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Influence of Proteins on the Absorption of Lipophilic Vitamins, Carotenoids and Curcumin – A Review

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While proteins have been widely used to encapsulate, protect, and regulate the release of bioactive food compounds, little is known about the influence of co-consumed proteins on the absorption of lipophilic constituents following digestion, such as vitamins (A, D, E, K), carotenoids, and curcumin. Their bioavailability is often low and very variable, depending on the food matrix and host factors. Some proteins can act as emulsifiers during digestion. Their liberated peptides have amphiphilic properties that can facilitate the absorption of microconstituents, by improving their transition from lipid droplets into mixed micelles. Contrarily, the less well digested proteins could negatively impinge on enzymatic accessibility to the lipid droplets, slowing down their processing into mixed micelles and entrapping apolar food compounds. Interactions with mixed micelles and proteins are also plausible, as shown earlier for drugs. This review focuses on the ability of proteins to act as effective emulsifiers of lipophilic vitamins, carotenoids, and curcumin during digestion. The functional properties of proteins, their chemical interactions with enzymes and food constituents during gastro-intestinal digestion, potentials and limitations for their use as emulsifiers are emphasized and data from human, animal, and in vitro trials are summarized.

1. Introduction

Proteins are of primary importance for the human body, as the individual amino acids (nine of them being essential, and six further being conditionally or semiessential[1]) are building blocks for all human cells and tissues. Since free amino acids cannot be stored by the human body for later specific usage, a regular consumption of dietary proteins is paramount, and the recommended dietary allowance (RDA) of 0.8 g kg^{-1} body weight (ca. 60 g day^{-1}) for healthy adults reflects this necessity.[1] Proteins do vary in their amino acid composition, and the well digestible ones comprising a high content in essential amino acids with relative ratios close to the cellular requirements have the highest biological value. These include egg, milk, animal meat, and fish flesh proteins, as well as proteins from some plant sources such as those from soy and derived products.[2]

Due to their different size and tridimensional structure,[3] proteins hugely vary in their properties such as water solubility, amphiphilicity, digestibility in the human digestive tract, net charge (depending on surrounding pH and the protein's isoelectric point (p*I*)), and interaction of parts of their structure (e.g., hydrophobic pockets) with other molecules.[4,5] Proteins may also contain nonamino acid components, including, for example, phosphorous and calcium, and plant proteins can be tightly associated with secondary plant compounds such as flavonoids/isoflavonoids, lectins, saponins, and phytates.[6] These may affect protein digestibility, as has been reported for food processing.[7]

During gastro-intestinal (GI) digestion, some proteins can act as emulsifiers, that is, stabilizing an emulsion by increasing the interaction between lipophilic constituents and water-soluble ones, thus reducing surface tension.[8] Proteins with suitable emulsifying properties are generally quite amphiphilic, that is, with botht polar and apolar groups.[9] These proteins are thus interesting natural candidates to enhance the bioavailability of liposoluble microconstituents, especially in the context of lipid-poor, calorie-restricted diets that may otherwise go along with malabsorption. Indeed, it is well known that the liposoluble vitamins, that is, vitamins A, D, E, and K, and other bioactive compounds with low polarity such as carotenoids, are poorly absorbed in the absence of dietary lipids,[10,11] which are needed to drive the formation of mixed micelles, consisting of fatty acids, phospholipids, mono- and diglycerides, bile salts, and cholesterol, among other.[12] It has recently been shown that well digested proteins, such as those present in whey protein isolate (WPI), are able to modify the bioavailability of liposoluble microconstituents, such as carotenoids from a juice, both in vitro[13] and in vivo.[14] Similarly, for vitamin D3, a positive effect on postprandial absorption in mice was found following the simultaneous intake of WPI.[15] The positive effects of proteins on the absorption of liposoluble microconstituents are thought to be related to the following features:

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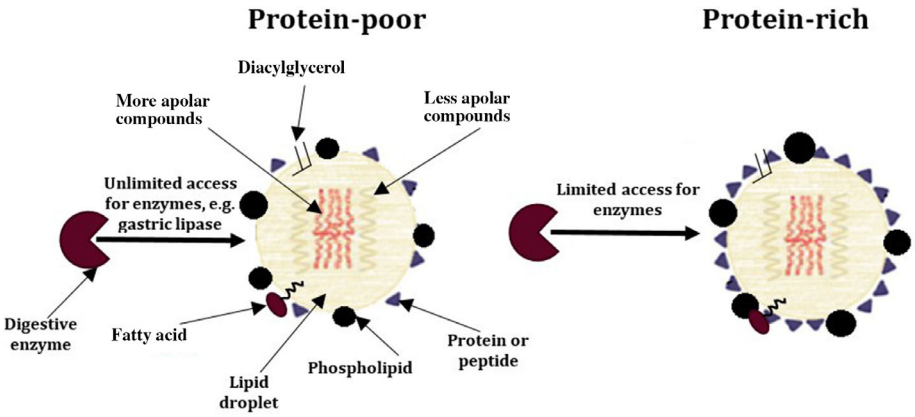

Figure 1. Action of proteins on enzymatic access of lipid droplets in the gastric phase.

