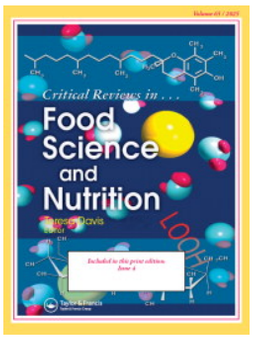

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Critical Reviews in Food Science and Nutrition

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/bfsn20

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To cite this article: Mengxiao Yang, Zhi Yang, David W. Everett, Elliot Paul Gilbert, Harjinder Singh & Aiqian Ye (21 Jan 2025): Digestion of food proteins: the role of pepsin, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2025.2453096

To link to this article: https://doi.org/10.1080/10408398.2025.2453096

Published online: 21 Jan 2025.

CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
https://doi.org/10.1080/10408398.2025.2453096

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REVIEW ARTICLE

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Digestion of food proteins: the role of pepsin

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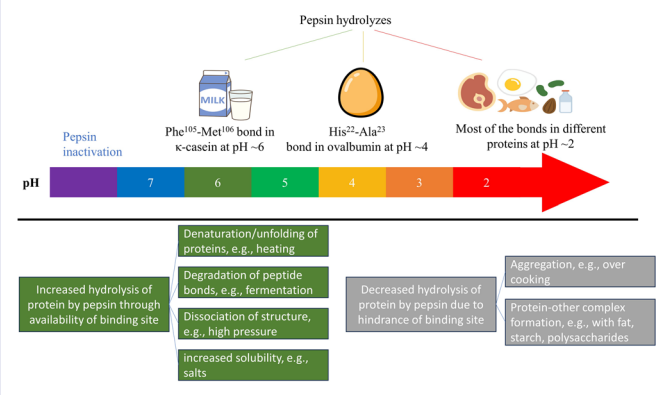
ABSTRACT

The nutritive value of a protein is determined not only by its amino acid composition, but also by its digestibility in the gastrointestinal tract. The interaction between proteins and pepsin in the gastric stage is the first step and plays an important role in protein hydrolysis. Moreover, it affects the amino acid release rates and the allergenicity of the proteins. The interaction between pepsin and proteins from different food sources is highly dependent on the protein species, composition, processing treatment, and the presence of other food components. Coagulation of milk proteins under gastric conditions to form a coagulum is a unique behavior that affects gastric emptying and further hydrolysis of proteins. The processing treatment of proteins, either from milk or other sources, may change their structure, interactions with pepsin, and allergenicity. For example, the heat treatment of milk proteins results in the formation of a looser curd in the gastric phase and facilitates protein digestion by pepsin. Heated meat proteins undergo denaturation and conformational changes that enhance the rate of pepsin digestion. This review provides new ideas for the design of food products containing high protein concentrations that optimize nutrition while facilitating low allergenicity for consumers.

GRAPHICAL ABSTRACT



KEYWORDS

Digestion; egg protein; meat protein; milk protein; plant protein; pepsin

Introduction

Proteins are macromolecules that contain various amounts of 20 different amino acids linked *via* peptide bonds (Wu 2021) and are a vital part of our diet (Joye 2019). Typical dietary proteins are derived from animal and plant sources. Animal-derived proteins are present in meat, poultry, fish, eggs, and dairy products. They are widely consumed or used as ingredients in the food industry, for example, casein, whey protein, muscle proteins, and egg proteins. However, the production of animal-derived protein sources is associated with higher greenhouse gas emissions, whereas plant-based protein sources are considered environmentally sustainable (Gorissen and Witard 2018). Plant proteins are divided into legume and cereal proteins, which are abundant, relatively low-cost, sustainable, less allergenic, and widely accepted by consumers (Goldstein and Reifen 2022). In addition, emerging protein sources, such as cultured meat, insects, algae, and microbial proteins have great potential for meeting future protein supply and demand, but few of these have been commercialized (Kaur et al. 2022). Generally, animal-derived proteins provide the essential amino acids in the ratios needed to sustain growth and metabolic processes in the human body (Kaur et al. 2022),

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whereas plant-based protein sources typically have suboptimal levels and ratios of the essential amino acids (Schimbator et al. 2020). The nutritive value of dietary protein should consider the amino acid composition and the digestibility and the absorption of the resultant hydrolysis products in the human gastrointestinal tract (Villamide et al. 2010).

Generally, three different phases of protein digestion can be distinguished throughout the gastrointestinal tract (Erickson and Kim 1990): cephalic, stomach, and intestinal. Gastrointestinal enzymes hydrolyze peptide bonds adjacent to specific amino acids in dietary proteins. The initial step in protein digestion is degradation at the acidic pH in the stomach and hydrolysis by the action of pepsin with varying substrate specificities (Whitecross et al. 1973; Silk, Grimble, and Rees 1985). Pepsin is the principal enzyme involved in protein digestion in the gastric phase. Pepsin is a nonspecific monomeric aspartic protease with broad substrate specificity, and it is most efficient in hydrolyzing peptide bonds between hydrophobic amino acids, and preferably aromatic amino acids, such as tryptophan, tyrosine, and phenylalanine (Mohseni-Shahri, Moeinpour, and Nosrati 2018; Inouye and Fruton 1967; Fujimoto et al. 2004), whereas it does not hydrolyze bonds containing valine, alanine or glycine (Monogioudi et al. 2011). Interactions between proteins and pepsin are strongly dependent on the pepsin-to-protein ratio. However, humans show wide variations in gastric and pancreatic secretions, which vary with the type of food consumed (da Silva Gomes et al. 2003; Armand et al. 1996). The pH in the fasting stomach is approximately 2, but can vary from 1.5 to 6 depending on the time since eating and the concentration of the different types of food components in the meal, greatly impact pepsin activity. Previous studies on protein digestion fall into four categories: *in vivo* human studies, *in vivo* animal studies, static *in vitro* studies, and (semi)-dynamic *in vitro* studies (van Lieshout et al. 2020).

Most ingested proteins are first digested by pepsin, which produces large polypeptides, a few smaller peptides, and a few free amino acids in the stomach (Joye 2019). Several techniques have been used to evaluate the progress of protein hydrolysis using the concept of degree of hydrolysis, defined as the percentage of hydrolyzed peptide bonds (Navarrete del Toro and García-Carreño 2003). A free amino group and a free carboxyl group are released each time a peptide bond is hydrolyzed. Spectrophotometric, potentiometric, chromogenic, or fluorometric techniques are usually carried out as time-course experiments to quantify the extent of hydrolysis (Navarrete del Toro and García-Carreño 2003). Various models have been developed to describe the kinetics of pepsin-mediated soluble protein hydrolysis. Numerous studies have been based on the Michaelis–Menten model (Ruan, Chi, and Zhang 2010; Luo, Zhan, et al. 2018; Luo, Chen, et al. 2018), which allows prediction of the reaction kinetics by considering the effects of the enzyme-to-substrate ratio, the affinity of the protease for the substrate, and several other parameters. Another commonly used model relies on the use of a first-order reaction; for example, a two-parameter kinetic model has been applied to simulate the digestion of milk proteins under conditions of moderate enzyme inactivation (Margot, Flaschel, and Renken 1997; Yang et al. 2022; Carlson, Hill, and Olson 1987a). To explore protein unfolding as a limiting factor in the gastric digestion of proteins, Herman, Gao, and Storer (2006) developed a theoretical approach with two consecutive exponential steps, one for protein unfolding and one for protein hydrolysis by pepsin. Kondjoyan, Daudin, and Santé-Lhoutellier (2015) developed a model based on first-order reaction kinetics that accounts for heat-induced changes in the number of hydrolysis sites in myofibrillar proteins to predict the kinetics of *in vitro* digestion by pepsin.

The products generated during protein digestion largely depend on the structural properties of the food, including protein solubility, accessibility of digestive enzymes, protein sources, and the processing history of foods (Kaur et al. 2022). In addition, the structure formed during digestion plays an important role (Ye 2021); for example, the proteins from cow milk will coagulate, whereas oat "milk" does not show substantial changes in structure and physical stability during *in vitro* gastric digestion with pepsin (Xin Wang et al. 2022). The diffusion of digestive enzymes can be impacted as a consequence of different protein structures formed during digestion, i.e., inhibition of pepsin diffusion within a solid compared to a liquid matrix (Q. Luo et al. 2017). This, in turn, affects the passage of gastric chyme, further degradation of proteins and peptides, and the absorption of free amino acids in the small intestine (Moughan 2009). Poor digestibility is associated with increased allergenicity when intact proteins or large fragments are exposed to the gut immune system, thereby inducing IgE-mediated food allergic responses (Astwood, Leach, and Fuchs 1996; Untersmayr and Jensen-Jarolim 2008). Besides, pepsin is one of the commonly employed as primary proteases in the enzymatic production of bioactive peptides. For these reasons, the interaction between proteins and pepsin as an initial step of digestion is important for nutrient release and reducing allergenicity. In this review, the interactions between pepsin and proteins from different sources are discussed, with a focus on the interactions between pepsin and milk proteins. In addition, as food, in most cases, undergoes processing and storage prior to consumption, the effects of certain common pretreatments (mainly thermal) on the physicochemical properties of proteins and their digestibility will be discussed.

Gastric environment

The stomach secretes an average of 2–3 L of gastric fluid each day, containing water, electrolytes, hydrochloric acid, enzymes, mucus, and glycoprotein intrinsic factor (Arora et al. 2005; Bornhorst and Paul Singh 2014). In a fasting state, gastric pH is approximately 3.5–5.0 for infants and approximately 1.5–2.0 for a healthy adult. Generally, after consuming a meal, the pH of the gastric content increases to approximately 5.5–7 and decreases with further gastric emptying, depending on the nature and amount of ingested food (Huppertz and Chia 2021; Ye 2021).

Pepsin

Digestive enzymes enable the efficient digestion of food proteins in the stomach. Pepsinogens are classified into five groups: pepsinogens A, B, and F; progastricsin; and prochymosin, which are precursors of pepsin A, B, and F; gastricsin; and chymosin, respectively (Table 1) (Kageyama 2002).

