Structural properties of food proteins underlying stability or susceptibility to human gastrointestinal digestion
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Is any specific structural trait that makes food proteins resistant or sensitive to proteolytic degradation in the gastrointestinal tract? In principle, elements at various hierarchical levels of protein structure may confer stability or susceptibility to hydrolysis. However, based on a critical revision of the recent literature, we emphasize the impossibility of providing absolute answers because protein digestion is affected by a complex series of factors ascribable to the food micro-/macrostructure and the intricacy of human digestion. Taking into consideration the uncertainty associated with the current in vitro models of food digestion/absorption and the capability offered by recent analytical advancements, we emphasize the need for suitable in vivo readouts for improving the comprehension of the fate of dietary proteins in our body and their effects on human health.

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Current Opinion in Food Science 2023, 50:100992
This review comes from a themed issue on Food Physics & Materials Science
Edited by Andrea Gomez-Zavaglia
For complete overview of the section, please refer to the article collection, “Food Physics & Materials Science 2023”
Available online 13 January 2023
https://doi.org/10.1016/j.cofs.2023.100992
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Introduction
Food proteins experience ‘harsh’ conditions throughout the human gastrointestinal (GI) tract. Generally, dietary proteins are split into large- and medium-sized polypeptides by pepsin in acidic gastric conditions. Downstream, in the duodenum, a set of pancreatic proteases and peptidases with varying cleavage specificity cooperate to degrade the products of peptic digestion, which are further processed by a complex arsenal of intestinal brush border membrane exopeptidases before absorption [1]. Peptides that eventually cross the intestinal barrier and reach the bloodstream may undergo hydrolytic attack by a series of plasma peptidases [2]. Such a coordinated and finely tuned process of protein degradation has evolved to pursue a dual physiological purpose: 1) breakdown of proteins into simple derivatives that can be absorbed and used by the body; 2) reduction of the risk of abnormal reactions toward non-self potentially harmful sequences. Nevertheless, some food proteins or their large proteolytic products may relevantly survive GI degradation. The possible existence of a direct link between protein digestion stability and the capability of inducing immunoglobulin E (IgE)-mediated adverse reactions has been questioned and practically refused [3,4]. However, the way and the extent to which food proteins are degraded during digestion have several implications on human health regarding the absorption and bioavailability of the resulting amino acids, and the survival of possible bioactive or immunogenic polypeptides.

Is any specific structural trait that makes food proteins stable or sensitive to proteolytic degradation in the GI tract? Despite the extensive research on the topic, this question remains substantially unanswered due to uncertainty associated with several orders of variability factors (Figure 1): i) effects of the food matrix on protein structure and aggregation; ii) variety of in vitro and in vivo digestion models and variability of conditions for any given model; iii) intra- and interindividual variability in the case of in vivo digestion; iv) analytical issues related to the characterization of the ‘digestomes’ [5].

Based on selected recent investigations, we survey the structural features affecting the ‘digestibility’ of food proteins and the possible implications for human health. Furthermore, we emphasize those still unresolved aspects that are driving the upcoming research trends in this field. In nutrition, protein digestibility is usually a measure of the degree of net absorption of nutrients in the digestive tract. Herein, digestibility is intended in a broader meaning, indicating the degree of hydrolysis
that food proteins undergo during digestion, which measures the overall number of peptide bonds cleaved at any stage of the GI degradation process.

**Structural traits affecting digestion stability of food proteins**

Protein stability depends on all the levels of hierarchical organization, that is, primary, secondary, ternary, quaternary structures, and supramolecular assembly. The structural levels of a protein are interrelated, as the secondary structure depends on the primary one and the resulting three-dimensional (3D) organization is, in turn, affected by intrinsic factors, such as intrachain disulfide bonds, hydrophobicity, hydrogen bonding, post-translational modifications, as well as by the interaction with the environment (e.g., solvent, ions, chaotropic agents, pH, and temperature). By determining protein folding and accessibility of proteases to cleavage sites, these factors affect the inherent resistance of food proteins against process-induced denaturation and GI hydrolysis and, therefore, food-protein digestibility.