(a) Emulsifying properties, that is, produced peptides during digestion could foster the solubility and stability of lipid droplets while interacting with their surface, promoting their processing into mixed micelles[16];

(b) Improving the enzymatic access to the surface of lipid droplets by altering surface tension and viscosity[17];

(c) Stabilizing the mixed micelles in the small intestinal phase (i.e., the chyme), by interacting with their surfaces, which prevents their aggregation[9];

(d) Improving solubility and perhaps preventing the oxidation of lipophilic constituents following their interaction with proteins, via hydrophobic pockets within peptides.[18,19]

Negative effects are likewise possible, and may be explained by the following mechanisms:

(a) Limiting access of lipases to lipid droplets, while forming a protein layer on their surface (**Figure 1**), preventing their processing into mixed micelles, even though the effect of proteins appears weaker compared to, for example, lecithin and Tween 20[20];

(b) Reducing gastric lipase activity by certain proteins, such as β-lactoglobulin,[21] explained by interfacial depletion of the substrate (lipids) into the interior of the droplets;

(c) Causing precipitation of poorly soluble proteins and those of lower digestibility, which could entrap liposoluble constituents[17];

(d) Interacting with microconstituents at the surface of mixed micelles (e.g., xanthophylls),[22] or even penetrating into the mixed micelles (**Figure 2**) causing their destabilization.[23]

Considering their health benefits, the bioavailability of liposoluble vitamins and nonnutrients such as carotenoids or curcumins is of great significance, and the investigation of the potential impact of their interaction with proteins on their absorption is meaningful. For instance, vitamin A deficiency is still the major micronutrient deficiency worldwide and a major cause for child mortality.[24] It is predominant in countries with low preformed vitamin A intake, where provitamin A in form of apolar carotenoids such as β-carotene is the most relevant source, and protein intake is likewise often critical in those places, though this can also be a consideration for vegetarians/vegans.[25] Other vitamins, such as vitamin D, have also been reported to be present at marginal to deficient concentrations in some populations, especially in persons living at higher latitudes where vitamin D formation by the sun is limited.[26]

Another aspect demonstrating the importance of proteins for the bioavailability of fat-soluble micronutrients is their use as encapsulation materials.[27] For instance, β-carotene incorporated into whey protein complexes by microencapsulation showed good water solubility,[18] as well as bioaccessibility, following in vitro GI digestion,[28] pointing out toward increased bioavailability of such combinations. Similar positive effects on the solubility and cellular transport (through Caco-2 cell monolayers as a model of the intestinal layer) of curcumin using β-lactoglobulin as a carrier have been reported.[29]

Therefore, in this review, we aimed to focus on the use of typically consumed proteins or those employed for encapsulation purposes, either from animals or plant sources, as effective emulsifiers of apolar vitamins and selected bioactive plant compounds during digestion. The functional properties of proteins related to their emulsification capacity, chemical interactions with digestive enzymes and/or plant bioactive compounds taking place in the stomach and small intestine, as well as challenges and limitations of the use of different protein matrices as emulsifiers, is emphasized in this review. We also summarized the results from human, animal and in vitro trials investigating the relationship between protein intake, digestion, and influence on the absorption of liposoluble vitamins (A, D, E, K) as well as carotenoids and curcumin (**Table 1**). Finally, we have highlighted the properties of proteins which are essentially linked to their emulsifying activity, and future considerations to better explore these properties and discuss gaps of knowledge regarding the use of proteins for improving the availability of a wide range of fat-soluble bioactive compounds.

