strategy.

3.2. Homologous functional peptides

Milk peptides identified were searched against known functional peptides for homology. Peptides with ≥80% homology were kept as matches (Table 1). An 80% match cut-off was employed rather than 100% match because peptides with a few extra or missing amino acids compared with the originally identified peptide may still be functional. Whether the original function is retained will be the subject of future testing. All functional peptide matches were found in both healthy and mastitic milks. Peptides were homologous with known antimicrobial, antihypertensive, opioid agonist and calcium binding enhancer peptides. In some cases, the number of different peptides matching a database peptide increased in the mastitic sample.

3.3. Milk peptidome of healthy and subclinical mastitis samples

Peptidome differences between subclinical mastitis and healthy samples were studied by comparison of the number of peptides (NP) found in each sample and the logarithm of their corresponding signal intensities (Log(I)). An increase in proteolytic activity is expected to be reflected in both measurements.

NP and Log(I) were obtained for each sample (Table S3) and the average values of the subclinical mastitis and healthy groups compared. Only peptides from the four most represented proteins were compared. The results are shown in Fig. 2. A positive value represents an increase in peptide release in the subclinical mastitis group over the healthy sample group.

To determine if the differences observed among groups are significant, NP and Log(I) were compared between the subclinical mastitis and healthy milk sample groups via paired, one-tailed *t*-tests (Table 2). Values with *p*-value ≤0.05 were deemed significantly different. The one-tailed *t*-test is justified by the expected increase on the proteolysis on the subclinically mastitic samples.

For the three subjects under study, both the NP and Log(I) were significantly higher in the subclinically mastitic milk samples than the healthy group (NP average 342 versus 234, $p < 0.05$; Log(I) average 8.51 versus 7.96, $p < 0.05$). However, although close to significance for CASB and CASA2, no protein reaches *p*-values <0.05 for NP. A different situation is observed when Log(I) values are compared. Significant differences are observed for increases in peptide abundances for all three casein proteins examined. Interestingly, this increase in proteolysis seen for the three caseins in subclinical mastitis is not apparent for GLCM1, which demonstrates a decreasing tendency (albeit not statistically significant) in the subclinically mastitic milk samples for both NP and Log(I). GLCM1 is known to be a peripheral protein, i.e., it is not integrated inside the membrane but has affinity for it. We hypothesize that the differences observed between the casein component (secreted in milk) and GLCM1 are explained by the different localization of these proteins in the mammary tissue.

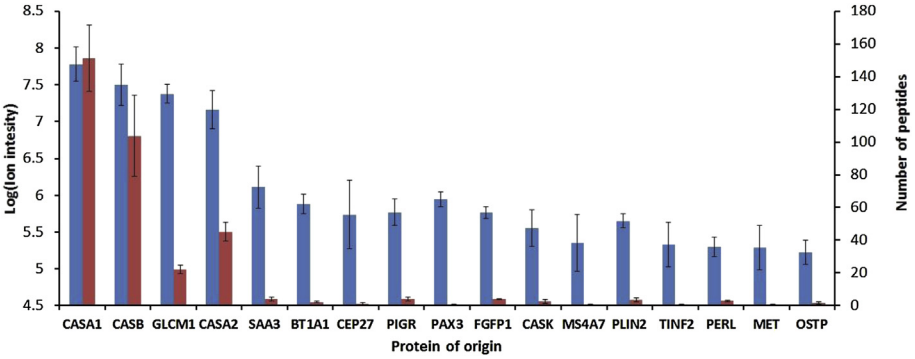

Fig. 1. Average ($n = 6$) logarithmic ion intensities (■) and number of peptides (■) by protein of origin; error bars show the standard deviation from the average. Protein abbreviations are expressed in Uniprot code.