A comparison of the primary structures of typical mammalian pepsinogens and pepsins has been reported by Kageyama (2002). For ease of use, most *in vitro* studies have utilized commercial porcine pepsin A (EC 3.4.23.1), referred to as "pepsin" throughout this review unless otherwise specified. Pepsin has broad specificity for hydrolyzing bonds adjacent to aromatic and hydrophobic amino acids, such as phenylalanine, tyrosine, tryptophan, and leucine (Dunn 2002; Inouye and Fruton 1967), with an optimum pH of approximately 2.0 (Kondjoyan, Daudin, and Santé-Lhoutellier 2015; Pletschke, Naudé, and Oelofsen 1995).

To explore the impact of protein substrates on the pH dependence of pepsin activity, a comparation of degree of hydrolysis with initial rates of pepsinolysis was reported in Figure 1 (Salelles, Floury, and Le Feunteun 2021). For consistency, the activity at pH 2 is arbitrarily set to 100%. The graph showed that pepsin activity increases as pH decreases at pH values ≥ 2, regardless of the substrate. However, it also highlights that pepsin activity under extreme acidic (pH < 2) or weakly acidic (pH ≥ 4) conditions is strongly influenced by the specific protein substrate. Notably, caseins emerge as the most susceptible substrates to peptic hydrolysis within the pH range of 6–3.

The amino acid sequence homology is 57.5% between chymosin and pepsin, and the active site sequences, Phe^{33}–Ser^{38} and Asp^{216}–Thr^{219}, of chymosin are fully conserved in pepsin (Phe^{31}–Ser^{36} and Asp^{215}–Thr^{218}) (Plowman and Creamer 1995). Both enzymes can rapidly hydrolyze the Phe^{105}–Met^{106} bond of κ-casein at pH above 5, and before the easy availability of cloned enzymes, porcine pepsin was often admixed with bovine chymosin for cheesemaking (Plowman and Creamer 1995; Kageyama 2002). Therefore, gastric pH is critical for pepsin action, which affects both the active site conformation and enzymatic activity, especially with milk proteins as a substrate. The pepsin activity against typical substrates (hemoglobin and milk) is shown in Table 2.

Interaction between milk proteins and pepsin

Milk is an excellent source of nutrients for humans that provides proteins, fats, vitamins, and minerals (Haug, Høstmark, and Harstad 2007). In most ruminant milk, nitrogen is distributed in the form of caseins, whey proteins, milk fat globule membrane proteins, and non-protein fractions (Swaisgood 1982). Studies on milk digestion, especially milk protein digestion, have recently attracted extensive attention (Thom Huppertz and Chia 2021; Ye 2021; Miranda and Pelissier 1983; Wada and Lönnerdal 2014; Tunick et al. 2016; Ye et al. 2016b; Mulet-Cabero et al. 2020). Different hydrolysis and digestion behaviors have been observed under gastric environmental conditions because of the structural differences between casein and whey proteins (Wang, Ye, et al. 2018), which affect allergenicity (Bernard et al. 1998; Busse et al. 2002; Cocco et al. 2003).

Table 1. Classification of pepsinogens [adapted from Kageyama (2002).

Group	Active form	Enzyme Commission number
Pepsinogen A	Pepsin A	3.4.23.1
Pepsinogen B	Pepsin B	3.4.23.2
Progastricsin	Gastricsin	3.4.23.3
Prochymosin	Chymosin	3.4.23.4
Pepsinogen F	Pepsin F	–

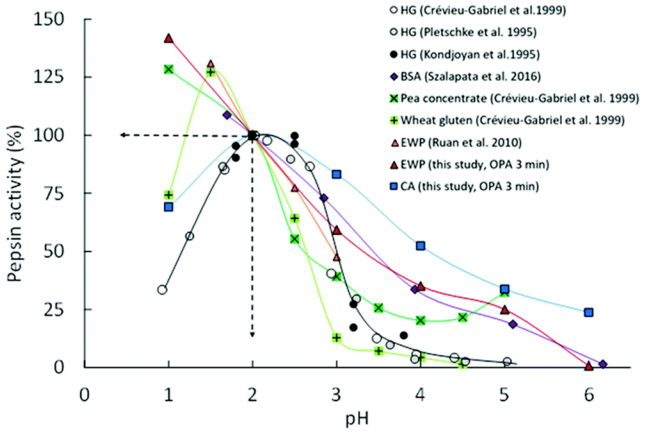

Figure 1. pH dependence of porcine pepsin activity at short times on different protein substrates: Hemoglobin (HG; [○], • and ○), bovine serum albumin (BSA; [●]), pea concentrate ([○]), wheat gluten ([◆]), egg white proteins (EWP; [✕] and [+]) and casein aggregates (CA; [▲]). Lines represent the guides to the eyes and activity values were set arbitrary at 100% at pH = 2.0. Adapted from Salelles, Floury, and Le Feunteun (2021).

Table 2. Pepsin activity against typical substrates[a] [adapted from Kageyama (2002).

Enzyme	Hydrolytic activity (%)[b] Hemoglobin	Milk clotting activity (%)[c]
Porcine pepsin A	55–59	25–66
Porcine pepsin B	1–3	0.4–1
Human gastricsin	100	43
Bovine chymosin	7–14	100

[a]The variation in a few values is due to differences reported in the literature.
[b]Hemoglobin digestive activity was determined at pH 2.0 (Foltmann 1992; Nielsen and Foltmann 1995; Tang et al. 1959).
[c]Assay at pH 6.3 (Foltmann 1992; Tang et al. 1959). The value of porcine pepsin B is estimated from a "caseogram" assay (Foltmann, Szecsi, and Tarasova 1985). The highest value is considered as 100%.

Whey proteins

Whey proteins mainly consist of β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) (ratio ca. 3:1) in milk (Visser, Slangen, and Rollema 1991). Both proteins have well-defined secondary and tertiary structures, with intermolecular disulfide bonds stabilizing the tertiary structure, but are prone to heat-induced unfolding and denaturation at >80 °C (Boland 2011).

In solution, native α-La is exposed to hydrolysis by pepsin. The rate of proteolysis of α-La increases during the gastric digestion process, resulting in the formation of lower molecular-weight products. However, the resistance of α-La to pepsin increases upon adsorption at an oil-water interface due to alterations in tertiary conformational structure (Nik, Wright, and Corredig 2010).

β-Lg has a three-dimensional structure consisting of an eight-stranded antiparallel β-hydrophobic barrel unit with an α-helix, accounting for the resistance to acid hydrolysis and pepsin action in the gastrointestinal tract (Dalgalarrondo et al. 1995; Chobert et al. 1995; Reddy, Kella, and Kinsella 1988; Schmidt and Poll 1991). The relative resistance of β-Lg to acid hydrolysis, as well as to proteases, allows a portion to remain intact after digestion, and this is subsequently absorbed through the intestinal mucosa and presented to immunocompetent cells, thus becoming one of the main allergens in cows' milk (Wal 2001). Heating at 80–90 °C induces structural changes in β-Lg that improve susceptibility to pepsin activity (Reddy, Kella, and Kinsella 1988). The reduction of disulfide bonds in β-Lg can increase its susceptibility to pepsin action (Kananen et al. 2000). Furthermore, adsorption-induced changes in β-Lg facilitate the hydrolysis of peptides by pepsin (Macierzanka et al. 2009). When present as an adsorbed layer on emulsion surfaces, the change in conformation due to unfolding at the emulsion interface exposes peptic hydrolysis sites, substantially decreasing the resistance of β-Lg to pepsin (Sarkar et al. 2009; Nik, Wright, and Corredig 2010; Peram et al. 2013).

Casein proteins

In the milk of most species, caseins constitute a class of four gene products, that is, α_{S1}-casein, α_{S2}-casein, β-casein, and κ-casein (Visser, Slangen, and Rollema 1991; T Huppertz 2013). The individual casein molecules lack a tertiary structure, but together with colloidal calcium phosphate (CCP), can assemble as large structures called casein micelles, with κ-casein bound to the micelle surface layer (Fox 1993; Dalgleish and Corredig 2012). Over the last few decades, different models have been proposed to characterize the internal and external structures of casein micelles, including the submicelle (Walstra 1990), dual binding (Horne 2006), and nanocluster (de Kruif and Holt 2003; Douglas G Dalgleish 2011) models. α_{S1}-casein, α_{S2}-casein, β-casein, and κ-casein have been classified as important cow milk allergens by the World Health Organization and International Union of Immunological Societies, with their allergenic potential in the micelle structure being conserved (Costa et al. 2022).

Caseins (without micellar structure)

Hydrolysis of individual caseins by the proteolytic enzymes normally occurs at pH <4 (in a thermostatically controlled water bath at 30 °C) (Tam and Whitaker 1972). α_{S1}-casein and α_{S2}-casein are rapidly hydrolyzed by pepsin during gastric digestion (Lisson, Lochnit, and Erhardt 2014), and no measurable amounts of intact protein can be detected at the end of this phase (Astwood, Leach, and Fuchs 1996; Dupont et al. 2010; Fu, Abbott, and Hatzos 2002). This is expected, as α_{S1}- and α_{S2}-caseins are poorly structured proteins with no defined tertiary structure, and therefore exposed to proteases, such as pepsin, trypsin, and chymotrypsin (Swaisgood 2003; Astwood, Leach, and Fuchs 1996). According to Lisson, Lochnit, and Erhardt (2014), after gastric digestion, almost no breakdown was observed in the 54–89 region of α_{S1}-casein, whereas pepsin-generated peptides were detected in the remainder of the protein sequence. For α_{S2}-casein, except for regions 34–59 and 198–207, which barely provided any proteolytic fragments, peptides were distributed throughout the entire protein sequence (Lisson, Lochnit, and Erhardt 2014). In addition, there are preferential hydrolysis sites for pepsin, for example, glutamic acid at position 192 in α_{S1}-casein B variant and phenylalanine in α_{S2}-casein variant B.