2. Protein Digestion and Its Impact on the Stabilization of Lipid Droplets and Formation of Mixed Micelles

Proteins consumed normally entail 20 genetically encoded amino acids, which are needed by the human body to function. The nine

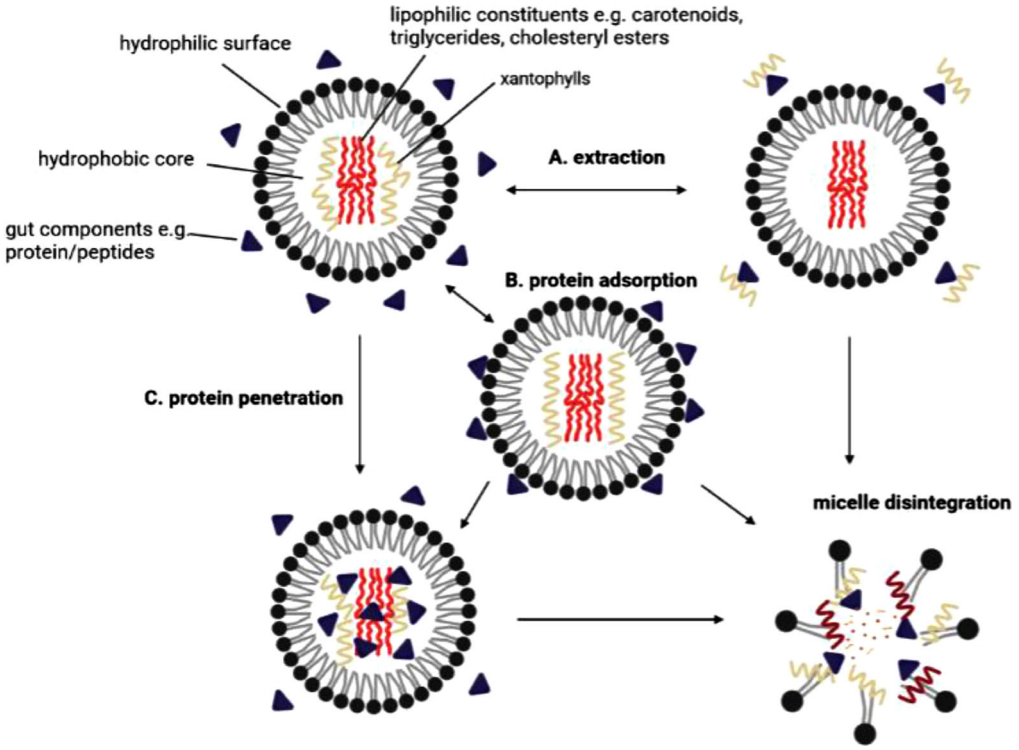

Figure 2. Degradation of mixed micelles depending on the presence of proteins. Adapted with permission.[23] Copyright 2020, Taylor and Francis Group.

essential ones are histidine, isoleucine, leucine, valine, lysine, threonine, phenylalanine, methionine, and tryptophan.[40] When two to ten amino acids are linked by peptide bonds, the term oligopeptide (or peptide) is used, including dipeptides, tripeptides, tetrapeptides, and so on. Between ten and 100, this is referred to a polypeptide (ca. >5.5–11 kDa), while above 100 amino acids, it is generally considered a protein (or as a long polypeptide).

2.1. Digestion of Proteins in the Gastric Phase

In the oral cavity, food items are mainly ground into smaller particles during chewing, and they are also wetted under the influence of saliva to prepare the food bolus by dispersing the formed particles and initiating enzymatic food breakdown. The food bolus is then passed on into the stomach, where the pH varies from about pH 1.3–2.5 under fasting conditions to pH 4.5 or even higher upon the entry of a meal, due to the buffering capacity of the food matrix. Upon further digestion, due to the secretion of additional hydrochloric acid by the parietal cells of the gastric mucosa, the pH of the stomach drops again toward pH 2.[41]

Following the ingestion of a meal, gastric chief cells secrete pepsinogen, which is converted into the proteolytic enzyme pepsin under the influence of the hydrochloric acid of the stomach. In addition, the gastric phase of digestion contains gastric lipase, electrolytes, and mucus, which all aid in the emulsification of lipophilic food constituents.[42] The amount of pepsin present in the stomach has been reported to vary largely, approximately between 20 and 1000 μg mL^{-1} gastric juice,[41] while its cleavage capacity has been reported to be between 200 and 7000 U mL^{-1}[41] (one unit defined as a change in absorbance of 0.001 at 280 nm at 37 °C, in 1 min, at pH 2.0, with hemoglobin as substrate). Similarly, in vitro digestion conditions employ between 160 and 12.500 U mL^{-1}[41]; the well-established INFOGEST protocol uses 2000 U mL^{-1}.[43]

Pepsin in the stomach has been reported to be active especially below pH 3, while being denatured at pH 5–6.[44] As reviewed by Ahn et al., pepsin, an endopeptidase, exhibits a broad cleavage specificity. Generally, hydrophobic amino acid residues, such as phenylalanine, methionine and leucine at the P1 position of the N-terminal, or phenylalanine, tryptophan, and tyrosine at the P1′ position of the C-terminal (cleavage occurs between P1 and P1′), increase cleavage probability, whereas the presence of positively charged amino acids such as histidine, lysine, and arginine at the P1 position, substantially reduce cleavage probability.[44]

