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Peptide release profiles from lactoferrin were tracked and characterised during simulated gastric digestion using a novel quantitative proteomic approach.

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Digestion proteomics: Tracking lactoferrin truncation and peptide release during simulated gastric digestion

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Abstract

The extent to which nutritional and functional benefit is derived from proteins in food is related to its breakdown and digestion in the body after consumption. Further, detailed information about food protein truncation during digestion is critical to understanding and optimising the availability of bioactives, in controlling and limiting allergen release, and in minimising or monitoring the effects of processing and food preparation. However, tracking the complex array of products formed during the digestion of proteins is not easily accomplished using classical proteomics. We here present and develop a novel proteomic approach using isobaric labelling to mapping and tracking protein truncation and peptide release during simulated gastric digestion, using bovine lactoferrin as a model food protein. The relative abundance of related peptides was tracked throughout a digestion time course, and the effect of pasteurisation on peptide release assessed. The new approach to food digestion proteomics developed here therefore appears to be highly suitable not only for tracking the truncation and relative abundance of released peptides during gastric digestion, but also for determining the effects of protein modification on digestibility and potential bioavailability.
1. Introduction

A balanced nutritional diet requires the intake of foods high in protein, which serve to provide essential amino acids for the proper growth and repair of the human body, as well as energy. In a global climate of expanding population and pressure on protein-based food production systems, it is increasingly important to derive optimum nutritional value from available protein food sources. The extent to which benefit is derived from a food is related to its breakdown and digestion in the body. Detailed information about the behaviour of foods during digestion will be critical to understanding and improving the availability of bioactives; in controlling and limiting allergen release; in enhancing nutritional benefit; in minimising or monitoring the effects of processing and food preparation on bioavailability; and in developing and evaluating targeted delivery technologies.

As food molecules are broken down and modified within the digestive tract, highly complex mixtures are generated. This has traditionally made monitoring the specific breakdown of food during digestion very difficult. The degradation of proteins during digestion may be monitored using amino acid analysis\textsuperscript{1} and polyacrylamide gel electrophoresis,\textsuperscript{2-4} techniques, which offer a holistic overview of the extent of digestion. Mass spectrometry, with its ability to specifically identify proteins and their fragments within complex mixtures, has become a preferred technique for characterising the products of protein digestion within \textit{in vitro} stomach and digestive tract simulations.\textsuperscript{5,6}

Advances in redox proteomics and quantitative peptide tracking\textsuperscript{7-11} have provided tools for quantitatively monitoring the digestive and oxidative degradation of proteins and the formation of their products. This paper describes the novel application of a proteomic-based approach to characterise and track protein digestion in an \textit{in vitro} stomach simulation. In a typical proteomic analysis, a proteolytic enzyme, such as a trypsin, is utilised to break whole proteins down into peptides for subsequent analysis. However, to track protein truncation and release of peptides during food consumption, it is critical that any digestion occurring is only that induced by the natural digestive process itself. In the approach described here, we have developed a novel application of isobaric labelling to track protein digestion without any prior artificial breakdown of the protein structure.

Key questions about the behaviour of proteins during digestion are addressed with this new approach, such as: Which peptides are released? How does truncation occur? What does the peptide profile look like during and after digestion? Does processing affect digestion?

This study examined the digestion of lactoferrin as the model protein, in a simulated stomach environment and also compared the effect of a typical milk pasteurisation protocol on this digestion. Lactoferrin was chosen as a model because of its status as a highly functional food protein. This iron-binding milk protein has attracted wide interest because of its antimicrobial\textsuperscript{12,13} and other putative bioactivities\textsuperscript{14,15} – in normal bovine milk, is is present at about 100 µg/ml, is thought to be relatively heat stable,\textsuperscript{16} and begins to break down under gastric digestion.\textsuperscript{17,18} However, until now, little has been discovered as to how lactoferrin breaks down into component peptides during digestion, and what influence prior processing has on this digestion – subtle protein denaturation (alterations in its three-dimensional shape) may alter how sections of the polypeptide sensitive to proteolysis are exposed.
2. Materials and methods

