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Digestomics: an emerging strategy for comprehensive analysis of protein catabolism

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When cells mobilize nutrients from protein, they generate a fingerprint of peptide fragments that reflects the net action of proteases and the identities of the affected proteins. Analyzing these mixtures falls into a grey area between proteomics and metabolomics that is poorly served by existing technology. Herein, we describe an emerging digestomics strategy that bridges this gap and allows mixtures of proteolytic fragments to be quantitatively mapped with an amino acid level of resolution. We describe recent successes using this technique, including a case where digestomics provided the link between hemoglobin digestion by the malaria parasite and the world-wide distribution of chloroquine resistance. We highlight other areas of microbiology and cancer research that are well-suited to this emerging technology.

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Introduction

Metabolism is the biological conversion of nutrients into energy, biomass, and waste products. The molecular details of metabolism have a direct impact on the types of nutrients organisms use, the amount of energy that can be harvested from finite resources, and the types of ecosystems organisms inhabit. Traditionally, metabolism has been studied from the perspective of small molecule nutrients. Almost everything that is known about metabolic pathways and regulation has been defined using a small collection of amino acids, sugars, nucleic acids, and fats. However, metabolites only represent about 3% of the dry mass of the cell whereas protein constitutes 50–60% [1,2]. Consequently, organisms that can use this resource for metabolism can have a significant competitive advantage.

Although protein catabolism is a well-recognized mechanism for satisfying nutritional needs, it is surprisingly difficult to study in a comprehensive manner. Amino acids are mobilized through the combined action of proteases and peptidases. This enzymatic digestion produces a complex mixture of related peptides ranging in size from dimers to nearly intact proteins. Decoding these mixtures is a significant technical challenge that requires high resolution mass spectrometry (MS), custom bioinformatics, and a strong understanding of biology. Until recently, these challenges have made comprehensive analyses of protein digestion impractical. However, a constellation of emerging techniques has made this formidable problem more tractable, and has opened the door to diverse applications ranging from elucidating selective pressures acting on drug resistant microbes to investigating metabolic adaptation in cancer cells [3••,4••]. This emerging digestomics approach has been previously defined in the narrow context of mammalian and insect digestion [5,6]. Here, we recast this concept to encompass the study of any living system that uses protein catabolism for nutrition.

Digestomics: the comprehensive analysis of protein catabolism and quantitative mapping of peptides to genomes at an amino acid level of resolution.

Barriers to digestomics

Digestomics exists in a middle-ground between metabolomics and proteomics that is poorly served by the existing analytical workflows. In the case of metabolomics, sample preparation conditions are optimized for the recovery of small molecules and have been specifically designed to minimize the solubility of proteins and large peptides [7]. Furthermore, mass spectrometry-based metabolomics is typically restricted to low molecular weight molecules, optimized for singly charged species, uses high-flow liquid chromatography conditions with condensed chromatographic separations, and is often conducted with the MS instrument in negative ion mode [8]. These conditions are poorly suited to analyses of peptides. Moreover, data analyses in metabolomics typically involve either targeted analyses of vetted species or untargeted assignments driven by database matches to libraries of metabolite standards, which only contain a few peptides [9–11].

Standard proteomic workflows are also inappropriate for digestomics. Proteomic sample preparation methods

target intact proteins, which leads to poor recovery of small peptides. In addition, proteomic sample processing often includes enzymatic digestion (e.g. trypsinolysis), which introduces non-biological peptides into the mixture [12]. Moreover, MS analyses in proteomics employ automated fragmentation strategies that typically exclude low mass and singly charged species. Finally, proteomic data analyses typically employ database searching and spectral matching algorithms that bias results towards larger peptides. These larger species are favored because they can be identified with higher confidence and are more likely to yield unique matches when aligned with a total proteome database [13]. This is problematic in the context of digestomics because small peptides are frequently biologically significant [4••,14,15•]. In summary, routine metabolomics methods exclude large molecules, routine proteomics methods exclude small molecules, and digestomics must encompass both. Not surprisingly, the emerging digestomics workflow is a hybrid of existing metabolomics and proteomics methods (Figure 1).