According to their structure, proteins differ largely in their digestibility in the stomach. While some proteins clump due to the acid pH of the stomach such as casein, likely increasing stomach residence time, others such as WPI and soy protein isolate (SPI), with better solubility properties under these conditions, may be more rapidly passed on into the duodenum to be further hydrolyzed in the small intestine.[6] Despite the differing effects of the pH on the various types of proteins, most are denatured in the stomach and partly unfolded.[45] Another important aspect, which is typically disregarded at least during in vitro simulation,[31] is the effect of peristalsis of the stomach, which considerably facilitates the creation of a more uniform mixture of the digesta, producing the chyme, aiding in the emulsification of the lipid phase and reducing particle size.[46] In essence,

Table 1. Individual studies investigating the relationship between protein intake, digestion, and influence on lipophilic food constituent absorption.

Liposoluble compound	Protein type	Study details	Effects found	Remarks	Ref.
In vitro studies					
Carotenoids	WPI, SPI, SC, GEL	WPI, SPI, SC, and GEL were co-digested with carotenoid-rich food matrices (tomato juice, carrot juice, and spinach), following the INFOGEST model[30] for static in vitro GI digestion.	Complex interactions, in bioaccessibility tendency positive for carotenes (+33%), rather negative for xanthophylls (−50%).	Bioaccessibility may depend on the amount of oil used during digestion, individual type of carotenoid, pancreatin, bile, and rpm as an indicator of gastric sheer force.	[13]
Carotenoids	WPI	The influence of WPI on β-carotene bioaccessibility at various gastric conditions, including altered simulated peristalsis, gastric phase duration, and varying concentrations of pepsin, bile extract, pancreatin, and the physicochemical properties of the digesta, were investigated.	20% increase in β-carotene bioaccessibility in vitro, especially under incomplete digestive conditions, i.e., under low digestive enzyme concentrations. WPI reduced β-carotene bioaccessibility by approx. 70% when lower concentrations of bile, enzymes rather compromised lipid digestion or reducing shaking speed.	Higher bioaccessibility with a reduced amount of dietary lipids. The addition of WPI during simulated GI digestion influenced the bioaccessibility of β-carotene positively and negatively, depending on the digestion conditions.	[31]
Carotenoids	SPI-PEP	β-Carotene was encapsulated in SPI-PEP conjugate-stabilized emulsion, and its gastrointestinal behavior and antioxidant activity were assessed in vitro.	Compared with β-carotene-SPI emulsion and β-carotene-mixture emulsion, the bioaccessibility (12%), absorption, and stability of β-carotene significantly increased in the β-carotene-conjugate emulsion. Therefore, β-carotene-conjugate emulsion had the highest bioavailability among the three groups.	SPI-PEP conjugate emulsion increased the bioavailability of β-carotene in the simulated gastrointestinal tract compared with β-carotene-SPI emulsion and β-carotene-mixture emulsion.	[32]
Vitamin D	WPI, SPI	VD encapsulated with different combinations of WPI and SPI were investigated in vitro.	5% WPI + 5% SPI mixture exhibited the highest encapsulation efficiency. All the encapsulates showed significantly higher stability and VD retention (> 93%) during storage at 4°C as compared to free VD.	The study concluded that the complex encapsulates presented stronger interactions for matrix development than a single protein that resulted in better protection and controlled release of VD.	[33]
Vitamin A	Caseinate complexes	The bioavailability of VA through sodium caseinate-VA complexes was evaluated by exposing Caco-2 cells to the digesta of milk fortified with various complexes.	Up to 37% higher uptake by Caco-2 cells compared to free VA. The total uptake of VA by Caco-2 cells was highest for milk fortified with RSNaCaS-VA followed by RNaCaS-VA, control milk, SNaCaS-VA, NaCaS-VA, and free VA.	During the formation of RNaCaS-VA and RSNaCaS-VA complexes, more hydrophobic sites were exposed, leading to the attachment of VA on the interior hydrophobic regions of sodium caseinate molecule. This led to higher stability of VA during GI digestion and further resulted in higher bioaccessibility and bioavailability of vitamin A in Caco-2 cells.	[34]
Vitamin E	WPI	The influence of plant-based (gum arabic and quillaja saponin) and animal-based (whey protein isolate, WPI) emulsifiers on the production and stability of VE-fortified emulsions was investigated in vitro.	85% increased bioaccessibility with WPI than in saponin- or gum arabic-emulsions.	Lesser effect for VE with saponin and arabic gum. Lipid digestion was slower in saponin-emulsions, presumably because the high surface activity of saponins inhibited their removal by bile acids and lipase.	[35]

(*Continued*)

Table 1. (Continued).