2.1. Chemicals and consumables

Sodium chloride was obtained from Mallinckrodt Baker (Phillipsburg, NJ), hydrochloric acid from Merck (Darmstadt, Germany), triethylammonium bicarbonate from Fluka (Buchs, Switzerland) ammonium bicarbonate from BDH (Poole, England), alpha-cyano-4-hydroxy-cinnamic acid from Bruker (Bremen, Germany). Pepsin was obtained as a lyophilised powder from Sigma-Aldrich (St Louis, MO). HPLC-grade isopropanol, trifluoroacetic acid (TFA), acetonitrile, and 200 µl C18 StageTips were acquired from Fisher Scientific. (Fair Lawn, NJ). 8-plex iTRAQ reagents were obtained from Applied Biosystems (Foster City, CA).

2.2. Lactoferrin extraction and purification

Pasteurised and non-pasteurised skim milk was obtained from a commercial dairy milk processor. The pasteurisation step was 72˚C for 15 seconds. The non-pasteurised milk was microfiltered (10 µm filter) in the laboratory. From these milks a basic protein fraction was prepared. The milk was loaded onto a cation exchange column (SPFF, GE Healthcare) and the bound material eluted with 1 M NaCl. The isolate was diafiltered with a 10 kDa ultrafiltration membrane to remove salt and freeze-dried. After resuspension in a 5% (w/v) solution in 20 mM phosphate buffer pH 6.5, the milk samples were passed through another SPFF column, but eluted this time with a NaCl gradient (0 to 1 M NaCl over 18 column volumes) to separate the components. The lactoferrin-containing fractions (~800 ml in 785 mM NaCl) were pooled and separated through a 14.3 cm x 2.6 cm, 75 ml phenyl sepharose (hydrophobic) column (GE Healthcare) with a gradient of 4 to 0 M/20 CV, pH 6.5. The lactoferrin fractions were pooled and ultrafiltered with a 50 kDa membrane to remove some of the lower molecular weight proteins, before freeze-drying.

2.3. Pepsin digestion

Unpasteurised and pasteurised lactoferrin was dissolved in HPLC-grade water at 5 mg/ml. Pepsin was dissolved in 150 mM NaCl/HCl (pH 2.0) at 13.2 µg/ml. The pepsin solutions (11.4 ml) were pre-warmed to 37˚C, and 600 µl unpasteurised and pasteurised lactoferrin solutions were added and stirred at 37˚C over the course of three hours. Aliquots of 400 µl of pepsin-digested lactoferrin were taken at 0.5, 2, 5, 10, 20, 30, 60, 90 and 180 minutes and added to 100 µl 250 mM NaHCO₃ in an ice-bath. All experiments were performed in quadruplicate. The time-point samples were surveyed using one-dimensional SDS-PAGE to determine appropriate time-points for more detailed, quantitative (iTRAQ) mass spectrometric analysis. SDS-PAGE demonstrated that extensive hydrolysis of the intact ~80 kDa lactoferrin protein occurred rapidly – a band at ~80kDa could not be detected even after 30 seconds’ digestion. A strong band in the 10 kDa and smaller range appeared at 0.5 minutes, and was observed more and more faintly over the course of digestion, indicating the formation of smaller and smaller truncation products (data not shown).
2.4. Isobaric labelling

Pepsin-digested lactoferrin samples (0, 0.5, 2, 5, 10, 20, 30 and 60 minute digestions) were passed through C18 StageTips (to remove salts and concentrate the samples: StageTips prepared with 50 µl 50% acetonitrile, 0.1% TFA followed by 50 µl 0.1% TFA; 150 µl sample loaded; washed with 2 x 50 µl 0.1% TFA, eluted in 50 µl 50% acetonitrile, 0.1% TFA. The samples were then reduced to dryness in a centrifugal concentrator and reconstituted in 30 µl 0.5 M triethylammonium bicarbonate, pH 8.5.