Digestomics workflow

The need to capture a diverse range of naturally-occurring peptides shapes the sample preparation and data analysis workflow. The methods outlined here are adapted from a previous study, which quantified peptides ranging from 2 amino acids to 32 amino acids in length [4••]. To achieve this, peptides can be extracted from cells using relatively high dilutions (1:20; cell:solvent volume) in 90% methanol containing 3% acetonitrile and 0.1% formic acid. The high dilution helps improve the recovery of sparingly soluble peptides, and the acetonitrile helps improve the extraction of hydrophobic species and ensures miscibility with the chromatographic mobile phase. Most peptides can then be separated on a capillary reversed-phase chromatography column (e.g. 75 μm × 25 cm, packed with 1.7 μm, 130 Å BEH C18 resin) and analyzed by nanoflow LC–MS using a linear gradient (120 min; A: 3% acetonitrile/0.1% formic acid; B: 97% acetonitrile/0.1% formic acid) at a flow rate of 400 nl/min. We have found that foregoing the use of a trapping column (which is frequently used in proteomics) significantly improves the recovery of small peptides. High resolution MS and fragmentation (MS/MS) data are then acquired in positive mode using a top-10 MS/MS strategy to facilitate peptide identification. These datasets must include +1 ions and low-molecular weight species, which are often excluded from conventional proteomic analyses. These complex spectra can then be interpreted using complete proteomes digested *in silico* via the 'no enzyme' cleavage specificity option available in existing proteomics software (e.g. Mascot and SEQUEST). These software tools can then identify peptides by matching the observed MS/MS patterns to the reference database. These assignments must be made using low scoring thresholds (e.g. Mascot ion score threshold $\leq$10) to allow for short peptide matching. Once the tentative assignments have been generated, peptides can be mapped to all of the possible proteins of origin and scored using the genome-wide coincidence mapping approach (Figure 1).

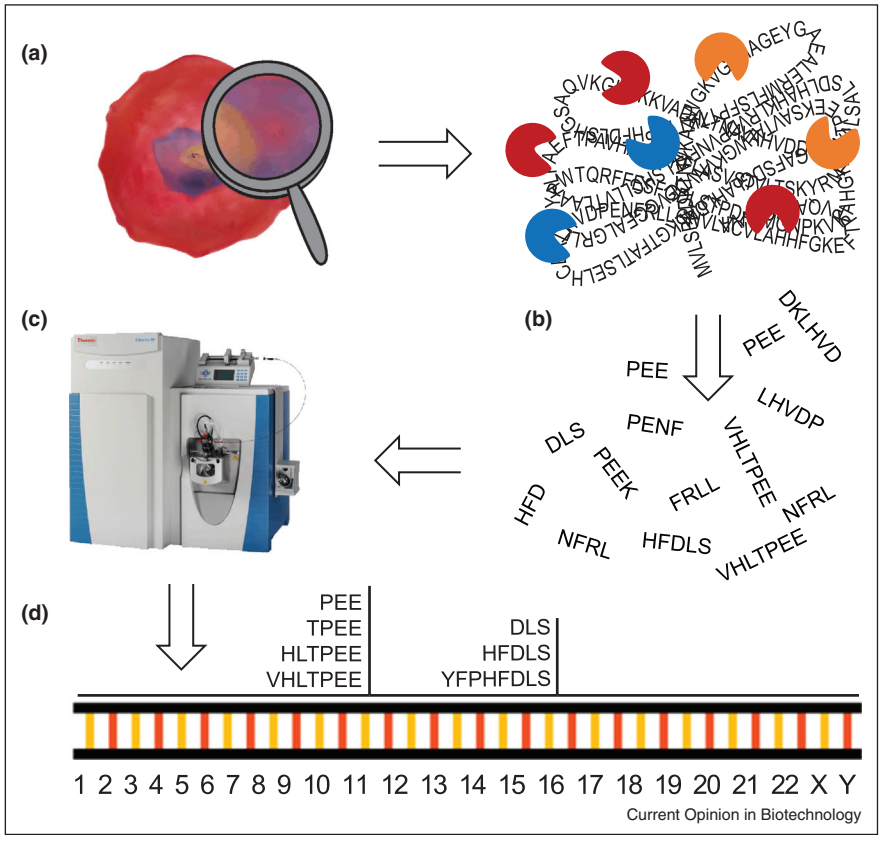
Bioinformatics in digestomics

There are significant underlying differences in data complexity and structure that differentiate digestomics from proteomics. Proteomics data generally contain diverse collections of peptides mapping to proteins widely distributed across the genome. In contrast, digestomics typically involves complex mixtures of related peptides originating from a relatively restricted set of proteins (i.e. a food source). For example, peptides found in malaria parasites are largely derived from hemoglobin [4••], analyses of wound exudates are heavily represented by clotting factors [16,17], and pancreatic cancer cells are associated with albumin catabolites scavenged from the extracellular environment [3••]. The low protein diversity in each of these cases reflects the fact that protein digestion generally targets a small subset of accessible proteins rather than the entire proteome. This observation has significant implications for the downstream bioinformatics.