Maize (*Zea mays* L.) storage proteins, that is, zein, are generally considered resistant to GI digestion. Zeins have an elongated axial ratio, with a 3D organization different from compact globular proteins. In hydroalcoholic solution α-zeins, which are the most abundant and hydrophobic ones of the family, have coiled-coil tendencies that result in complex aggregates with a central section of nine helical segments constituted by nonpolar residue side chains connected by glutamine-rich loops or turns, forming a hardly accessible hydrophobic surface inside a triple superhelix [6]. Secondary structure of α-zeins is solvent-dependent and their amphiphilic character is the main driving force for their self-assembly properties that ultimately produce protein structures functionally analogous to a block copolymer. In the GI tract’s water milieu, the overall organization of zein might be even more compact. Thus, zeins represent a paradigmatic example of food proteins with characteristic primary structure, determining the formation of secondary and tertiary structural elements with a subsequent supramolecular assembly that hinders digestibility (Figure 2). In this context, specific cysteine residues are critical for contributing to the formation of large protein aggregates [6]. The factors that control the supramolecular assembly of food proteins are still underexplored, even though they influence the sensory and nutritional quality of foods and could be modulated through processing [7]. Some seed storage proteins, such as the allergenic soybean 7S β-conglycinin subunits, natively assemble into amyloid aggregates that exhibit resistance to digestion [8]. However, even small conformational changes can drastically modify protein digestibility. For example, the so-called S-ovalbumin (S-Ova), a conformer thermodynamically more stable than native ovalbumin (Ova) generated by slight spontaneous conformational transitions, denatures at higher temperatures and is more proteolytically resistant than Ova, especially toward pepsin. Upon denaturation, Ova and S-
Ova share comparably increased *in vitro* digestibility and similar immunological properties [9].

Environmental parameters, such as solution pH, are primary thermodynamic variables determining the structure, function, and dynamics of proteins by settling the charge of the polypeptides. Native β-lactoglobulin (β-Lg) is resistant to pepsin because of exceptional structural stability at gastric pH. Many potential cleavage sites for pepsin are buried inside the hydrophobic core of the native protein and are not readily accessible. Unlike β-Lg, α-lactalbumin (α-La) is pepsin-sensitive [10], despite a more compact 3D structure consisting of 123 amino acid residues stabilized by four disulfide bridges. In the gastric environment, also due to the loss of affinity for a Ca$^{2+}$ ion at acidic pH, α-La undergoes conformational transitions populating the so-called ‘molten globule state’, which are relatively stable folding conformations, intermediate between native or denatured states. In these conformations, proteins maintain several native-like secondary structure elements but have a loose tertiary structure that involves the exposure of the hydrophobic core to the solvent and proteases. The substantial insensitivity of native α-La (holo form) to trypsin at nearly neutral pH [11] confirms the pH dependence of protein conformations and related digestibility. The pH-dependent structure of food allergens directly influences the kinetics of the proteolytic digestion after endosomal uptake by antigen-presenting cells. The subsequent exposure of the major histocompatibility complex (MHC) class-II molecules, which can vary as a function of pH, determines the T-cell polarization and the immune response [12].

Nonprotein food compounds affect GI protein degradation. Starch and fibers synergistically reduce protein digestibility by hindering the accessibility to proteases and reducing hydration [13]. Food lipids alter...
**Digestion stability of food peptides**

Dietary bioactive peptides often are very short sequences (<10 residues or shorter), and the determinants of food allergies are, in general, proteolytic products of food allergens, that is, peptides, and not the proteins themselves. Nevertheless, small-/medium-sized peptides resulting from protein hydrolysis escape the detection by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which is commonly used to monitor protein degradation. For this reason, we believe that an informative characterization of the food-protein ‘digestomes’ requires necessarily a combination of mass spectrometry-based peptidomics and proteomics [5].