After bringing a set of 8-plex iTRAQ reagents to room temperature, 100 µl isopropanol was added to each vial, which was then vortexed and centrifuged. Reconstituted samples were randomly assigned to iTRAQ reagents (randomisation was performed separately for each sample and replicate), and 50 µl of the appropriate isopropanol/iTRAQ reagent mixtures were added to each vial. Labelling was performed for two hours at room temperature, and the reaction stopped by the addition of 100 µl water to each tube and incubation at room temperature for 30 minutes.

The labelled samples were pooled and excess iTRAQ reagents were removed using C18 StageTips as described above. The samples were then evaporated to dryness, combined with 100 µl water, and evaporated to dryness once more before reconstitution for LC-MALDI in 3% acetonitrile, 0.1% TFA (20-fold dilution). Mean quantitative (iTRAQ) data from the four replicates was used to establish peptide digestion profiles.

2.5. LC-MALDI-MS/MS

Using a Proxeon Easy-nLC (Bruker, Bremen, Germany), 5 µl samples were loaded on a trap (22 mm, 100 µm i.d.), and separated on an analytical (150 mm, 75 µm i.d.) column using a 2-55% B (acetonitrile/0.1% trifluoroacetic acid) gradient over 80 minutes with a flow rate of 300 nl/min). The trap and column were in-house packed with Microsorb C18 300-5 media (Varian, Palo Alto, CA). Using a Proteineer fc fraction collection robot, 384 fractions were collected from 10-74 minutes in 10 second time slices on 800 µm Anchor-Chip plates by post-column mixing at a flow rate of 700 nl/ minute with ethanol/acetone/ 10 mM NH₄H₂PO₄ in 0.1 TFA /saturated CHCA matrix solution in acetone (ratio 6:3:1:1). A Peptide Calibration Standard (Bruker) containing angiotensin I (m/z 1296.6848) and II (m/z 1046.5418), substance P (m/z 1347.7354), bombesin (m/z 1619.8223), ACTH-clip [1-17] (m/z 2093.0862), ACTH-clip [18-39] (m/z 2465.1983) and somatostatin (m/z 3147.471) was diluted 50-fold with matrix solution and spotted onto the plates manually.

Automated MS/MS was performed using an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker, Bremen, Germany) in positive ion mode, using WarpLC software. Settings were adjusted for SILE chemistry as appropriate.

3. Results and Discussion

Samples of both pasteurised (72°C for 15 seconds) and unpasteurised lactoferrin were subjected to simulated gastric digestion and sub-samples collected over a time course (three hours). The in vitro conditions involved digestion with pepsin (a proteolytic stomach enzyme) of lactoferrin in a pre-warmed acidic sodium chloride solution. The digestion reaction was terminated in the aliquots taken at various time points by increasing the pH (by adding NaHCO₃) and cooling in an ice-bath.
Sub-samples taken as the digestion progressed were then evaluated using a novel proteomic approach. The goal was to map and track the pepsin-driven release of peptides from the lactoferrin over the course of the digestion, and evaluate their relative abundance over time. It is important to note that prior to proteomic analysis the samples were not digested with trypsin, and analyte quantitation was performed on single peptides. This is a significant difference to the approach followed in classical shotgun proteomics, where proteins are cleaved prior to mass spectrometry using trypsin, and quantitative data from multiple peptide identifications is used to provide relative abundance data for parent proteins.