In proteomics, bioinformatics strategies have been optimized for making confident protein assignments from relatively sparse collections of peptides. This process begins with the digestion of proteins using well-defined proteases (e.g. trypsin) to generate predictable cleavage patterns that can be matched to *in silico* reference libraries of protein digestion [18]. This strategy minimizes false discovery by restricting the bioinformatics search space [13]. Proteins can then be identified by matching observed MS/MS fragments to the reference library [13]. Since the matching score from existing algorithms increases proportionally with peptide length, there is an intrinsic bias against small peptides (Mascot; URL: http://www.matrixscience.com/help/feb2000.html). Although this strategy increases the confidence of protein identification, it de-emphasizes the small, but biologically significant, peptides generated through proteolysis [4••]. Once peptides have been assigned a score, a threshold is established for reducing false positives. These false discovery thresholds can be empirically determined by conducting peptide matches against a decoy database [19]. Generally, the highest match score of all peptides associated with a particular protein is a significant factor in the confidence of the protein identification. Although this strategy is optimized for the practical constraints of proteomics, it fails to leverage the significant data redundancy implicit to digestomics analyses.

The distribution of peptides observed in digestomics differs considerably from those seen in proteomics. Proteolytic cascades often involve the semi-ordered degradation of proteins via the ensemble action of multiple

Figure 1



Digestomics workflow. The generalized workflow for digestomics presented here is adapted from a previous study [4]. **(a)** Cells mobilize peptides and amino acids via proteolysis. The net action of these enzymes creates **(b)** a characteristic mixture of peptides that can **(c)** be decoded using high-resolution mass spectrometry. Naturally occurring peptides are extracted from biological samples using sample preparation methods adapted from metabolomics. Briefly, cells are extracted in acidified 90% methanol containing 3% acetonitrile. Mass spectra are acquired using a modified proteomics approach including nanoflow reverse phase ultrahigh performance liquid chromatography and top-ten MS/MS fragmentation in positive mode. Once these fragment data have been generated, they are assigned using Mascot and aligned to the appropriate genome at an amino-acid level of resolution. **(d)** Coincidence scores are then generated to reflect the aggregate dataset.

proteases. The overlapping specificity of proteases and nonlinear action of digestion produces a complex mixture of related peptides, each of which can be mapped to the same parent protein(s). Whereas spurious peptide matches occurring from false discovery map randomly across the genome, true positives map linearly to specific loci (Figure 2). This overlap creates an opportunity for improving signal-to-noise through signal averaging (Eq. 1), which is analogous to the routine data processing methods used in nuclear magnetic resonance spectroscopy [20]. These coincidence scores can be generated regardless of peptide length, uniqueness, or matching score, which circumvents many of the traditional barriers to integrating data from both large and small peptides. Our analyses show that coincidence-based scoring can introduce a significant improvement in the signal-to-noise of protein assignments relative to traditional proteomics algorithms for certain datasets (Figure 2). This data analysis approach may be portable to a variety of other proteomics applications that require similar peptide alignment strategies [21–27].

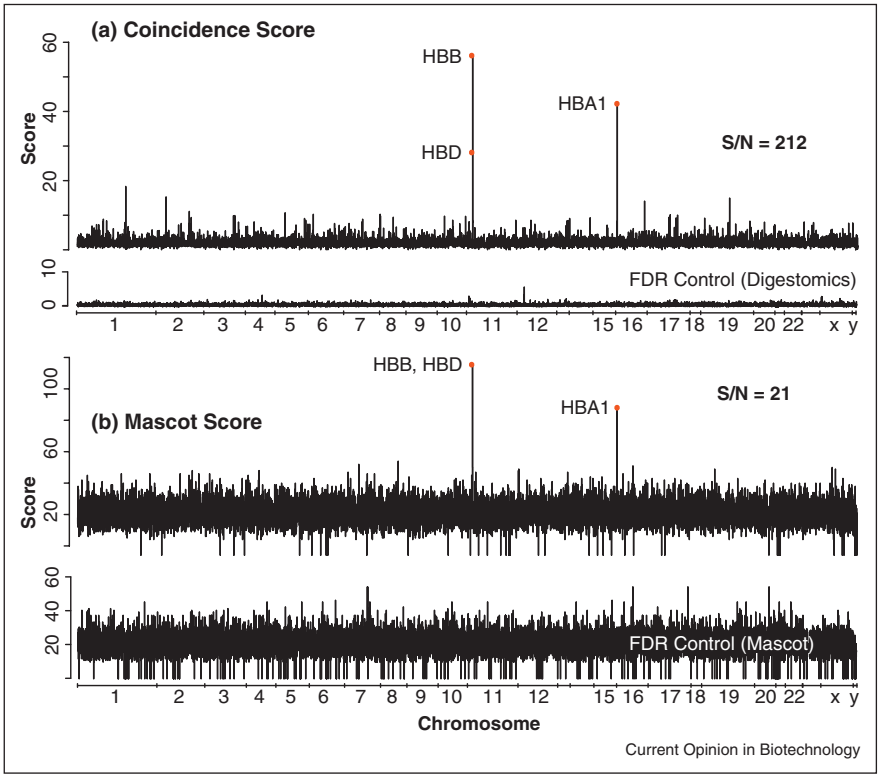
$$\text{coincidence score} = \frac{1}{n}\sum_{i=1}^{n} c_i \quad (1)$$