Liposoluble compound	Protein type	Study details	Effects found	Remarks	Ref.
Animal studies					
Vitamin A	β-Lactoglobulin (β-LG)	In vitro and in vivo assessment of VA encapsulation in a liposome–protein delivery system. Animals: 72 male CBA/C57 mice; Group A: pure VA in soybean oil, group B: VA + liposomes.	The % EE of VA in liposomes in absence and presence of β-LG was 50.64% (±1.29%) and 56.17% (±1.90%), respectively. The stability of incorporated VA in protein/liposome complex was significantly enhanced by protein presence during the whole period of storage, compared to that of bare VA.	The most important determinant of the encapsulated VA in liposome–protein complex was the in vivo bioavailability evaluation. Phospholipid–sterol-protein-membrane resembling system may be one of the promising approaches to enhance the absorption of VA.	[36]
Vitamin D	Pea protein isolate (PPI)	Weaned male albino rats ($n = 35$) were fed either normal diet (VD 1000 IU kg^{-1}) (control group; $n = 7$) or a VD-deficient diet (<50 IU kg^{-1}) for six weeks (VD-deficient group; $n = 28$).	2.3 times higher absorption (increase in serum 25(OH)VD) compared to control without PPI emulsion. Consumption of VD dispersed in PPI nanoemulsion created by ultrasound and pH shifting improved its circulating status, which influenced the levels of bone turnover biomarkers in VD-deficient rats.	The presence of canola oil did not result in increased absorption. PPI can be used to encapsulate other fat-soluble micronutrients and bioactive needed in fortification and supplementation programs such as VA, VE, and carotenoids.	[37]
Vitamin D	WPI, SC	Sprague-Dawley rats ($n = 78$) were administered 840 IU VD3 dissolved in ethanol and either (a) complexed with whey protein isolate (protein: vitamin ratio 2:1), (b) complexed with caseinate (protein: vitamin ratio 2:1), or (c) provided in a water solution.	Compared to noncomplexed and caseinate complexed, serum VD3 concentrations were higher across the different time points in rats that received the VD3 complexed with whey protein.	Using an in vivo rat model, the study demonstrated that complexation with milk proteins is an efficient strategy to enhance the bioaccessibility of VD3. Both complexation with whey protein isolate and caseinate, respectively, enhanced bioaccessibility of VD3, but complexation with whey protein isolate was found to be more potent than complexation with caseinate.	[15]
Carotenoids	SC	Male Sprague-Dawley rats aged 3 weeks were divided into groups receiving VA free diet and an experimental diet.	The serum retinol content in the low β-carotene diet groups was about 7%–12% of that in the mid-level β-carotene diet groups. However, the liver retinol content in the low β-carotene diet groups was 0.12%–0.18% of that in the mid-level β-carotene diet groups. Fat-soluble β-carotene and retinol accumulation decreased with a higher fat intake (with a 10% fat diet than with a 2% fat diet) rather than increased with an increase in fat intake. This study shows that the mid-level dietary protein groups were influential for retinol accumulation and metabolism of β-carotene.	The low β-carotene diet groups (0.006 mg β-carotene/100 g diet) showed a higher serum retinol accumulation rate, CDO activity, and liver β-carotene accumulation rate than the mid-level β-carotene diet groups. These results suggest the marked effect of protein, fat, and β-carotene level in diets on the absorption and metabolism of β-carotene.	[38]

(*Continued*)

Table 1. (Continued).

Liposoluble compound	Protein type	Study details	Effects found	Remarks	Ref.
Human studies					
Carotenoids	WPI, SPI	A cross-over, randomized human trial with 24 healthy adults ($n = 12$ males, $n = 12$ females). After 2-week washout periods, 350 g of a tomato–carrot juice mixture was served in the absence/presence of WPI or SPI (50% of the recommended dietary allowance, RDA ≈ 60 g day^{-1}).	Considering total popualtion (both genders) and combining carotenoids/TAGs, the AUC (in plasma TRL-fraction) increased by 45% with WPI compared to control meal	SPI-supplement meal had no significant effect	[14]
Vitamin E	Milk protein	Included three experiments: (a) ($n = 48$), compared delivery of 100 mg all-rac-α-tocopheryl acetate/d, 1%-fat milk containing soybean oil, milk fat, or both (1:1); (b) ($n = 24$), compared delivery of RRR-α-tocopheryl acetate and all-rac-α-tocopheryl acetate in milk with the delivery of all-rac-α-tocopheryl acetate in orange juice (200 mg day^{-1} in each group), (c) ($n = 7$), compared delivery of 30 mg all-rac-α-tocopheryl acetate/day in milk with and without added VA and VD.	Microdispersion of VE in milk increased the molar ratio of plasma tocopherol to cholesterol by >2-fold compared with the molar ratio after consuming VE capsules. In contrast, the molar ratios were comparable after ingestion of orange juice and capsules. VA and VD did not affect vitamin E delivery by milk.	Milk augments VE transport by human lipoproteins at intakes of 100–200 but not 30 mg day^{-1}. This augmentation depends on the presence and type of fat in milk, its VA and VD contents, and whether VE is natural or synthetic.	[39]