When β-casein from each of the variants (A1, A2, B, and I) was subjected to *in vitro* digestion using pepsin at pH 2.0, Petrat-Melin et al. (2015) observed minimal intact β-casein presented using SDS-PAGE after 60 min of pepsin digestion (with little difference between different variants), following Dupont et al. (2010). According to Jinsmaa and Yoshikawa (1999) and Haq, Kapila, and Kapila (2015), pepsin hydrolyzes the Leu^{58}-Val^{59} peptide bond of β-casein, and leucine aminopeptidase removes valine from the N-terminus.[67] His in the β-casein variant A1 residue is more susceptible than the ^{67}Pro in β-casein variant A2 residue toward pepsin-induced hydrolysis, with the ^{66}Ile residue following (Thiruvengadam et al. 2021). In both cases, hydrolysis produced a peptide with Ile at the C-terminus. The one amino acid difference in residue 67 is responsible for releasing the seven amino acid N-terminus fragments to form β-casomorphin-7 in β-casein variant A1 (Thiruvengadam et al. 2021). The effect of dairy products on gastrointestinal dysfunction could be at least partially attributed to the proteolytic release of the bioactive peptide β-casomorphin-7 from β-casein (Sheng et al. 2019). Compared with A1 variant, the A2 variant of β-casein has a much slower rate of proteolytic digestion; therefore, its consumption results are nil or not physiologically relevant, much lower yield of β-casomorphin-7 (Boutrou et al. 2013; Haq, Kapila, and Kapila 2015; De Noni 2008) and substantially less severe gastrointestinal symptoms for individuals who consume only A2 β-casein (Sheng et al. 2019).

κ-casein has nine variants, containing galactose, N-acetylgalactosamine, and N-acetylneuraminic acid or sialic acid, which occur as either trisaccharides or tetrasaccharides attached to threonine residues in the C-terminal region (Vreeman et al. 1986). In the presence of pepsin, the hydrolysis of κ-casein is highly pH-dependent. Tam and Whitaker (1972) investigated the rates and extents of hydrolysis of κ-casein by pepsin at pH 3.0, 3.5, 5.5, and 6.0. The reaction between pepsin and κ-casein at pH 6.0 differs from that at pH 3.0: pepsin hydrolyzes κ-casein into para-κ-casein and caseinomacropeptide (CMP, also referred to as glycomacropeptide or caseinoglycopeptide) at pH 6.0 ($\kappa-\text{casein} \xrightarrow[pH6]{pepsin} \text{para}-\kappa-\text{casein}+\text{CMP}$) (Manso and López-Fandiño 2004), whereas pepsin hydrolyzes

κ-casein into peptides or amino acids at pH 3.0 ($\kappa-\text{casein} \xrightarrow[pH3]{pepsin} \text{peptides/amino acids}$) (Tam and Whitaker 1972). At pH 6.0, pepsin has the fastest initial rate of hydrolysis on κ-casein, followed by α_{s1}-casein and β-casein (Tam and Whitaker 1972). However, the extent of hydrolysis of κ-casein at pH 6.0 was lower than that at pH 3.0. The intact κ-casein acts as a steric stabilizing "hairy" layer on the casein micelle surface, providing both steric and electrostatic repulsion between micelles, thus preventing aggregation by hydrophobic association. After a defined proportion of κ-casein is hydrolyzed (usually approximately 90%) to form CMP (while other caseins are not hydrolyzed at pH 6.0), the destabilized casein micelles coagulate. This is discussed further in Section 3.2.2.

Casein micelles

The stability of casein micelles depends on the presence of κ-casein on the surface, which functions as an interface between the hydrophobic caseins in the micelle interior and the aqueous environment. κ-casein can be specifically hydrolyzed by pepsin at a relatively high pH (>5) (Plowman and Creamer 1995; Kageyama 2002); therefore, several changes occur for milk proteins in the gastric environment with decreases in pH during gastric digestion (Figure 2):

1. At pH >6, hydrolysis of the Phe^{105}-Met^{106} bond in κ-casein results in the destruction of the protective effect toward aggregation that κ-casein has on the casein micelle surface.
2. After a certain proportion of κ-casein "hairs" are clipped off as CMP, the destabilized casein micelles coagulate.
3. When the pH further decreases to 4–5, the dissociation of CCP (which is attached electrostatically to the phosphorylated serine of casein in the micelle interior and contributes to micellar stability at higher pH values (e.g. 6.7 in fresh milk) from the casein micelles results in the structural rearrangement of the curd.
4. At pH <4, proteolysis of all casein proteins leads to the release of peptides into the serum phase.

As structured casein curd can affect the subsequent proteolysis of proteins at low pH, pepsin-induced hydrolysis of κ-casein and coagulation of casein micelles will be discussed further. The hydrolysis and aggregation phases differ kinetically but are not fully separated over time (Frederiksen et al. 2011; Yang et al. 2022). To date, there has been no simple and "real-time" method reported in the literature for analyzing the hydrolysis of κ-casein. To quantitatively estimate the extent and rate of pepsin hydrolysis of κ-casein, the most common methods are to analyze either the formation of CMP or para-κ-casein, or the disappearance of intact κ-casein. Classical methods of CMP analysis include electrophoresis and chromatography (Manso and López-Fandiño 2004). According to Yang et al. (2022) and Carlson, Hill, and Olson (1987a), pepsin-induced hydrolysis of κ-casein follows a combined kinetic model of first-order hydrolysis and putative pepsin denaturation as follows:

$$ln\left(1-\frac{H_t}{100}\right)=\frac{K_{enz}\cdot C}{K_{den}}\cdot\left[exp\left(-K_{den}\cdot t\right)-1\right] \quad (1)$$

where H_t is the percent hydrolysis of κ-casein at time t, K_{den} (min^{-1}) is the reaction rate constant for the denaturation reaction, C (U/mL) is the pepsin concentration, K_{enz} (min^{-1} $U^{-1}mL$) is the reaction rate constant for the enzymatic reaction per unit quantity of enzyme solution, and $K_{enz}\cdot C$ is the overall reaction rate constant K (min^{-1}). This is similar to the chymosin hydrolysis of κ-casein (van Hooydonk 1987).

During the coagulation of milk, the curd is mainly formed from the casein matrix with fat globules entrapped within the casein network (Fox et al. 2004; Joshi et al. 2004). Several important parameters describe the casein aggregation phase, including the coagulation time, curd firmness, and curd microstructure. Numerous studies have determined the milk coagulation properties with different apparatus, which are summarized by Troch et al. (2017) and Lucey (2002), including rheology, microscopy (Everett 2007), the Formagraph (Kübarsepp et al. 2005; Cipolat-Gotet et al. 2012), diffusing-wave spectroscopy (Alexander, Corredig, and Dalgleish 2006; Alexander and Dalgleish 2004; Sandra, Alexander, and Dalgleish 2007) and scattering techniques (Lopez-Rubio and

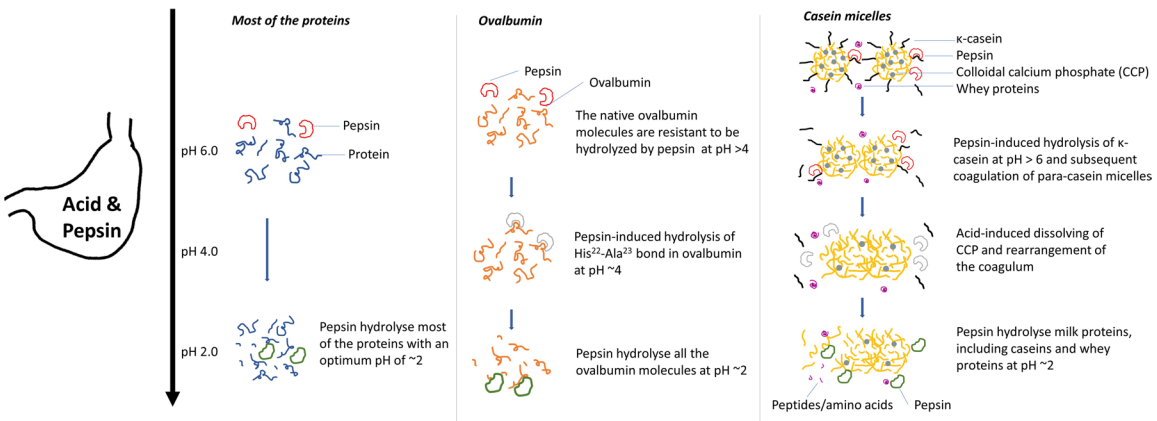

Figure 2. Mechanism of protein digestion in the gastric environment.

Gilbert 2009; Gilbert 2019; Adams et al. 2019; de Kruif 2014; Gilbert 2023; Bayrak et al. 2021; Yang et al. 2025).

The coagulation behavior of proteins depends on several underlying phenomena: the rate of enzymatic action, the critical degree of hydrolysis of κ-casein for curd formation, and the rate of aggregation of destabilized casein micelles (Garnot and Corre 1980). These phenomena are affected by several factors, such as the pepsin concentration and pH in the gastric environment (Culioli and Sherman 1978; Kameswaran and Smith 1999; Mohamed A Mehaia and El-Khadragy 1998), heat treatment of proteins (Wilson and Wheelock 1972; Anema, Kim Lee, and Klostermeyer 2007), and the type of mammals from which the milk is obtained (Roy et al. 2020b). The effects of these conditions and milk processing on pepsin-induced hydrolysis of κ-casein, and coagulation of casein micelles are discussed **in Section 3.2.2.1** and summarized in Figure 3, Table 3.

Factors influencing pepsin-induced hydrolysis of κ-casein and coagulation of casein micelles

Pepsin secretion in adults is estimated to be between 20 and 30 kUnits of enzyme activity/24 h at 37 °C (Moreno 2007). The pH decreases to different extents depending on the buffering capacity of the ingested food. The temperature of a human gastric environment is approximately 37 °C in a fasting state but can change depending on the temperature of ingested food (Sun et al. 1988). Under these dynamic changes in the gastric environment, the hydrolysis of κ-casein by pepsin is fast to be determined, particularly because of the rapid decrease in pH to <4, which accelerates the general hydrolysis of all proteins (Tam and Whitaker 1972; Ye 2021). Therefore, to investigate the kinetics of the hydrolysis of κ-casein and the consequent coagulation of milk proteins, it is necessary to use low pepsin concentrations [<23.71 (U/mg)/100 mL milk] under controlled pH and temperature conditions (Yang et al. 2022; Roy et al. 2020b).