Table 1
Homology search for functional peptides.[a]

Avg. healthy	Avg. mastitis	Found peptide protein of origin	Position in bovine protein	Known functional peptide	Activity	Reference
1.3	1.0	Bovine α_{S1}-casein	105–111	RYLGLE	Opioid agonist	(Gobbetti, Stepaniak, De Angelis, Corsetti, & Di Cagno, 2002)
7.7	7.3	Bovine α_{S1}-casein	95–105	HIQKEDVPSER	Antihypertensive	(Wu, Pan, Zhen, & Cao, 2013)
1.0	1.3	Bovine α_{S1}-casein	114–124	LRLKKYKVPQL	Antimicrobial	(McCann et al., 2006)
0.7	2.3	Bovine α_{S1}-casein	172–179	DAYPSGAW	Antihypertensive	(Pihlanto-Leppälä, Rokka, & Korhonen, 1998)
1.0	0.3	Bovine α_{S1}-casein	36–44	LRFFVAPF	Antimicrobial	(Hayes, Ross, Fitzgerald, Hill, & Stanton, 2006)
8.3	10.0	Bovine α_{S1}-casein	45–53	EVFGKEKVN	Antimicrobial	(Hayes et al., 2006)
12.0	20.7	Bovine α_{S1}-casein	195–208	SDIPNPIGSENSEK	Antimicrobial	(Hayes et al., 2006)
7.3	7.0	Bovine α_{S1}-casein	16–38	RPKHPIKHQGLPQEVLNENLLRF	Antimicrobial	(Lahov & Regelson, 1996)
3.7	3.3	Bovine α_{S2}-casein	189–197	FALPQYLK	Antihypertensive	(Tauzin, Miclo, & Gaillard, 2002)
2.0	1.7	Bovine α_{S2}-casein	204–212	AMKPWIQPK	Antihypertensive	(Maeno, Yamamoto, & Takano, 1996)
4.7	9.7	Bovine β-casein	214–224	GPVRGPFPIIV	Antihypertensive	(Nakamura et al., 1995)
3.0	5.3	Bovine β-casein	16–39	RELEELNVPGEIVESLSSSEESIT	Increases calcium bioavailability	(Gobbetti et al., 2002)
3.3	13.7	Bovine β-casein	192–198	AVPYPQR	Antihypertensive	(Maruyama, Nakagomi, Tomizuka, & Suzuki, 1985)
6.7	13.0	Bovine β-casein	208–217	YQQPVLGPVR	Antihypertensive	(Gobbetti et al., 2002)
0.7	5.7	Bovine β-casein	75–81	YPFPGPI	Opioid agonist	(Gobbetti et al., 2002)
0.7	5.7	Bovine β-casein	75–85	YPFPGPIPNSL	Opioid agonist	(Gobbetti et al., 2002)
5.3	9.7	Bovine β-casein	208–222	YQEPVLGPVRGPFPI	Antimicrobial	(Birkemo, O'Sullivan, Ross, & Hill, 2009)
3.3	7.3	Bovine β-casein	208–224	YQEPVLGPVRGPFPIIV	Antimicrobial	(Sandre et al., 2001)
7.0	13.7	Bovine β-casein	129–136	YPVEPFTE	Antihypertensive	(Perpetuo, Juliano, & Lebrun, 2003)

[a] "Avg. healthy" and "avg. mastitis" are the average number of peptides found in the healthy and subclinical mastitis samples, respectively, with ≥80% homology to a known functional peptide.

3.4. Intra-molecular proteolytic analysis

An in-house script called PepEx was used to visualize the proteolysis inside each protein and sample. PepEx compiles the total abundances associated with each amino acid of the protein sequence by summing the endogenous peptides that contain them. The proteolytic maps of the casein fraction (CASA1, CASA2 and CASB) and GLCM1 for the mastitic and healthy milk sample of one of the subjects are shown in Fig. 3. In the horizontal axis, the sequence of the protein is represented from the N-terminus to the C-terminus (left to right). In the vertical axis, the logarithm of the ion intensities is plotted. Similar results were obtained for the other two subjects under study (Fig. S2).