AUC, Area-under (time-vs. concentration) curve; CDO, carotene 15,15′-dioxygenase; EE, encapsulation efficiency; GEL, gelatin; NaCaS, sodium caseinate; PEP, pleurotus eryngii polysaccharide; PPI, pea protein isolate; RSNaCaS, reassembled succinylated sodium caseinate; SC, sodium caseinate; SNaCaS, succinylated sodium caseinate; SPI, soy protein isolate; TGA, triacyl-glycerides; VA, vitamin A; VD, vitamin D; VE, vitamin E; WPI, whey protein isolate.

the results of protein digestion in the stomach are generally shortened and denatured polypeptides, with low amounts of free amino acids. However, it has also been stated that this step is not crucial, as persons who had their stomach removed were still able to digest and make use of proteins from the diet.[47] Thus, protein digestion in the stomach is far from being considered complete, depending on the protein type, the codigested food matrix, and the volume of the food, as a larger volume may slow down gastric passage, leading to prolonged exposure of the food matrix to the milieu of the stomach. Digestion times in the stomach can vary between 15 and 20 min for small liquid meals up to 3–4 h for more voluminous and solid meals.[48] Particle size is reduced to typically 1–2 mm,[49] which is important, as too large particles would delay further emptying of the stomach via the pylorus into the duodenum.

Regarding their physicochemical properties, proteins have been reported to increase the viscosity of a meal during digestion, due to their partial solubility in the aqueous phase. Some can possibly form gel-like structures such as gelatin, more drastically increasing viscosity.[22] In addition, many proteins may reduce surface tension of the aqueous phase in the stomach,[50,51] due to their amphiphilic nature, though also increased surface tension has been reported in in vitro trials, due to coprecipitation and general clearance of other surface tension reducing agents.[22]

Due to their amphiphilic properties, proteins may foster stabilization of lipid droplets in the gastric phase of digestion, fostering fatty acid release, and transition of lipid droplets into smaller droplets. At least this has been observed in vitro for certain proteins such as WPI regarding its effect on lipid droplet size in the intestinal phase of digestion,[22,31] and similar effects are expected for the gastric phase, possibly enabling a better access of gastric lipase to lipid droplets. This is less likely the case for proteins that precipitate in the stomach such as casein.[6] Moreover, proteins, perhaps especially at high concentrations (Figure 1), may also inhibit access of gastric lipase to lipid droplet surfaces, similar to what was reported for pancreatic lipase (see following section 2.2).

2.2. Digestion of Proteins in the Small Intestine

The secretion of bile and pancreatic juices containing hydrogen carbonate neutralizes the acid from the stomach. More specifically, the intraluminal pH is rapidly increasing to about 5.4–7.5 in the duodenum, 5.3–8.1 in the jejunum, and 7.0–7.5 in the ileum.[41]

The majority of the digestive processes take place in the small intestine, including proteolysis. Indeed, additional enzymes

result in the further digestion of the proteins released into the duodenum, while the jejunum is considered the major place of amino acid (and small peptide) absorption.[47] The following secreted zymogens (inactive precursors, secreted by the pancreas) are turned into active proteases (under the influence of trypsin):

(a) Trypsinogen, which is activated into trypsin (endopeptidase) following its cleavage by enteropeptidase (secreted in the duodenum). Once activated, trypsin cleaves the peptide bond on the carboxyl side of arginine and lysine;

(b) Chymotrypsinogen, which is activated into chymotrypsin (endopeptidase), favorably cleaving peptide bonds that are on the carboxyl side of tryptophan, phenylalanine, tyrosine, or leucine;

(c) Proelastase, which is activated into elastase (endopeptidase), cleaving especially peptide bonds on the carboxyl side of small, hydrophobic amino acid residues such as glycine, valine, leucine, isoleucine, and alanine;

(d) Procarboxypeptidases, which are cleaved to exopeptidases called carboxypeptidase A, B, and other aminopeptidases. In contrast to aminopeptidases, which cleave peptide bonds at the N-terminus of proteins, carboxypeptidases A has a stronger preference for cleaving peptide bonds at the carboxyl side of hydrophobic and branched chain amino acids such as phenylalanine, tryptophan, tyrosine, or leucine/isoleucine, while carboxypeptidase B preferably cleaves the peptide bonds on the carboxyl side of positively charged amino acids, such as arginine and lysine.