The protein/peptide mixtures resulting from the digestion were labelled by isobaric tagging of the N-termini. Isobaric tags are of equal mass and chemical functionality (differing only in their isomeric composition), which means that peptides labelled with any of the tags will behave identically during sample processing and separation. A commercially available variant, iTRAQ™ (used in this project), comes in up to eight forms – peptides in one sample are chemically derivatised with one of the eight labels, and are then combined with other samples labelled with their own labels. The distinctly labelled samples are then processed as a single sample, separated using liquid chromatography, and then analysed using tandem mass spectrometry (MS/MS). During the fragmentation step of tandem mass spectrometry, each of the eight labels fragments in a distinct manner, so that eight peaks appear in each peptide’s MS/MS spectrum. The area of each peak can be used to provide relative quantitation, while the ions resulting from fragmentation of the peptide itself can be used to decipher the sequence of the peptide as usual.

The use of different iTRAQ labels for each stage of digestion permitted the release of each peptide from lactoferrin to be individually tracked. Each peptide’s MS/MS spectrum contained quantifiable iTRAQ reporter ion peaks corresponding to the peptide’s relative abundance at each time point.

Full proteomic mapping was performed for each time-point of the digestion, generating a complex profile of released peptides. Examples of individual peptide abundance changes are shown from Figures 5 - 8, but first, an overall picture of lactoferrin release is presented. Figure 1 illustrates the amino acid sequence of lactoferrin, and indicates (in red) the stretches of sequence that were quantified.
Figure 1  Primary sequence of bovine lactoferrin, related to the abundance maps in Figures 2 and 3. Residue 1 (methionine) is at the N-terminal end, and residue 708 (arginine) is at the C-terminal end. The portion of the sequence that was tracked in this experiment is rendered in red.

Figures 2 - 4 show visual summaries of the relative abundance of all locations within lactoferrin observed to be released over the digestion. Note that relative abundance information is drawn from the eight iTRAQ reporter ions, each specific to a certain time-point, as described in the previous paragraph. The abundance of a certain peptide at a given time-point is thus relative to its abundance as measured over all the time-points, not to the abundance of separate peptides.

The summary maps allow visualisation of when various locations of the lactoferrin sequence are most bioavailable. Rapidly-released sections of the polypeptide are broken down to such small units that they are no longer recognised as peptides under the standard constraints of bio-informatic software, which in a biological digestive system would be augmented by absorption across digestive membranes and emptying of the stomach into the duodenum.

The amino acid sequence of lactoferrin (Figure 1) is represented by the x-axis on Figures 2 and 3 (the lactoferrin sequence is represented as starting at residue 62 because profiling data was not obtained for the extreme N-terminal end of the polypeptide). The scale of these diagrams prevents the sequence being written as a marker below the axis, but this can be approximated in an enlarged portion of the data presented in Figures 2 – 4.
Figure 2  Relative abundance summary map of unpasteurised lactoferrin. The x-axis represents the polypeptide sequence of lactoferrin (from residues 62 to 708), with indicative sequence positions marked. Gaps indicate areas in the sequence where abundance data was not obtained. The y-axis represents the time point in the digestion. The grey-scale represents relative abundance at each time point, with progressively darker shades representing higher and higher relative abundance. Each amino acid residue in the peptide is represented by a (very narrow) vertical bar along the x-axis.

Figures 2 and 3 are relative abundance summary maps of unpasteurised and pasteurised lactoferrin, respectively. These maps represent the abundance (as determined by quantitative iTRAQ analysis) of the entire protein at each digestion time point in a visual manner (at least, those portions of the protein that were observed by mass spectrometry). Each vertical bar represents a small stretch of the lactoferrin polypeptide and the grey-scale (on the y-axis) represents the relative abundance of that section of the polypeptide at each time point (see also Figure 4, an enlarged section of the summary map for unpasteurised lactoferrin). The relative abundance of each point in the polypeptide is calculated from all peptides containing that amino acid residue. For example if two overlapping peptides were detected (peptide A from residues 1-10, and peptide B from 5-15), the relative abundance of residues 5-10 reflects the contribution of both peptides A and B.
Relative abundance summary map of pasteurised lactoferrin. The x-axis represents the polypeptide sequence of lactoferrin (from residues 62 to 708) – with indicative sequence positions. Gaps indicate areas in the sequence where abundance data was not obtained. The y-axis represents the time point in the digestion. The grey-scale represents relative abundance at each time point, with progressively darker shades representing higher and higher relative abundance. Each amino acid residue in the peptide is represented by a (very narrow) vertical bar along the x-axis.