For the casein fraction composed by CASA1, CASA2 and CASB, the proteolytic maps show a clear increase in the proteolysis all over the sequence of the proteins for the mastitic sample over the healthy one. Only in a few regions (between amino acid residues N_{53}-A_{68}, H_{95}-K_{117} of CASA1 and K_{214}-L_{222} of CASA2) the proteolysis is higher in the healthy sample compared with the subclinically mastitic sample. The shape of the maps—with almost perfectly overlapping cluster regions—suggests that the proteases involved on the proteolysis of both samples are the same. For GLCM1, a decrease in the central peptide cluster was observed for the mastitic samples in the three subjects.

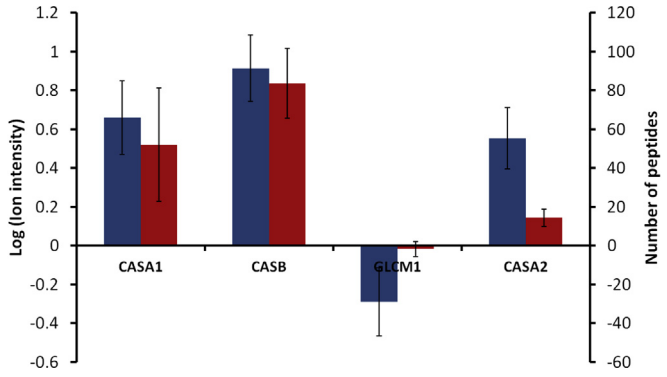

Fig. 2. Peptidome comparison (■, logarithmic ion intensities; ■, number of peptides) between healthy and mastitis bovine milk samples; error bars indicate standard deviation from the mean.

3.5. Estimation of protease activity

Protease activity was determined for all the samples using PEnTab (Fig. 4). As expected, plasmin is the main enzyme involved in the proteolysis. Plasmin's relative participation is the highest in both mastitic and healthy milk for all proteins, except for CASA1, where cathepsin D and other undefined enzymes are more prevalent. CASA2 contains only a few cathepsin D and elastase cleavage sites, which explains the dominant participation of plasmin in the hydrolysis of this protein. A couple of considerations should be made about the meaning of the enzymatic group "others". PEnTab is unable to estimate the participation of exoproteases that may be present in milk. The evidence for exoproteases in milk, however, remains weak (O'Mahony, Fox, & Kelly, 2013). Additionally, as PEnTab works with a user-defined list of proteases, the presence of unspecified enzymes (i.e., bacterial enzymes) cannot be predicted. As a consequence, the group "others" is constituted by these two potential sources of proteolytic activity.

Comparison of the subclinical mastitic and healthy sample enzyme activity graphs demonstrates that there is an increase in the standard deviation for the mastitic average values compared with those of the healthy samples. This increase in the standard deviation may be explained by the differences in the inflammatory process among individuals.

Table 2
T-test analysis of the mastitic and healthy peptide content.

Protein	*p*-Value (NP)	*p*-Value [Log(I)]
CASA1	0.131	0.047
CASB	0.054	0.022
GLCM1	0.373	0.144
CASA2	0.052	0.047
Overall	0.015	0.016