As temperature affects pepsin activity (Zhao et al. 2011) and enzyme-induced milk coagulation behavior (Nájera, De Renobales, and Barron 2003; Dalgleish 1983; Horne and Lucey 2014), milk temperature has a profound effect on the structural organization of pepsin-induced curd. Yang et al. (2023a) reported that the optimum temperature for pepsin to specifically hydrolyze κ-casein is approximately 37 – 40 °C, and extensive pepsin denaturation occurs at 48 °C (Figure 3A1). Additionally, protein aggregation is markedly dependent on temperature (Dybowska and Fujio 1996; Gunasekaran and Ay 1996), with a temperature coefficient (Q_{10}) of approximately 12 (McMahon and Brown 1984). The coagulation time decreases as set temperature increases (Figure 3B1) (Panthi et al. 2019; Yang et al. 2023a), and coagulation occurs with a lower requirement for the extent of κ-casein hydrolysis at a higher temperature (Figure 3C1), in agreement with chymosin-induced coagulation results (Carlson, Hill, and Olson 1987c; Garg and Johri 1994).

Pepsin concentration. At a fixed pH (>5), the rate of the hydrolysis of κ-casein is positively related to the enzyme concentration (Figure 3A3) (van Hooydonk, Olieman, and Hagedoorn 1984; Yang et al. 2022). Consequently, milk coagulation time is inversely dependent on enzyme concentration (Figure 3B3) (Dalgleish 1993; Carlson, Hill, and Olson 1987a; López, Lomholt, and Qvist 1998; Foltmann 1959; Hyslop, Richardson, and Ryan 1979). At each pH value, the degree of hydrolysis of κ-casein required for coagulation was independent of the pepsin concentration (Figure 3C3) (Yang et al. 2022).

pH. At a defined pepsin concentration, Yang et al. (2022) reported the optimal pH for pepsin-induced hydrolysis of κ-casein is pH 6.0 (Figure 3A2), similar to that for chymosin-induced hydrolysis (van Hooydonk 1987). The coagulation time and degree of hydrolysis required for coagulation to occur decreased markedly with a decrease in pH (Figure 3B2, C2), which was observed in chymosin-induced milk coagulation (van Hooydonk 1987; Carlson, Hill, and Olson 1987b, 1987d); this was due to partial charge neutralization of the negatively charged para-casein micelles and an increase in the concentration and activity of calcium ions in milk serum at lower pH (van Hooydonk 1987; Zoon 1988; Choi, Horne, and Lucey 2007). Curd at a lower pH is more prone to microsyneresis (Mellema et al. 2002), with larger pores in the microstructure (Yang et al. 2022).

Ca^{2+}. Yang et al. (2023c) suggested that the hydrolysis of κ-casein induced by pepsin was not substantially affected by an increased concentration of calcium up to 22.5 mM at pH 6.0 (Figure 3A5). However, the coagulation of casein micelles is positively correlated with calcium concentration (Mehaia and Cheryan 1983; van Hooydonk, Hagedoorn, and Boerrigter 1986; Walstra and Jenness 1984). A schematic representation of the effect of calcium concentration on the hydrolysis of κ-casein, the aggregation of para-casein micelles, and the curd structure is shown in Figure 4. A minimum concentration of Ca^{2+} exists for casein coagulation below which coagulation will not occur, even when the degree of hydrolysis of κ-casein is high (Yang et al. 2023c). The coagulation time and critical hydrolysis level of κ-casein decreased with increasing $CaCl_2$ concentrations (Figure 3B5, C5). However, excessive calcium had a negative effect on curd formation due to charge repulsion or reduced κ-casein solubility (Udabage, McKinnon, and Augustin 2001; Choi, Horne, and Lucey 2007; Fox et al. 2017; Lucey and Fox 1993; Patel and Reuter 1986).

Heat treatment. Heat treatment is commonly applied in milk processing to enhance the desirable properties of dairy products, such as taste and texture, or to extend the shelf life and ensure safety. In the presence of whey proteins, β-Lg aggregate through thiol/disulfide interchange, hydrophobic interactions, and electrostatic and ionic interactions with themselves and with κ-casein, leading to

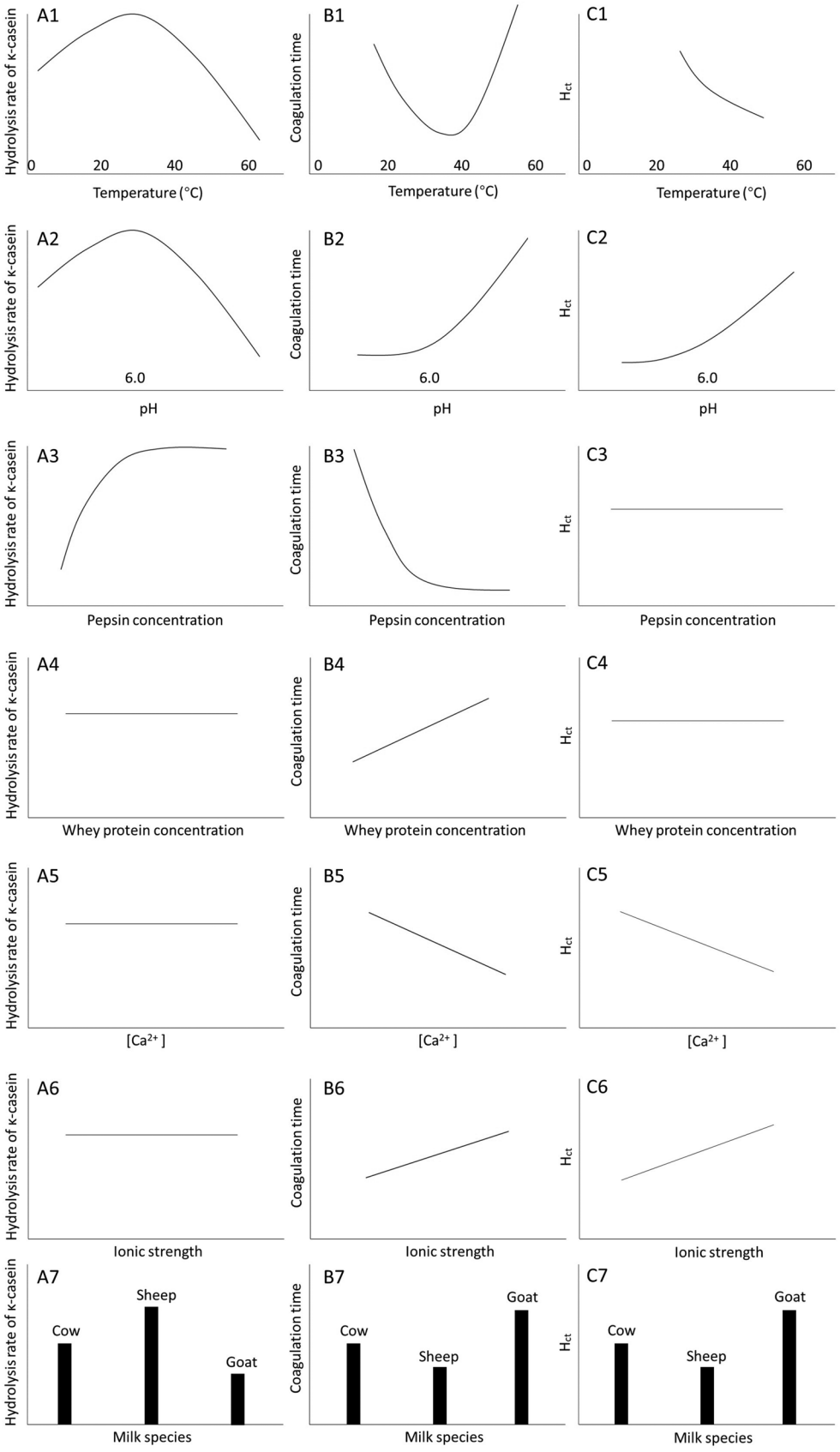

Figure 3. Summary of the effects of conditions and milk processing on pepsin-induced hydrolysis of κ-casein and coagulation of milk proteins. H_{ct}, critical hydrolysis degree for coagulation.

so-called whey protein/κ-casein complexes (Guyomarc'h, Law, and Dalgleish 2003; Jang and Swaisgood 1990; Singh and Creamer 1991). After heat treatment (95 °C for 5 min), the Phe^{105}-Met^{106} bond of κ-casein was reported to be less accessible to pepsin, leading to inhibition of the hydrolysis of κ-casein (Yang et al. 2023b). The attachment of denatured

Table 3. Summary of the effects of conditions and milk processing on gastric digestion of milk proteins

	Effect on the *in vitro* dynamic gastric digestion	
Factors	Coagulation and digestion behavior	References
Temperature	4 °C milk showed a delayed coagulation compared to milk at 37, 50, and 60 °C, which obtained a looser and softer structure with a substantially higher moisture content at the initial stage of digestion which, in turn, facilitated the breakdown and hydrolysis of the caseins by pepsin.	(Yang et al. 2023a; Fitzpatrick et al. 2024)
Whey protein concentration	More extensive coagulation of the caseins was observed at a higher CN:WP ratio from a study on the digestibility of infant formula powder using an *in vitro* digestion model.	(Phosanam et al. 2021; Ye, Cui, et al. 2019)
Heat-treatment	Heat treatments that induce substantial denaturation of whey proteins and the association with casein micelles (e.g., UHT and 90 °C for 20 min) lead to the formation of more fragmented clots and accelerate gastric emptying and hydrolysis of the caseins.	(Mulet-Cabero et al. 2019; Ye, Liu, et al. 2019; Ye et al. 2017, 2016b, 2016a; Tunick et al. 2016)
Ca^{2+}	Increases in [Ca^{2+}] results in denser and firmer curds, with slower release of proteins. Gastric proteolysis of proteins increases with increasing decalcification.	(K. Wang et al. 2023; Yang et al. 2023c)
Ionic strength	Increases in ionic strength (through the addition of NaCl) lead to curds forming a looser and fractured structure, resulting in a quicker release of proteins during the initial stage of digestion. No substantial difference in protein content within the curd is observed after 180 min of digestion.	(Yang et al. 2023c)
Milk species	The extent of moisture retained in a sheep skimmed milk clot is lower than that for cow and goat skimmed milk clots. The relative firmness of sheep milk clots is higher than that of cow and goat milk clots at the end of gastric digestion. The pattern of protein hydrolysis by pepsin is similar for the milk of all species, despite differences in the proportions of different proteins.	(S. Li et al. 2022; Roy et al. 2022)
High-pressure processing	High-pressure processing treatment led to the formation of a coagulum with a fragmented and crumbled structure, consequently leading to faster hydrolysis of the proteins by pepsin during gastric digestion.	(X. He et al. 2022; Aalaei et al. 2021)

Abbreviations: CN:WP: casein:whey protein; UHT: Ultra-high temperature processing.

whey proteins to the surface of casein micelles upon heating has a profound effect on the coagulation process of milk clotting, but this depends on the pH (Yang et al. 2023b).