Where c_i is the number of peptides aligned to each locus in a protein containing n amino acids.

Biological applications and future directions

Malariology is one of the most active emerging areas in digestomics. The malaria parasite, *Plasmodium falciparum*, digests approximately 75% of the hemoglobin present in the red blood cell over the course of its 48-hour intraerythrocytic lifecycle [28]. Recent reports have found that chloroquine resistance is linked to seemingly minor defects in the parasite's ability to digest hemoglobin [4••]. These perturbations result in a dramatic reduction in parasite fitness and may explain why drug

Figure 2

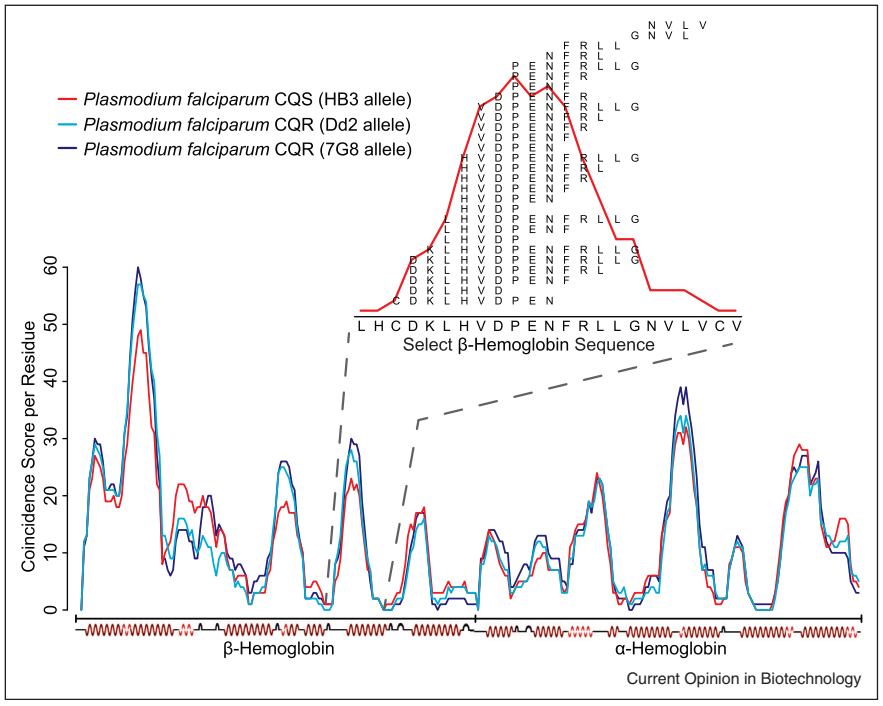


Genome-level maps of the *Plasmodium falciparum* digestome via traditional proteomics analysis versus the coincidence scoring algorithm. Proteolytic digestion profiles of human erythrocytes infected with late trophozoite stage *Plasmodium falciparum* analyzed using established proteomics methods and compared to the coincidence mapping approach. These data were adapted, with permission, from previously published work [4]. Each mapping strategy is shown relative to an empirical false discovery control generated by decoy searches against a sequence-scrambled human proteome. Both strategies start by translating MS/MS fragmentation data into putative peptide identities using Mascot searches. **(a)** Coincidence scores are then generated by aligning all of the putative assignments — irrespective of peptide length and including all peptides with Mascot scores of 10 or greater — to their respective genomic loci. The number of peptides aligned to each locus is quantified and expressed as an average representation for each gene. Correctly identified peptides consistently map to the same loci whereas false discovery resulting from incorrect peptide assignments are randomly distributed across the genome. Consequently, gene-by-gene averages of the total number of peptides mapped to each locus improves the signal to noise. **(b)** In traditional proteomics, the maximum Mascot score of all peptides matched to a particular protein is a significant factor in the confidence of the protein assignment. Fixed matching thresholds are used to minimize false discovery. Here, the maximum Mascot score associated with every gene is plotted to illustrate the improvement in signal to noise that can be captured via coincidence mapping. The signal to noise ratio was calculated by dividing the maximum match score by the standard deviation of the corresponding FDR control.