The peptide patterns obtained from the digestion of a food product depend on the process technology. For example, digestion kinetics and peptide patterns resulting from *in vitro* or *in vivo* digestion differ among differently manufactured cheese types and from those of yogurt and raw milk [28,29]. Similarly, isolated gluten protein fractions and whole-wheat flour produce different peptides, regardless of the wheat genotype [30]. On the other hand, some specific peptide ‘core’ sequences appear intrinsically stable toward digestive degradation [31]. A recent meta-analysis has individuated some traits of the primary structure common to many
peptides that have been described as resistant to GI digestion [32]. These traits include lower hydrophobicity, higher positive net charge at intestinal pH, branched-chain aliphatic N-terminal residues, absence of C-terminal leucine, and higher histidine and proline content, especially at the C-terminal, compared with susceptible peptides. However, there is no straightforward correlation between physiochemical properties and peptide stability; thus, the current in silico approaches are inadequate to predict the behavior of peptides exposed to hydrolytic enzymes. For example, low-sized peptides generally may show better structural stability than longer ones. Nevertheless, low-molecular-weight angiotensin converting enzyme (ACE)-inhibitor fish-derived peptides were more promptly hydrolyzed by the pepsin–pancreatin system under simulated conditions of GI digestion than their high-molecular-weight precursors [33]. Similarly, several casein-derived small molecular weight fractions appear less stable than longer casein-derived peptides during simulated GI digestion [32]. The supposed antihypertensive Val–Pro–Pro and Ile–Pro–Pro lactotripeptides are among the most studied bioactive peptides with well-assessed digestion stability [34]. Anyway, size does not appear to be a determining factor per se, since the pea-derived ACE-inhibitor tripeptide Leu–Arg–Trp fails to lower blood pressure in spontaneously hypertensive rats due to chymotrypsin-mediated hydrolysis, unlike its egg-derived isomer Ile–Arg–Trp, which exhibits antihypertensive and multifunctional activities in vivo [35].

Several in silico attempts have been made to predict the potential blood stability of food-bioactive peptides [36]. A high frequency of negatively charged (Glu) and small-sized residues (Ala, Glu, Ile, and Leu) correlates with long half-life in mammalian blood. In contrast, aromatic (Tyr and Phe) and other near-neutral amino acids (Gly, His, and Ser) predict a short peptide half-life. A Trp-to-Val substitution drastically reduces the peptide stability to blood peptidases [36]. However, the predictive power of these approaches based on the analysis of primary structure alone is limited and experimental validation is required.

Gluten peptides are exceptionally resistant to processing by GI digestive enzymes because of the high content of proline and glutamine residues [37]. However, Pro (formally an amino acid and not an amino acid) increases disorder and flexibility in Pro-rich peptides through cis-/trans-isomerization [38], thus potentially reducing peptide stability. Pro-rich domains are not intrinsically resistant to enzymatic hydrolysis since many microbial or mold-derived endoproteases are able to cleave gluten peptides in pseudogastric conditions [37]. Intestinal mucosa does include prolyl- or dipeptidyl-peptidases, which can cleave Pro-containing peptides [32]. Therefore, peculiar conformational arrangements of proline-rich peptides and their relatively low water solubility might contribute to explaining why these enzymes are the rate-limiting ones in the digestive breakdown [39,40]. The macropeptides of gliadins and low-molecular-weight glutenin subunits promptly released by pepsin contain hepta- and dodecapeptide repeat proline-/glutamine-rich motifs. Circular dichroism and nuclear magnetic resonance (NMR) determinations have confirmed that gliadin macropeptides, including the α2-gliadin-derived 33-mer immunodominant peptide in celiac disease, adopt a polyproline-2 (PP2) conformation both in water and in hydroalcoholic solvents [38]. At high concentrations and depending on the temperature, the PP2 conformation can convert toward a β-parallel structure, inducing a self-assembly process into macroaggregates that proteases could hardly access.

Heating or other food processing can modify several amino acids or induce the formation of unnatural compounds. Nonenzymatic glycation decreases protein digestibility, masking some amino acids, especially lysine, to protease recognition. Other chemical modifications may influence protein digestion, including oxidation, racemization, dephosphorylation, and covalent cross-linking. The overall impact of these modifications on protein and peptide bioaccessibility/bioavailability and their relationship with human health require urgent assessment [41,42], especially for formula-fed infants in early life, when severely processed milk or milk substitutes are the sole or prevalent source of food proteins [23,41].