Milk species. Milk from different species varies in composition, structure, and physicochemical properties, resulting in different digestive properties. Goat, sheep, and cow milk have similar casein micelle structures but differ in composition, size, hydration, and mineralization (Selvaggi and Tufarelli 2012). Para-κ-casein from sheep and goat milk differs from that of cows by ten and eight amino acids, respectively (Jollès et al. 1974; Mercier et al. 1976). A review by Roy et al. (2020a) discussed the composition, structure, and digestive dynamics of milk from different species. Milk from different species responds differently to pepsin and acid (Roy et al. 2020b). At the same pepsin-to-κ-casein ratio, sheep milk has the fastest rate of κ-casein hydrolysis, followed by cow and goat milk (Figure 3A7) (Yang et al. 2024). A range of factors, such as casein micelle size, CCP content, proportions of different caseins and whey proteins, mineral content, genetic variants, and complex interactions are responsible for the observed rheological behavior of coagulated milk from different species (Roy et al. 2020b).

Recombinant caseins. As dairy products are a source of high-quality protein and many micronutrients, synthesizing nature-identical milk proteins has been considered a possible solution to replace milk proteins (Hettinga and Bijl 2022). Not all four caseins may be required to construct a casein micelle, as human milk and elephant milk do not contain either of the α_s-caseins (Lönnerdal 1996, Madende et al. 2015). Human milk β-casein and bovine milk κ-casein are sufficient to produce reassembled casein micelles (Sood, Erickson, and Slattery 2002). No reports exist on the interactions between pepsin and non-animal whey or casein proteins.

Milk protein curd

Hydrolysis of curd proteins depends on the structure formed during gastric digestion (Ye 2021). In general, protein hydrolysis is considerably slower in densely structured clots than in loose clots with larger voids. In the soft curds formed from heated milk (72 °C for 15 s or 140 °C for 4 s), casein proteins are hydrolyzed faster than in hard clots formed from unheated milk (Ye et al. 2016b, 2017; Ye, Liu, et al. 2019; Miranda and Pelissier 1983). High-pressure processing results in a loose and fragmented clot structure in milk, leading to the faster hydrolysis of caseins (X. He et al. 2022). Pepsin-induced hydrolysis of milk curds depends on the milk species. Goat milk-based infant formulas form smaller protein aggregates, leading to faster hydrolysis of proteins compared with those from cow's milk formula (Maathuis et al. 2017; Ye, Cui, et al. 2019).

Structured curd is formed during milk digestion, and the matrix and type of curd structure before ingestion affect the digestion of milk proteins. A "hard" whey protein-based gel structure can slow down the disintegration of the protein matrix, whereas a "soft" whey protein-based gel structure can be completely broken down after 4 h of digestion (Guo et al. 2014; N. Luo et al. 2021). Qazi et al. (2021) reported a faster gastric emptying of peptides produced from an acid-induced (1.6% w/w Glucono-delta-lactone) curd, whereas the restructuring of the rennet-induced curd during gastric digestion reduced the flux of proteins and peptides into the digesta.

Interaction between egg protein and pepsin

Egg proteins are generally considered a valuable protein resource that provides all essential amino acids for human nutritional requirements (J. Wang, Chi, et al. 2018). Egg proteins are highly digestible: the true ileal digestibility of

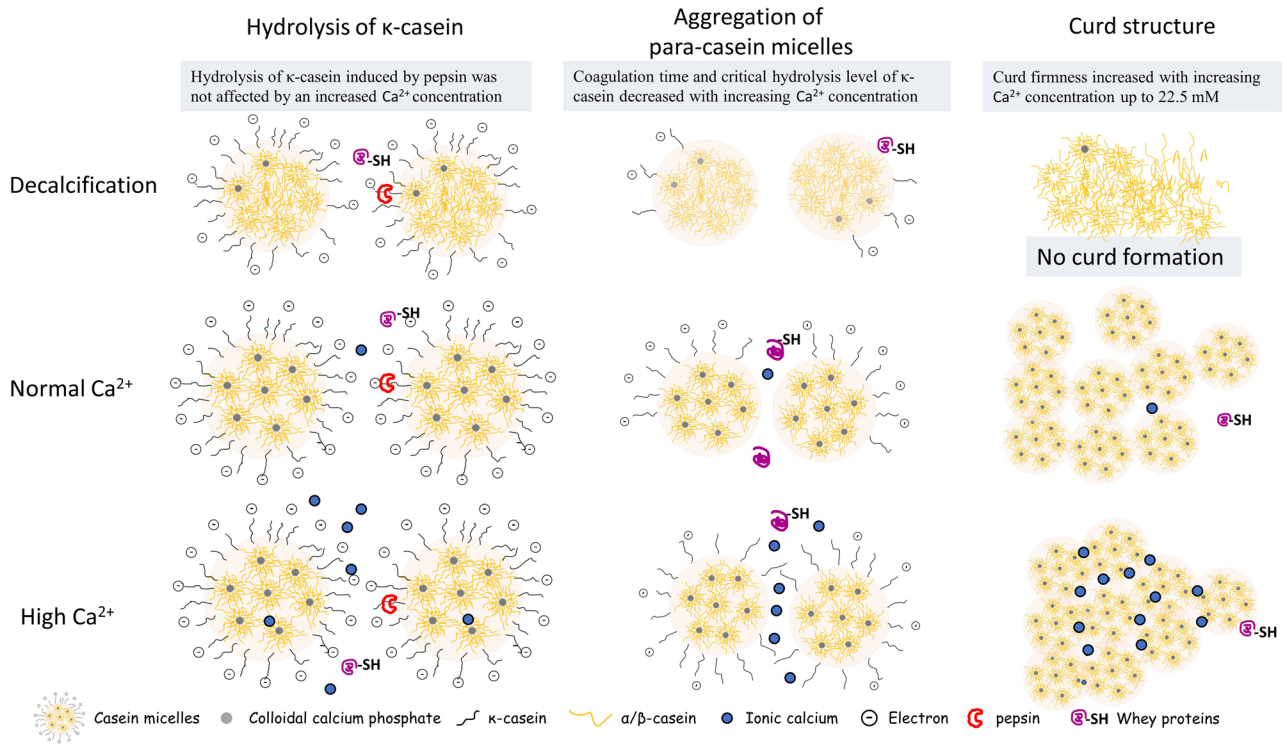

Figure 4. Schematic representation of the effect of calcium concentration on the hydrolysis of κ-casein, the aggregation of para-casein micelles, and curd structure. The addition of ionic calcium does not impact upon hydrolysis of κ-casein by pepsin, but it facilitates the coagulation of para-casein micelles through calcium bridges, resulting in a stronger network.

cooked whole egg proteins has been observed to be 90.9% in humans (Evenepoel et al. 1998). The proteins observed in the eggs are mainly in egg yolk (40%) and egg white (50%).

Egg yolk protein

The major proteins observed in egg yolk include low-density lipoprotein (68%), high-density lipoprotein (16%), phosvitin (4%), and livetin, which exist in a homogeneously emulsified fluid (Anton et al. 2003). α-Livetin (Gal d 5) is commonly associated with allergies (Dona and Suphioglu 2020). Egg yolk is widely used as a functional and nutritional ingredient in food products (Juneja and Kim 2018; Y Mine 2002). Egg yolk proteins can be hydrolyzed by food-grade proteinases from *Bacillus sp.* (Sakanaka et al. 2004). The interaction between pepsin and egg yolk proteins has not been studied in detail, but a slight increase in susceptibility to hydrolysis of egg white proteins was observed in the presence of egg yolk during *in vitro* digestion (Martos, López-Fandiño, and Molina 2013).

Egg white protein

Egg white consists of 40 different types of proteins, including ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), lysozyme (3.5%), ovomucin (3.5%), and globulins (8%) (Réhault-Godbert, Guyot, and Nys 2019). Ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), and lysozyme (Gal d 4) are commonly associated with allergies (Dona and Suphioglu 2020).

Before consumption, eggs are often processed and stored. Most thermal, non-thermal, and emulsification processes can affect the structural properties of egg proteins; the gelation mechanism of egg white protein is typically described as follows: native protein → denatured protein → soluble aggregated protein → gel or curd (Yoshinori Mine 1995). The altered physicochemical properties and structure of the proteins impact digestibility within the gastric and intestinal tract (Jiménez-Saiz et al. 2011). Egg white protein heated at 65 °C for 30 min showed the highest peptic hydrolysis compared to samples heated at 56 °C for 32 min and 100 °C for 5 min. This was attributed to the denaturation of egg white protein at 65 °C, which exposed previously buried hydrolysis sites. Conversely, heat treatment at 100 °C resulted in extensive aggregation, potentially hiding a few hydrolysis sites (Figure 5) (Wang, Qiu, and Liu 2018).

The effects of different methods of processing on the digestibility of egg proteins and allergenicity have been reviewed by Bhat et al. (2021a) and Farjami et al. (2021). The interactions between individual native/processed egg proteins and pepsin are summarized below.