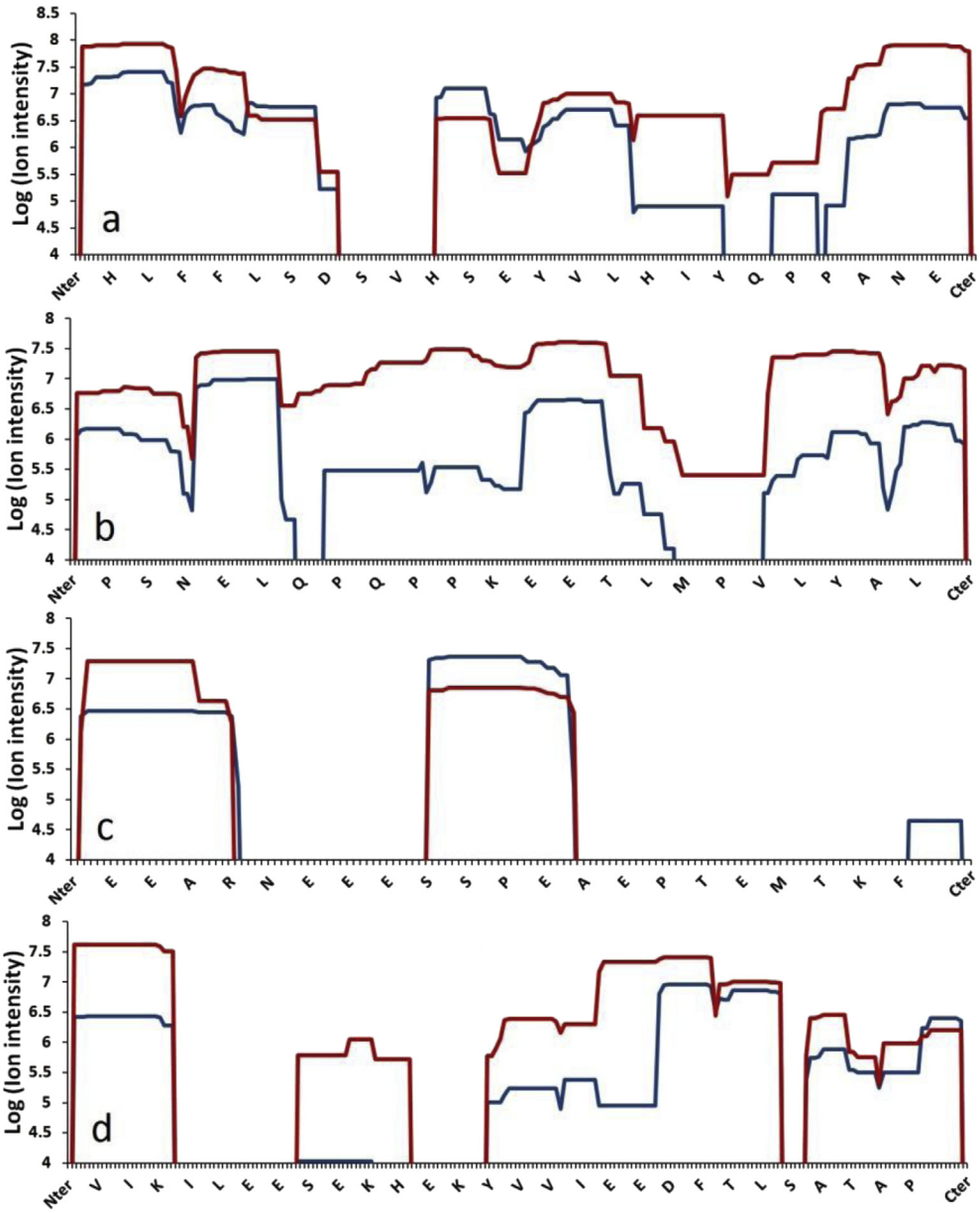

Fig. 3. Proteolytic maps of (a) CASA1, (b) CASB, (c) GLCM1 and (d) CASA2 for the mastitic (red line) and healthy (blue line) milk sample of the same subject. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To determine if the differences observed among groups are significant, Log(I) of the different enzymatic activities were compared between the subclinical mastitis and healthy milk sample groups via paired, two-tailed *t*-tests (Table 3). Peptides with *p*-value $\leq$0.05 were deemed significantly different.

The statistical analysis of the estimated proteolytic activity shows significant differences for all the enzymes but for plasmin. As cathepsin D and elastase share cleavage specificities (they both can cleave after A, V, G, L and I), it is difficult to differentiate between both enzymatic activities, which may explain why these two proteases seem to be the ones that change more significantly. The "other" proteolytic system that is composed (at least partially) by possible milk exopeptidases and unspecified enzymes also increases significantly. The unexpected non-significant increase of predicted for plasmin in the subclinically mastitic samples may be due to actions of other proteases, such as exopeptidases after the plasmin peptides are released.

4. Conclusion

Our results demonstrate that in bovine subclinical mastitis, the number and abundance of milk protein-derived peptides increases significantly. This result agrees with earlier work (Mansor et al., 2013) which demonstrated 48 sequence-identified peptides were significantly different in mastitic milks compared with healthy milks. However, this previous work did not determine whether the total number and abundance of peptides were increased. The present paper is the first to describe the bovine subclinical mastitis peptidome with extensive peptide sequence identifications and analysis of overall peptide count and abundance changes, as well as changes by protein.