In both pasteurised and unpasteurised lactoferrin, peptides covering most of the protein primary sequence were released and increased in abundance as the digestion progressed, with many locations peaking in the 30 minute and 60 minute samples. The majority of the lactoferrin sequence was relatively unaffected by pasteurisation, and displayed a similar release profile. In contrast, however, some stretches stood out as having different abundance profiles. See the summary map source data in the Supplementary Data section to observe specific areas in the sequence with notable differences in release patterns in pasteurised and unpasteurised lactoferrin (e.g. residues 418-430).
Figure 4  An enlarged portion of a summary map of unpasteurised lactoferrin, showing relative abundance data for residues 215 to 249 (x-axis, see sequence indicator). The y-axis represents the time point in the digestion. The grey-scale represents relative abundance at each time point, with progressively darker shades representing higher and higher relative abundance.

Figure 4 illustrates the information that can be gleaned from close inspection of summary maps. Residues 215-226 (FKCLQDGAGDVA) are observed more abundantly in the two minute fraction than at any other time-point. A nearby stretch of residues (235-245, ENLPEKADRDQ) is most abundant at the 30 minute time-point. To see more specific examples in tabular form, see the summary map data in the Supplementary Data section.

This summary of the released peptide profiles also highlights the complexity of the protein digestive process, with a complex range of peptide products forming that changes dynamically as the digestion proceeds. Using our approach, to analyse changes in peptide abundance throughout digestion, however, it is also possible to examine any given peptide individually, and track its release and breakdown throughout the digestion.

As one example, the peptide VVKGSNFQDLQ (in which seven out of the 13 residues are essential dietary amino acids), located at position 116-128 in lactoferrin, was observed at eight time-points during a simulated stomach digestion. Isobaric labelling such as iTRAQ allows the abundance of a specific peptide to be compared across samples (in this case, time-points). Of the total signal attributed to VVKGSNFQDLQ, about 4% of this was detected 30 seconds into digestion, and about 25% was detected 10 minutes into digestion. The release can be visualised relatively easily in graphical form (Figure 5). The abundance of the peptide
initially increases as it is released from the intact lactoferrin protein via digestion and then falls subsequently as it itself is truncated into smaller length peptides (see also Figure 6).

Figure 5  The abundance of peptide, VVKKGSNFQLDQL, from position 116-128 in unpasteurised lactoferrin, as released during a simulated stomach digestion. Error bars represent SEM, where n=4.

Figure 6  Digestion profile of a set of related peptides. VLKGEADALNLDDGY is a peptide from position 403-417 in unpasteurised lactoferrin. VLKGEADALNL, VLKGEADAL, and KGEADALNL are truncations of that peptide. Error bars represent SEM, where n=4.

The release profiles of related peptides were able to be directly compared and evaluated, as demonstrated in Figure 6  A fifteen-residue peptide, VLKGEADALNLDDGY, shows a digestion profile similar to that of VVKKGSNFQLDQL (Figure 5): a rapid release from the parent polypeptide (peaking 5 minutes into digestion), followed by a decrease in abundance due to further degradation. The digestion profile of three truncated products is also charted.
The shorter, truncated, products increase in abundance more slowly than the parent, decreasing in abundance as they, in turn, are cleaved.

This new approach to digestion proteomics was able to show the relationships between protein digestion product formation, and their peak times of potential bioavailability, which has notable importance in the evaluation of protein bioactive or potential allergen release. For instance, in the related peptide set shown in Figure 6 the primary digestion product (VLKGEADALNDGGY) was only briefly available as an intact polypeptide, whereas its truncated products were released and retained over a longer period – VLKGEADAL and KGEDALNL were present at relatively high abundance even after 60 minutes’ digestion. The length of time foods remain in the stomach compartment varies according to the food-type and other factors, but two hours is a common time used in in vitro digestion models of the stomach, duodenum and small intestine based on studies such as that by Hunt and Spurrell in 1951 that show the bulk of the gastric contents to be removed from the stomach to the duodenum by one hour and the remainder almost entirely removed by two hours.