Ovalbumin

The digestive behavior of egg white proteins is strongly affected by ovalbumin, a major protein constituent. Short hydrolysis was observed at pH 4 (Kitabatake, Indo, and Doi 1988) where only a single peptide bond (His^{22}-Ala^{23}) in the original ovalbumin (MW 45,000 Da) was hydrolyzed, releasing a peptide with a molecular weight of approximately 3000 Da. Both the released peptide and residual protein

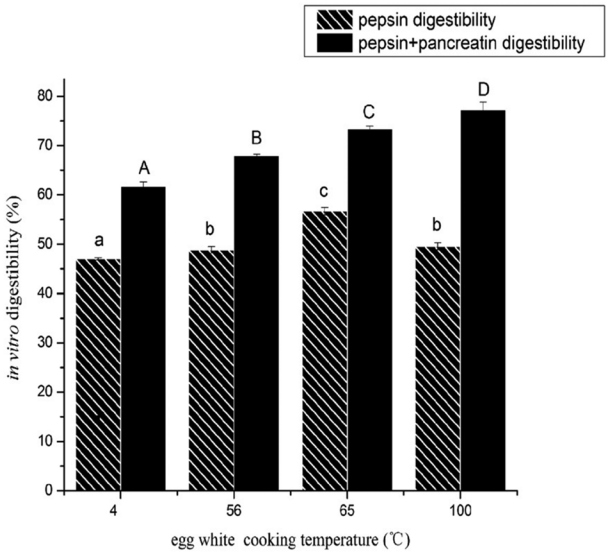

Figure 5. *In vitro* digestibility of egg white proteins treated under different cooking temperature conditions. Different letters indicate statistically substantial differences among the values ($p < 0.05$). Adapted from Wang, Qiu, and Liu (2018).

(MW 42,000 Da, P-ovalbumin) were resistant to further hydrolysis by prolonged incubation or the addition of additional pepsin at pH 4 (Figure 2). By changing the pH to 2, native ovalbumin could be more extensively hydrolyzed by porcine pepsin (Kitabatake, Indo, and Doi 1988). This finding could explain the result from Martos et al. (2010) that native ovalbumin was resistive to pepsin action at an enzyme/substrate ratio at 1:20 w/w (172 units/mg) at pH values above 2. The acidity of the infant stomach is much lower, with a pH of approximately 4, which may lead to the poor and slow degradation of allergenic epitopes (Martos et al. 2010).

According to Nyemb et al. (2014), by heating ovalbumin solutions under different pH and ionic strength conditions (the concentration of ovalbumin was below the critical concentration for gel formation), distinctly different aggregate morphologies were obtained, including linear, linear-branched, spherical, and spherical-agglomerated aggregates. In the *in vitro* digestion model, non-aggregated ovalbumin appeared to be the most resistant to pepsin digestion. All types of ovalbumin aggregates in thermally processed samples were more susceptible to digestion compared with those in unheated samples (Van der Plancken et al. 2003). The degree of unfolding before aggregation is the main factor affecting the digestibility of ovalbumin, with the lowest unfolding recorded in spherical agglomerated aggregates and the highest in linear aggregates (Nyemb et al. 2014; Van der Plancken et al. 2003). However, Maillard glycation impairs digestibility and IgE binding to ovalbumin (Jiménez-Saiz et al. 2011; Farjami et al. 2021).

Ovomucoid

Ovomucoid comprises three tandem domains, each cross-linked by three intradomain disulfide bonds, which lead to resistance to proteolytic digestion and heat denaturation (Matsuda et al. 1985; Kovacs-Nolan et al. 2000; Kosti et al. 2013; Shin, Han, and Ahn 2013). Ovomucoid is considered one of the main allergens in egg whites, known as a "trypsin inhibitor" (Abeyrathne et al. 2015). During *in vitro* gastric digestion, pepsin hydrolyzes the ovomucoid almost completely, producing free amino acids and di- and tri-peptides (Jiménez-Saiz et al. 2011; Matsuda et al. 1985; Kovacs-Nolan et al. 2000; Abeyrathne et al. 2015). The trypsin inhibitory activity of ovomucoids is reduced by pepsin digestion (Konishi et al. 1985), but the residual activity may help maintain the integrity of ovomucoid peptide fragments (Kovacs-Nolan et al. 2000). Neither heat treatment nor Maillard reaction affected ovomucoid digestibility. Although the Maillard reaction reduced the binding of IgE to ovalbumin, it increased the binding of IgE to ovomucoid, suggesting resistance to denaturation and digestive enzymes.

Ovotransferrin and lysozyme

Native ovotransferrin and lysozyme can be rapidly hydrolyzed by pepsin (Liu et al. 2017; Liu et al. 2018; Yoshino et al. 2004), whereas ovotransferrin is more susceptible to pepsinolysis than ovalbumin and lysozyme (Liu et al. 2017; Fu, Abbott, and Hatzos 2002; Moreno 2007). According to Liu et al. (2017), denaturation/aggregation at 60 °C, which mainly involves ovotransferrin, has no effect on the susceptibility to pepsin, either for ovotransferrin or lysozyme. Heating at 80 °C substantially enhanced the susceptibility of ovotransferrin and lysozyme to pepsin hydrolysis.

Ovomucin

Ovomucin is a glycoprotein characterized by high molecular weight and is responsible for the gel-like properties of thick egg albumen (Hiidenhovi 2007). According to Hiidenhovi et al. (2005), pepsin hydrolyzes ovomucin efficient at pH 2.0. Hydrolysis for 1 h produces peptides of small molecular masses (<10 kDa) encompassing up to 70% of all peptides, whereas only a small portion of peptides (≤6%) have a molecular mass >100 kDa.

Egg white gels

The processing-induced unfolding of ovalbumin proteins can improve the digestibility of egg proteins by exposing them to hydrolytic sites and increasing access to digestive enzymes. However, certain processes may cause egg white proteins to gel, depending on their composition and concentration. For example, insoluble aggregates form in an ovotransferrin/lysozyme mixture and lysozyme/ovalbumin mixture when heated above 70 °C, at pH 9 (Matsudomi, Takasaki, and Kobayashi 1991; Matsudomi, Yamamura, and Kobayashi 1986). According to Liu et al. (2017), differences in the digestibility were observed in the two systems (i.e. colloidal dispersion versus gel), where enzyme diffusion was a decisive factor in the gel network (Luo, Boom, and Janssen 2015; Nyemb et al. 2016). Four different egg white gel structures with different combinations of pH and ionic strength before heating were prepared by Nyemb et al. (2016), who reported that the overall extent of protein digestion, ranging from highest to

lowest, was granular-spongy, intermediate, smooth-rigid, and breakable gels. This observation can be explained by changes in enzyme diffusion due to the microstructural characteristics of the gel, or an alteration in digestive enzyme adsorption or catalytic activity due to local differences in pH within the gel matrices. P-ovalbumin (the product of limited proteolysis of ovalbumin by pepsin at pH 4) produces a clearer gel than the original ovalbumin solution upon heating (Kitabatake and Doi 1985); however, there is no information on the digestibility of this transparent gel.

Interaction between meat proteins and pepsin

Meat proteins can be categorized into three groups: myofibrillar proteins (50%–55%), sarcoplasmic proteins (30%–34%, mostly enzymes, and myoglobin), and connective tissue (10%–15%, mostly collagen and elastin fibers embedded in mucopolysaccharides) (Baldwin 2012). Most meat proteins are susceptible to digestion, whereas myoglobin and collagen are less digestible (Li, Zhao, et al. 2020; Zhang et al. 2020). Physicochemical processes and pretreatments, such as pulsed electric fields, high-pressure processing, and cooking, affect digestive processes, thereby affecting the release and bioavailability of amino acids (Gong et al. 2020; Bhat et al. 2021b). In general, the unfolding of buried residues through denaturation (e.g. moderate heating) can improve protein digestibility (Bax et al. 2013). However, extensive aggregation of proteins can decrease proteolytic susceptibility and hinder protein digestion (Lee et al. 2021), for example, excessive heating (Santé-Lhoutellier et al. 2008), or dry curing (Du et al. 2018). In this review, we briefly discuss the interactions between meat proteins and pepsins.

Myofibrillar proteins

Myofibrils mainly consist of myosin heavy chain (200 kDa), actin (42 kDa), and other minor proteins, such as α-actinin (100 kDa), tropomyosin, troponins, and myosin light chains (Liu et al. 2011). During *in vitro* gastric digestion (porcine pepsin, 272 U/mg protein), the basic sarcomere structure of raw meat myofibrils is quickly lost. Actin and myosin heavy chains are rapidly degraded within a short period of time under simulated gastric digestion conditions (Liu et al. 2011); whereas tropomyosin is relatively resistant to pepsin digestion (Liu et al. 2011) and even after heat treatment (Leung et al. 1994; Shanti et al. 1993).

Cooking at low temperatures generally induces structural and conformational changes and mild oxidation, leading to partial unfolding of the polypeptides (Bhat et al. 2021b). Collectively, these changes enhance pepsin accessibility to hydrolysis sites and improve the digestibility of myofibrillar proteins (Marie-Laure Bax et al. 2012). In contrast, high temperatures (>100 °C) can induce oxidation, disulfide bond formation, and protein aggregation or gelation, which can bury the active sites deep into the tertiary structure, thereby decreasing accessibility to pepsin (Sante-Lhoutellier, Aubry, and Gatellier 2007; Marie-Laure Bax et al. 2012; He et al. 2018). The effect of heating on the digestibility of myofibrillar proteins under static *in vitro* digestion conditions is shown in Figure 6 (Chen et al. 2023).

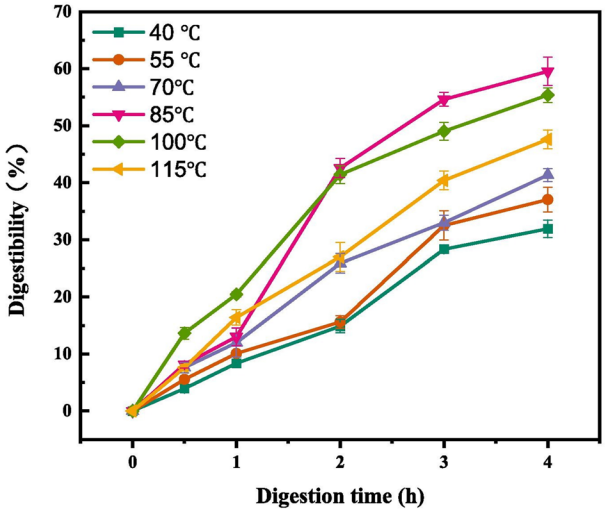

Figure 6. Effect of heating temperature (40, 55, 70, 85, 100, and 115 °C for 30 min) on digestibility of myofibrillar proteins. Adapted from Chen et al. (2023).