Role of proteases and in vivo bioavailability

In addition to the intrinsic stability, the digestibility of food proteins depends on the efficiency of GI proteases/peptidases [43]. Under physiological conditions, GI proteases/peptidases cover broad ranges of enzymatic activities and cleavage specificities [32].

Human digestion efficiency and absorption can vary according to numerous factors, such as genetics, age, sex, assumption of drugs, dietary pattern, oral chewing, and physical and psychological status. As for the protein substrates, the chemical environment affects the activity of digestive enzymes. Furthermore, GI proteolytic action is modulated by endogenous protease inhibitors and exogenous food components (e.g. plant protease inhibitors and polyphenols) [44]. The complexity of food peptide patterns found in vivo, even evidencing unexpected cleavage specificities [45], suggests the possible contribution of additional factors to digestion, such as GI microorganisms. The in vitro models of food-protein digestion cannot account for the intra- and interindividual variability of digestion and absorption capacity, which should be investigated as key ‘primary endpoints’ in nutritional research [46].
For this reason, in vivo bioavailability of food-protein components should be considered the main goal for establishing the relationship between dietary proteins and human health. Few investigations have addressed the bioavailability of food-derived peptides due to many technical challenges [5,47]. However, some recent studies have demonstrated that dietary peptides are stable enough to be monitored in biological fluids. Peptides from peanut allergens have been detected in both human plasma and breast milk by immunochromatographic techniques [48]. More reliably, circulating milk-derived peptides have been characterized by high-resolution mass spectrometry [49]. It has been demonstrated that food-derived peptides can survive digestion and are then excreted into breast milk [50–52]. Immunological active gluten peptides have been identified in the urine of children who had eaten cereals [53]. Milk peptides have been identified in the stool of breastfed infants, demonstrating that some sequences can survive the entire process of GI degradation, including the proteolysis by colon microorganisms, and may even influence infant gut development [54]. These results have been obtained with small cohorts of individuals and need to be supported by studies on extended populations of healthy and diseased subjects. Under physiological conditions, dietary peptides occur in human biological fluids at very low abundance. The hypothesis that peptide concentration might be underestimated because part of them is converted into bioactive nonpeptide metabolites deserves further exploration [55].

Conclusions

The bioavailability and excretion peptide patterns emerging from the preliminary studies in humans reflect the extremely high variability and complexity of protein digestion. Many knowledge gaps still prevent us from offering a concrete perspective of preparing food products that meet specific nutritional and functional requirements [56]. Most of the current studies carried out with in vitro digestion models are focused on the detailed kinetics of food-protein degradations rather than on the fate of food-protein-derived products in our body. The structural and mechanistic factors that rule the organization of nutrients within raw and processed food matrices, their digestion, absorption, distribution, effects on target organs, and ultimate impact on human health should be addressed with opportunely designed investigations, which combine molecular assessments with in vivo clinical interventional trials and observational studies.

Conflict of interest statement

Nothing declared.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


This review surveys the impact of technological processes on milk protein modifications and their stability to GI digestion.


This study attempted to determine the half-life of known food bioactive peptides in blood using in silico approaches, based on structural factors such as primary structure, molecular weight, and hydrophobicity.


This review surveys the impact of technological processes on milk protein modifications and their stability to GI digestion.

This review summarizes capabilities and challenges of peptidomics for characterizing food-derived peptides in biological fluids and tissues.


In this article, hundreds of dietary peptides were purified from the plasma of human volunteers and characterized by mass spectrometry.


In this article, dietary gluten peptides have been purified from the urine of healthy and celiac disease individuals and, for the first time, characterized by mass spectrometry.


This article demonstrates that some food-derived peptides can survive the degradation along the entire GI tract, also including the microbial fermentation in the colon.


This article hypothesizes the metabolic transformation as an alternative mechanism through which dietary peptides exert systemic bioactive effects.


This review surveys the structural and compositional aspects of foods that modulate macronutrient digestibility, the mechanistic basis to elucidate the effects of nutrition on human health and the possibility of designing foods with improved functionality.