The typically resulting di- and tripeptides are then in part further cleaved at the brush border by aminopeptidases into amino acids, prior to their absorption by the enterocytes.

GI protein digestibility is assumed to play an important role given the potential interactions of the intermediate and resulting products with other dietary ingredients. Well soluble and partially cleaved proteins may result in more interactions with lipid droplets and later with mixed micelles than with largely undigested proteins, as proposed by improved emulsification activity of certain protein hydrolysates.[52] Other proteins, for example, those rich in proline stretches such as gluten, have been shown to be of low digestibility, due to the reduced structural flexibility and inaccessibility to digestive proteases.[47] Proteins with high levels of β-pleated sheet are likewise poorly accessible for digestive enzymes, such as shown for certain wheat flour doughs.[53] Also, cross-linking of proteins, that is, via disulfide bridges, reduces the digestibility of proteins.[47] Such proteins are presumed to have low emulsification properties. While food processing, such as high-pressure homogenization and ultrasound treatments,[54,55] can improve digestibility of proteins, the intake of large amounts of proteins or the presence of external factors, earlier termed "antinutrients," can prevent or drastically retard the breakdown of proteins. Many are trypsin and chymotrypsin inhibitors, such as tannins and other phenolics, phytates, hemagglutinins/lectins, as well as dietary fiber (reducing protein digestion by rather physical effects, i.e., occlusion) among other, as reviewed by Joye.[47]

Contrarily, rapid digestion starting in the gastric phase and ongoing in the intestinal phase may foster potential positive interactions of peptides with mixed micelles. For instance, WPI did show good and consistent stabilization of lipid droplets, both during the gastric phase (with a slight increase of droplet size explained by partial proteolysis of the interface) and the intestinal passage (by reducing again droplet size to around 2 μm). The stabilized lipid droplets were smaller than those of lecithin-stabilized droplets, perhaps as the β-lactoglobulin fraction of the WPI is resistant to pepsinolysis,[56] despite the fact that WPI has been proposed to have one of the highest digestibilities in the ileum, about 97%–98% (versus 94% for caseins[57]).

2.3. Potential Interactions Between Proteins and Apolar Dietary Constituents During Digestion

Most important regarding the digestion of liposoluble food compounds such as fat-soluble vitamins and secondary plant metabolites (e.g., carotenoids and curcumin) would be likely their interaction with lipid droplets, which are then further processed under the influence of pancreatic lipase and bile acids into mixed micelles, a prerequisite formation for the absorption of liposoluble compounds.

In this respect, both positive and negative effects on the micellization processes can be envisioned, either due to enhanced stabilization of lipid droplets and faster micelle formation, or reduced access of digestive enzymes and slowed down process of micellization. In an in vitro study by Mun et al.,[20] the effect of different surfactants on the resistance of lipid droplets against the digestion by pancreatic lipase followed the order nonionic surfactant (Tween 20 at 13 mM), phospholipids (lecithin at 75 mM), and finally proteins (caseinate or WPI, 220 and 212 mM, respectively), suggesting that compared to other surfactants, proteins did not strongly hamper lipid digestion. More recent results comparing the effect of Tween 20, β-lactoglobulin, and lecithin at similar concentrations (2.5%) showed only small differences in the release of free fatty acids (FFAs) from an oil–water (o/w) emulsion,[58] emphasizing that differences can depend on the type of proteins digested, their concentrations, and the specific digestive conditions.

In line with potential negative effect of proteins on the processing of lipophilic constituents during digestion, in simulated in vitro experiments, the addition of proteins (WPI and SPI) rather reduced the bioaccessibility of several carotenoids, which may be related to a hampering effect of proteins on FFA release, though this remains somewhat speculative.[59] Effects may further depend likely on the aqueous solubility of the micellized compounds. Some protein–lipophilic drug interactions (Figure 2) have shown that proteins can specifically interact with the surface of mixed micelles, which may explain negative effects found on xanthophyll bioaccessibility that rather resides at the outer layer of mixed micelles.[13] Proteins may thus deplete the embedding of lipophilic constituents in mixed micelles, or even disrupt mixed micelles entirely.