Sarcoplasmic proteins

Myoglobin is a sarcoplasmic protein that contributes to meat color (Suman and Joseph 2013). Li, Zhao, et al. (2020) observed poor digestibility of myoglobin owing to its low susceptibility to pepsin (from porcine gastric mucosa, CAS No. 9001-75-6) digestion, brought about by the rigid structure of the hydrophilic surface and hydrophobic pocket (Kaur, Banipal, and Banipal 2017). The aggregation and gelation of sarcoplasmic proteins begin at approximately 40 °C and finish at approximately 60 °C (Baldwin 2012). Sarcoplasmic proteins are more rapidly hydrolyzed during gastric digestion following heating at lower temperatures (55 °C) compared with that at higher temperatures (70–90 °C); this arises due to protein denaturation causing a loss of solubility and reduction in interactions among sarcoplasmic or myofibrillar proteins (Sayd, Chambon, and Santé-Lhoutellier 2016).

Connective tissue (collagen)

Collagen is the main component of the endomysium and perimysium, which determines meat tenderness and digestibility (Purslow 2018). Native collagen is resistant to hydrolysis by digestive enzymes (pepsin from porcine gastric mucosa, CAS No. 9001-75-6; pancreatin from porcine pancreas, CAS No. 8049-47-6) (M. Zhang et al. 2020; Li, Zhao, et al. 2020), because of its tight tertiary structure (Ravikumar and Hwang 2008). Its complex triple-helical structure (Exposito et al. 2010) and high levels of glycine and (hydroxyl) proline reduce the flexibility of the protein backbone, which affects the binding of enzymes to active sites on proteins (Kaur et al. 2010).

Moderate heating can induce the exposure of buried aromatic residues (tyrosine and phenylalanine), reduce the conformational stability of the type I collagen triple helix, and

increase pepsin-treated digestion; however, heating at higher temperatures has the opposite effect (Zhang et al. 2020). Connective tissues tend to gelatinize at 80 °C and form a gel that fills the spaces between muscle fibers (Roldán et al. 2013); this can decrease the digestibility of meat proteins by reducing the diffusion of digestive enzymes into the compact myofibrillar structure (L. Kaur et al. 2014).

Interaction between plant proteins and pepsin

Compared with animal proteins, plant proteins are believed to have lower digestibility, principally because of the presence of anti-nutritional factors in plant proteins (Mariotti 2017). Legumes, cereals, pseudocereals and oilseeds are important sources of plant protein for humans (Zhang et al. 2024). Most plant protein-based foods are consumed by humans after simple cooking and complex fermentation. Cooking legumes has been shown to cause protein denaturation, reducing their resistance to enzymatic attack (Alajaji and El-Adawy 2006; Wiesinger et al. 2018). Sá, Moreno, and Carciofi (2020) observed that cooking facilitates the leaching of unfavorable compounds and destroys protease inhibitors, thereby improving legume protein digestibility. When peas cooked at 100 °C for 40 min, increased *in vitro* protein digestibility was found when compared to uncooked ones. This improvement results from heat-induced alterations to the three-dimensional protein structure and reductions in trypsin inhibitors, phytic acid, and tannins (Habiba 2002). However, depending on heating conditions, excessive heat could cause the formation of large protein aggregates, which block enzymatic reactions (Sá, Moreno, and Carciofi 2020). When heating promotes interactions between proteins and non-protein compounds, the binding of pepsin to active sites may also be inhibited. Legume proteins become less soluble after heating and the resulting thermal aggregation may impair their digestibility (Carbonaro et al. 2000; Ruiz et al. 2016). As shown in Figure 7, the degree of hydrolysis of 5% w/w suspensions of heated quinoa protein isolates was lower than that of the unheated samples (Ruiz et al. 2016).

Based on the solubility differences in various solvents, plant proteins have been distinguished as water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins, and dilute alkali/acid-soluble glutenins, respectively (Osborne 1924; Fallahbaghery et al. 2017). Among them, globulin and albumin are mainly observed in legumes (such as soy and pea) while the prolamin and glutenin constituted the major protein fractions in monocotyledonous crops (Adebowale et al. 2007).

Albumin

Albumins are defined as water-soluble, globular proteins, presenting as a 2S albumin storage protein in seeds, e.g., as leucine in barley, wheat, and rye, as legumelin in pea, soybean and cowpea, as phaselin in kidney bean, and as ricin in castor bean (Rasheed et al. 2020). Under acidic conditions (pH 2.0), 2S albumins (Brazil nut Ber e 1, peanut Ara h 2, mustard Sin a 1, and hazelnut Cor a 14) are stable and retain a three-dimensional structure owing to the presence of disulfide bridges (Koppelman et al. 2005). There is a high

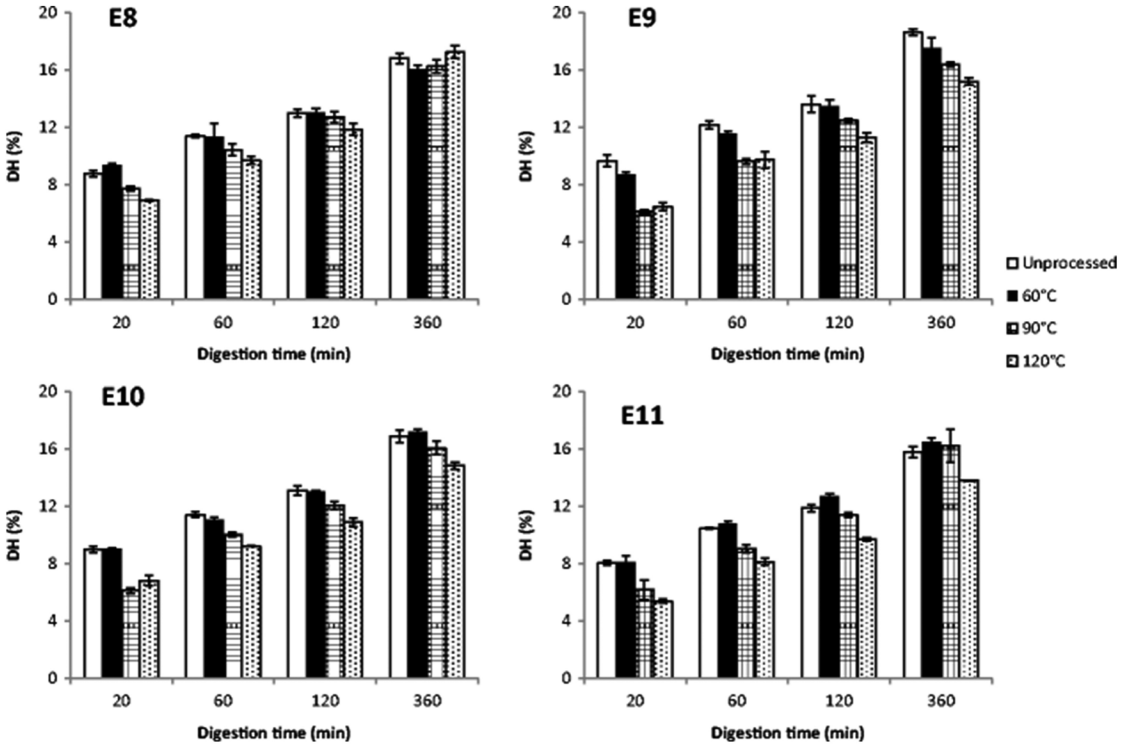

Figure 7. Degree of hydrolysis of 5% *w*/w suspensions of quinoa protein isolate E8 (protein isolated at pH 8), E9 (protein isolated at pH 9), E10 (protein isolated at pH 10) and E11 (protein isolated at pH 11) processed at different temperatures and subsequently digested for different time periods. Error bars are standard deviation. Adapted from Ruiz et al. (2016).

resistance of 2S albumins to pepsin digestion in simulated gastric fluid due to their compact structure, a high number of disulfide bonds, and their capacity for self-association (Moreno et al. 2005; Murtagh et al. 2003; Pantoja-Uceda et al. 2004; Koppelman et al. 2005; Carbonaro, Maselli, and Nucara 2015). Consequently, poorly hydrolyzed proteins and large fragments that exist after prolonged proteolysis can induce an allergic immune response, even after thermal treatment.

Globulins

In plants, globulins are present as storage proteins in both, dicots and monocots, making them the most common group of storage proteins (Rasheed et al. 2020). The hydrolysis rate and extent of a globulin protein fraction by pepsin (from hog stomach mucosa, 3200 units mg^{-1}) was at least twice that of the albumin protein fraction (Bhatty 1988). The major globulins observed in pulse legumes can be subdivided into 11S and 7S globulins, for example, legumin (11S) and vicilin (7S) in pea, and glycinin (11S) and β-conglycinin (7S) in soybean (Han et al. 2022). The 7S globulin, either in pea (vicilin) or in soybean (β-conglycinin), is resistant to pepsin (porcine pepsin, P7012, 2500 U/mg protein) proteolysis under gastric conditions (Han et al. 2022). The 11S globulins are slightly less resistant to proteolysis and undergo partial degradation after gastrointestinal digestion. Legumins (11S) are degraded more by pepsin than by trypsin, leading to a small decrease in IgE binding capacity (van Boxtel et al. 2008; Bavaro et al. 2018; Mattison, Grimm, and Wasserman 2014; Orruño and Morgan 2011). However, legumins are highly organized structures that present immunogenic subunits even after digestion, thus contributing to the partial retention of their allergenic potential (Costa et al. 2022).