The pattern of release of related peptides was also tracked and contrasted between the unpasteurised and pasteurised samples. Figure 7 shows the release of the lactoferrin peptide, IEAKKADAVTLDDGGM, and its subsequent truncation to IAEKKADAVTL, with significant different patterns of truncation observed between the unpasteurised and pasteurised samples. In the unpasteurised control, the longer peptide (blue line) is released from lactoferrin early in the digestion, and decreases in abundance from 20 minutes onwards. The truncation product (brown line) is released later and is still relatively abundant at 60 minutes. In the pasteurised sample, the truncation product forms much earlier, peaking 10 minutes into digestion. This result indicates that thermal modification during pasteurisation is altering the digestion kinetics of lactoferrin, likely through denaturation and potentially some degree of protein backbone cleavage. This also suggests generally that the bioavailability of protein bioactive peptides will be strongly influenced by thermal processing of the food prior to consumption, with some peptides becoming more available and others less available.
Figure 7  The digestion profiles of lactoferrin peptides IAEKKADAVTLDGGM (position 68-82) and its truncation product, IAEKKADAVTL (position 68-78) from unpasteurised and pasteurised lactoferrin. Error bars represent SEM, where n=4.

For thermally-induced redox modification at any given amino acid residue in any given released lactoferrin peptide, the relative abundance of the oxidised product relative to the unmodified peptide was tracked. Figure 8 illustrates this, showing the release and relative abundances of both the unmodified native peptide WVIPMGIL and the equivalent peptide with the tryptophan residue oxidised to hydroxytryptophan, over the course of lactoferrin digestion. In this case, the modification is shown to have a significant effect on peptide release. Here, the lactoferrin peptide, WVIPMGIL (position 126-133) is seen to increase in availability quite late in the digestion time-course (compared with the peptide profiles in Figure 5 and 6). Its oxidised equivalent, which contained a methionine sulfoxide residue in place of the methionine residue, was released earlier.

Figure 8  The digestion profiles of lactoferrin peptide WVIPMGIL (position 126-133) and its equivalent modified peptide with methionine oxidised to methionine sulfoxide. Error bars represent SEM, where n=4.

This new approach to digestion proteomics therefore appears to be highly suitable not only for tracking the truncation and relative abundance of released peptides during digestion, but also for determining the effects of post-translational modification on digestibility and potential bioavailability.

4. Conclusions

Tracking the truncation of proteins and peptides during gastric digestion has traditionally been very difficult due to the complex array of products formed. Classical proteomic approaches also miss critical information with respect to protein truncation and peptide release. Here we have here successfully demonstrated proof-of-principle for a new digestion proteomics approach specifically targeting the mapping of protein truncation during digestion. Utilising isobaric labelling, the release of peptides from bovine lactoferrin during a simulated gastric digestion time course was tracked and the relative abundance of related peptides evaluated. This approach has wide applicability in evaluating the gastric digestion of
food proteins, and, in particular, for determining the potential bioavailability of specific peptides including known functional bioactive peptides and known allergenic sequences.

This new approach was also shown to be useful in assessing the effect of processing, in this case pasteurisation, on the modification and release of peptides. After pasteurisation, the truncation of lactoferrin during the simulated digestion was altered, demonstrating that the bioavailability of specific peptides is influenced by thermal processing of the food prior to consumption, with some peptides becoming more available and others less available during the time-course of digestion.

The approach to food digestion proteomics developed here therefore appears to be highly suitable not only for tracking the truncation and relative abundance of released peptides during gastric digestion, but also for determining the effects of protein modification on digestibility and potential bioavailability.
5. References