sensitive strains have re-emerged in regions of the world that have discontinued chloroquine therapy [4••,14]. The three-way association between drug resistance, parasite fitness, and metabolism was uncovered through an untargeted metabolomics screen, which identified several small peptides (2–4mers) that were consistently elevated in drug-resistant strains. Connecting these peptides with an underlying biological mechanism required a new analytical approach, digestomics, which unambiguously linked the small peptides to hemoglobin catabolism (Figure 3). Since the original report, several other studies have shown that mutations in the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) modulate both drug sensitivity and hemoglobin catabolism [14,29–32].

Other exciting applications of digestomics have been emerging from microbiology. A recent article by the Juillard laboratory employed this technology to decode the complex mix of peptides found in the media of *Lactococcus lactis*. Detailed characterization of these peptides clarified both the source of these molecules and the cell surface proteolytic mechanism through which they are generated [15•]. Similar extracellular proteolytic activities have been found in other microbes and may play a significant role in host-pathogen dynamics. *Lactobacillus plantarum* colonization of the *Drosophila*, for example, elevates proteolytic activity in the gut that accelerates fly development [33•]. Similarly, both the lungs of cystic fibrosis patients and wounds contain rich mixtures of proteins and peptides [34,35,17]; the pathogens that

Figure 3



Gene-level view of proteolytic coincidence maps from chloroquine resistant versus sensitive strains of *Plasmodium falciparum*. Proteolytic profiles of isogenic parasite lines containing either drug sensitive (CQS; Hb3) or resistant alleles (CQR; Dd2 and 7G8) of the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) were analyzed. These profiles, which were derived with permission from previously published data [4], were analyzed using the digestomics approach and the coincidence scores from β-hemoglobin and α-hemoglobin are shown. The figure inset depicts how detailed peptide alignments contribute to the per-residue coincidence score. Quantitative differences between the CQS and CQR lines shown here recapitulate published results, which linked elevated peptides from certain loci to both drug resistance and reduced fitness.

colonize these sites are known to secrete proteases that have been linked to pathogenesis and metabolism [36,37]. Other interesting host-pathogen examples include enteroaggregative *Escherichia coli* and *Shigella flexneri* colonization of the colon, which has been linked to mucin digestion by Pic protease [38]; and aspergillosis, which requires the secretion of fungal proteases to mobilize biosynthetic precursors [39,40]. Although it is clear that several host-pathogen dynamics are mediated through proteolytic mechanisms, these connections are still poorly understood. The emerging digestomics technology offers a promising new avenue for investigating these clinically-relevant phenomena.

Cancer biology is another application where digestomics has showed clear relevance. Tumorigenesis in several types of cancer has been directly linked to protein scavenging. Pancreatic ductal adenocarcinoma cells, for example, access extracellular pools of albumin through macropinocytosis and can use this protein as their sole source of essential amino acids [3••]. Similarly, peptidomics analysis of plasma from breast cancer patients showed a greater than 4000-fold enrichment in certain peptides [41]. These cancer/proteolytic connections are even more pronounced when intracellular processes are considered. Autophagy — the lysosomal degradation of intracellular components — is an active area of research and a promising therapeutic target [42–45]. Digestomics is of obvious relevance to these efforts and may help shape our understanding of tumorigenesis.

Beyond these health-related applications, digestomics remains pertinent to the food-related conception of its original definition [5,6]. Recent articles have harnessed this approach to examine the mixtures of peptides generated from *in vitro* digestion of meat and the role Maillard reactions play in this process [46,47]. In summary, understanding the details of protein digestion may provide critical insights into diverse subjects ranging from cooking to cancer.

Conclusions

Mixtures of proteases generate a characteristic fingerprint of peptides that reflects nutritional preferences and enzymatic activities. The emerging digestomics approach allows these complex mixtures to be decoded and quantitatively mapped on a genome-wide basis. These detailed maps offer direct insights into the fundamental selective forces that shape organisms and, as the malaria example shows, can link seemingly minor molecular

details to world-wide population dynamics [4••]. We anticipate that this strategy will eventually become a routine technique that fills the gap between metabolomics and proteomics.

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