These increased levels of peptides found agree with previous reports that activity levels of many milk enzymes (based on colorimetric enzyme-substrate assays) are higher in bovine mastitis. Our findings agree with previous articles that plasmin

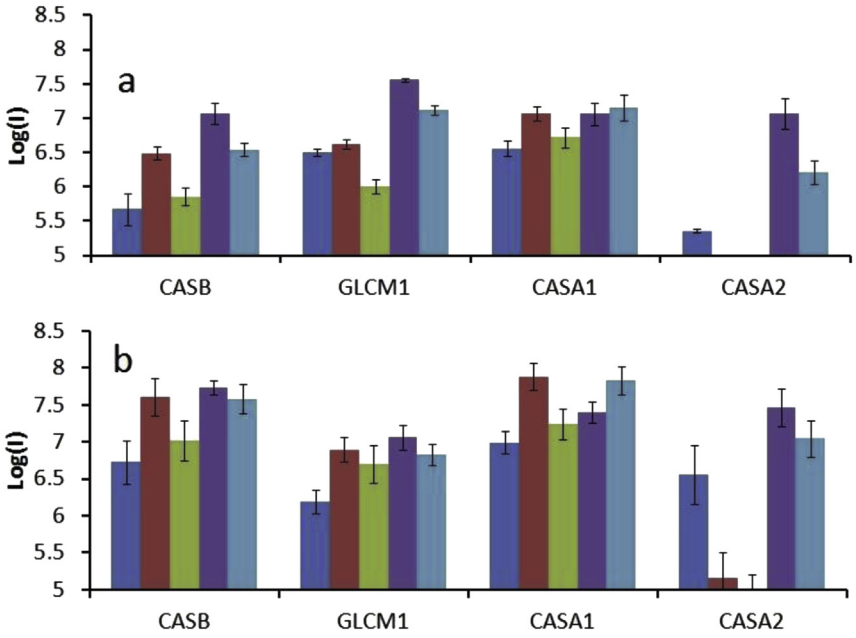

Fig. 4. Average participation of the proteases on the formation of endogenous peptides (left to right: ■, cathepsin B; ■, cathepsin D; ■, elastase; ■, plasmin; ■, other) by protein in (a) healthy and (b) mastitic samples. Error bars indicate standard deviation from the mean.

Table 3
T-test analysis of the mastitic and healthy peptide content.

Protease	*p*-Value [Log(I)]
Cathepsin B	0.021
Cathepsin D	0.005
Elastase	<0.001
Plasmin	0.146
Other	0.012

activity is increased in bovine milk with mastitis (Heegaard et al., 1994). We detected a significant increase in cathepsin D activity in subclinical mastitis samples, which agrees with previous work showing that cathepsin D activity increases with increasing SCC (Larsen et al., 2006; O'Driscoll, Rattray, McSweeney, & Kelly, 1999). However, interestingly, Larsen et al. (2006) demonstrated by immunoblotting that this increased cathepsin D activity was not due to cathepsin D, but rather procathepsin D. They suggested that procathepsin D is auto-activated into pseudocathepsin D. During the inflammatory response, cathepsin D can be secreted from macrophages (Owen & Campbell, 1999). Though cathepsin D has not activity ≥pH 7, at sites of inflammation, the pericellular pH may be low enough to activate cathepsin D (Owen & Campbell, 1999).

Early proteomic comparison of mastitic to healthy bovine milk revealed that, along with the caseins, levels of intact α-lactalbumin and β-lactoglobulin were lower in the mastitis milks (Hogarth et al., 2004). We originally hypothesized that these decreases in intact protein were due, at least in part, to increased protease activity in the mastitis milks. This hypothesis aligned with the significant increases in peptide release for the casein proteins. However, for α-lactalbumin and β-lactoglobulin, no peptides were identified in either healthy or mastitic samples. This finding suggests that production of these intact proteins may be lower during mastitis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.idairyj.2014.09.006.

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