Prolamins

The prolamins found in wheat are called gliadins, while the nomenclature of the prolamins in other cereals is based on their Latin names; zein in maize, hordein in barley, secalin in rye etc. (Shewry and Tatham 1990). Prolamin is classified into four subgroups: 10 kDa cysteine-rich, 13 kDa prolamin, 13 kDa cysteine-poor, and 16 kDa prolamin (Shewry 2023). These proteins contain a high proportion of hydrophobic amino acids (e.g., proline, leucine, alanine) and some polar amino acids (e.g. glutamine) (Taylor, Anyango, and Taylor 2013). Due to this composition, prolamins are easily dispersed in alcoholic solutions. Ogawa et al. (1987) demonstrated that the 13 kDa prolamin band in raw rice persisted after pepsin digestion, even as glutelin subunit bands (37–39 kDa and 22–23 kDa) disappeared, suggesting that prolamin is an indigestible protein. However, recent work indicates that structural changes induced by alkali extraction improve the *in vivo* digestibility of prolamin (Kubota et al. 2010; Kumagai et al. 2006; Kubota et al. 2014). Kubota et al. (2014) indicate that rice prolamin is not indigestible by nature, but is rendered indigestible by cooking (Kubota et al. 2014). A decrease in prolamin protein digestibility upon cooking is different amongst the various grain varieties and does not correlate with uncooked digestibility values (Nunes et al. 2004).

Glutelin

The most commonly found glutelin is that found in wheat (glutenin), although glutelins are also present in barley and rye (Shang et al. 2005). Glutelin, which consists of α and β subunits linked through disulfide bonds, represents the dominant dietary protein fraction in rice (Amagliani et al. 2017). Glutelin (10–500 kDa) has low water solubility owing to its compact structure with extensive disulfide bonds and hydrophobic interactions (Phongthai, Homthawornchoo, and Rawdkuen 2017). According to Zhang et al. (2022), the formation of cross-links between glutelin molecules generated aggregates during storage, and the aggregation restricted access of pepsin and trypsin to their sites of action in glutelin, which eventually reduced the *in vitro* digestibility of glutelin.

Interactions in animal/plant (hybrid) protein systems

Alves and Tavares (2019) reported that animal and plant protein-mixed food systems are efficient in modulating the texture of protein gels, forming low-cost edible films, and producing stable emulsions and foams. However, limited information on the digestion of such mixed systems has been reported, with one study reporting that coagulation of an oat "milk"-bovine skim milk blend slowed gastric emptying of macronutrients, leading to a delay in the release of protein and lipids into the small intestine compared to that with oat "milk" (Figure 8) (Xin Wang et al. 2022). Further research in this area could have substantial implications for the development of alternative protein sources and their applications in various food products.

Conclusions and future perspectives

Many studies on protein digestion have been conducted spanning various protein sources through human trials, animal experiments, and *in vitro* models. Gastric pepsin plays a pivotal role in this process by breaking down proteins into peptides of varying sizes under specific protein and gastric conditions. Understanding the interplay between proteins and pepsin is crucial for elucidating the fundamental mechanisms underlying gastric protein digestion. The role of pH is pivotal, as pepsin typically demonstrates optimal hydrolytic activity at a pH of approximately 2. However, two notable exceptions exist (Figure 2): the peptide bond (His^{22}-Ala^{23}) in egg ovalbumin, which is optimally hydrolyzed at pH ~4, and the Phe^{105}-Met^{106} bond in κ-casein within casein micelles, which undergoes specific hydrolysis at pH ~6, resulting in the aggregation of milk proteins to form a structured curd. (while during dynamic digestion, the pH gradually decreases to ~2, facilitating the rapid and complete hydrolysis of general protein bonds by pepsin). Further exploration is warranted to reveal additional proteins that

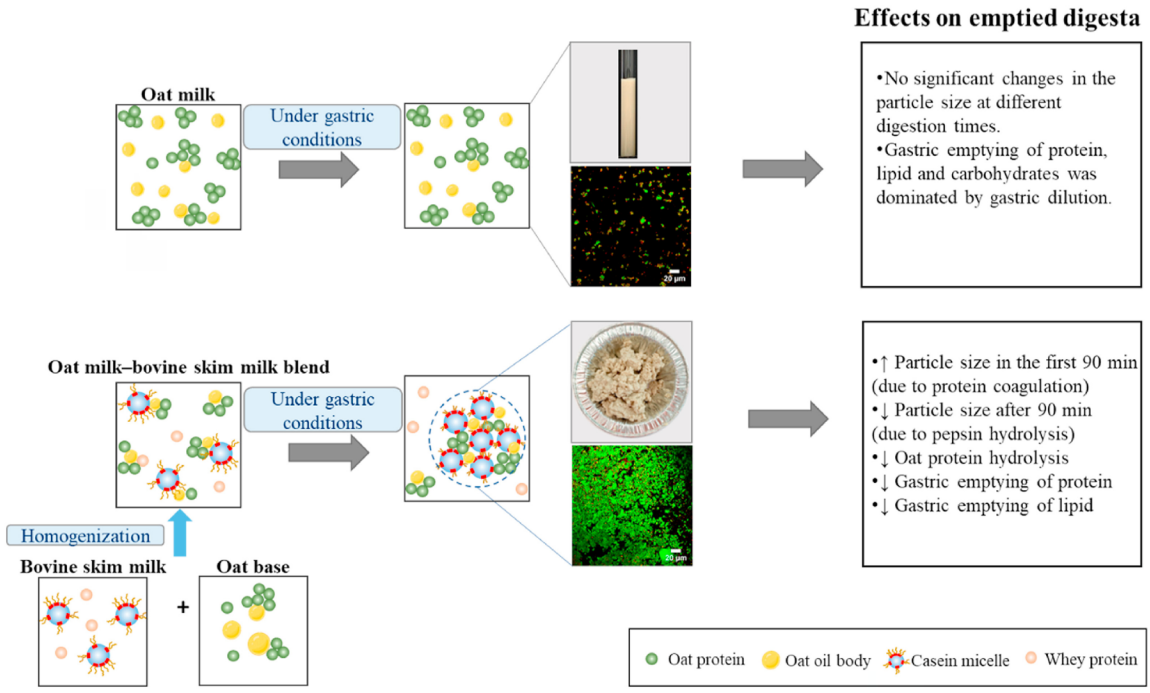

Figure 8. A schematic diagram of the gastric digestion behavior of oat "milk" and oat "milk"-bovine skim milk blend. Adapted from Wang et al. (2022).

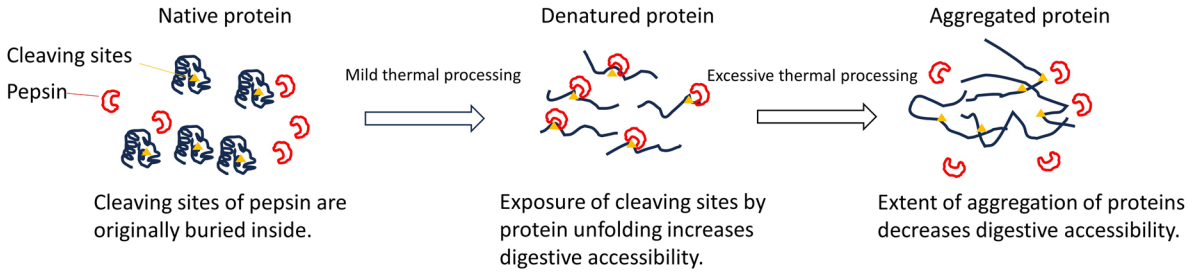

Figure 9. Mechanisms depicting changes in protein structure induced by thermal treatment leading to changes in the exposure of hydrolysis sites.

interact with pepsin at distinct optimal pH levels, such as insect, cultured, and algal proteins.

Food structure, both before and during digestion under gastrointestinal conditions, substantially influences nutrient availability. These structures are greatly influenced by the processing treatments applied during food manufacturing. Generally, the denaturation and unfolding of proteins enhance digestion by pepsin, whereas insoluble protein aggregation may hinder digestion (Figure 9). In addition to physical treatments or processing, exploring the impact of chemical treatments on proteins, such as the Maillard reaction, holds promise for further investigation. Standardizing the treatment conditions is crucial to enable a more precise comparison of proteins from diverse food sources.

Understanding the interactions between proteins and pepsins may have promising implications for food innovation. In the future, the biological signatures of the interaction between protein and pepsin and its subsequent processes, such as coagulation or the rate of degradation of the protein/protein coagulum structure, need to be extensively investigated in both *in vitro* and *in vivo* studies. In addition, the application of pepsin as an enzymatic agent could potentially change the solubility of proteins (e.g. pepsin-induced milk curd for cheese or yogurt production). The use of partially hydrolyzed protein by pepsin in functional food could be one of the strategies to prevent protein allergy.

AI-based tools are increasingly utilized to predict the cleavage patterns of proteases such as pepsin, offering significant advancements in understanding protein digestion. Traditional bioinformatics tools, such as PeptideCutter, provide rule-based predictions based on known cleavage preferences for hydrophobic and aromatic residues but lack flexibility for complex substrates (Du and Li 2022; Tamam et al. 2020). Emerging machine learning and deep learning models, such as DeepCleave and PROSPER, improve predictive accuracy by training on extensive cleavage datasets and incorporating sequence and structural features (Li, Chen, et al. 2020; Song et al. 2012). These tools leverage databases such as MEROPS and PRIDE to enhance the characterization of cleavage sites, while advanced algorithms, including recurrent neural networks and transformers, capture sequence and structural dependencies (Rawlings, Barrett, and Bateman 2010; Jones et al. 2006). As these AI-driven approaches evolve, they promise to deepen our understanding of pepsin-protein interactions and guide applications in food science, allergenicity research, and proteomics.

Acknowledgments

The authors would like to acknowledge the New Zealand Milks Mean More (NZ3M) program and the Riddet Institute Centre of Research Excellence, Tertiary Education Commission, New Zealand for financial support. Mengxiao Yang thanks the Australian Institute of Nuclear Science and Engineering (AINSE) for a Postgraduate Research Award.

Declaration of competing interest

There are no conflicts of interest to declare.

Funding

This work was supported by the New Zealand Ministry of Business, Innovation and Employment (Wellington, New Zealand), via the programme "New Zealand Milks Mean More" (MAUX1803), and the Tertiary Education Commission, via the Riddet Institute–New Zealand Centre of Research Excellence.

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