Interestingly, an impact of bile salts on protein digestion has also been found, showing drastically improved pancreatic proteolysis in vitro for β-lactoglobulin, bovine serum albumin (BSA), myoglobin, and a commercially available dietary protein supplement,[60] perhaps via the emulsifying properties of bile acids and the resulting unfolding of proteins.[61] However, it is

Table 2. Overview of frequently consumed proteins and properties related to their emulsification properties.

Protein	Water solubility (pH 7) [%]	Water solubility (pH 2–4) [%]	Chain lengths [Da]	Iso-electric point (pH)	Digestibility [%] – global after GI	Other characteristics (solubility in salt solution...) and main proteins	Ref.
WPI	92.6	87.1	11 000[a)]	4.5	100	Major subunits: β-lactoglobulin (ca. 50%), α-lactalbumin (ca. 20%), immunoglobulins (ca. 10%), BSA (ca.6%), lactoferrin	[66, 71, 72]
SPI	80.2	80.5	9300	4.5	84.0	Major subunits: conglycinin (7S) and glycinin (11S)	[73–75]
SC	>90.0	<20.0	23 200	4.6	76.25	α-Casein and β-casein (4:1), k-casein	[76–78]
GEL	87.0	Ca. 70.0	220 000[b)]	4.9[c)] 9.0[d)]	98.0	α-Chain, β-chain	[79–81]
Collagen[e)]	95.0	≈100	300 000	7.0–8.0	98.0	α-Chains	[82–84]
Rice	25.0-30.0	<20.0	25 000[f)]	4.5	93.0	Globulin, α-gluteolin	[85, 86]
Pea	70.0	30.0	320 000–380 000[g)]	4.3	86.0	Legumin (11S), vicilin (7s)	[87, 88]
Egg white	73.9[h)]	34.8	45 000	4.5	90.9[i)]	Ovoalbumin	[89, 90]
Chicken[j)]	94.0	21.3	16 800	5.5	90.3	α-2,3,4,6 and β2,3,5	[91, 92]
Cod	85.0	80.0	41 000	5.5	86.0	Gad c I, parvalbumin	[93–96]
Salmon	≈10	90 (pH 2) 10 (pH 4)	205.000[k)]	5.5	92.8	Myosin, actin	[94, 97]

BSA, bovine serum albumin; GEL, gelatin; GI, gastro-intestinal; SC, sodium caseinate; SPI, soy protein isolate; WPI, whey protein isolate. [a)] 1 A.A. equals 110 Da; [b)] Bovine skin collagen; [c)] Isoelectric point of acidic gelatin; [d)] Isoelectric point of basic (alkaline) gelatin; [e)] Hydrolyzed collagen; [f)] Brown rice protein; [g)] Molecular weight of pea legumin; [h)] Solubility analyzed at pH 8; [i)] Cooked egg-white; [j)] Chicken breast; [k)] Myosin.

somewhat unclear whether proteins have an effect on the apparent concentration and activity of bile salts, but precipitation of bile salts by binding peptides during digestion has been reported.[62] This may contribute to the negative effects of proteins at low bile salt concentrations, as observed for impaired bioaccessibility of carotenoids.[31]

3. Emulsifying Properties of Various Proteins

The chemical and biological properties of proteins do vary considerably. With respect to acting as emulsifiers, molecular weight, water solubility, net charge at a given pH, shape, and digestibility are among the most important attributes.[6,63] Food-derived proteins have been differentiated according to their water solubility into albumins (soluble), globulins (insoluble, but soluble in diluted salt solution), prolamins (insoluble, soluble in 70%–80% ethanol), and glutelins (insoluble, soluble in diluted alkaline solution).[64] The solubility of proteins is related to the net charge at a given pH. If the p*I*, where proteins have a net outer charge of zero, is close to the surrounding pH, then solubility is minimum. It has been emphasized that plant-based proteins generally exhibit a lower water solubility than animal-derived ones in the GI tract[65] (**Table 2**). For instance, the water solubility varies from, for example, 85 g/100 g (40°C, pH 3.5–7.5) for WPI[66] to only about 20 g/100 g (pH 2–7, 0.1 M NaCl) for SPI,[67] while above pH 7, solubility of the latter increases. Indeed, proteins with a low p*I* such as 4.5 for SPI (Table 2,[68]) tend to have limited solubility during the gastric phase of digestion, though other factors such as structure may affect water solubility more strongly. In fact, globular proteins (e.g., albumins, globulins), in contrast to fibrous proteins (e.g., collagen/gelatin), have been emphasized to undergo unfolding and rearrangements in o/w films, forming a viscoelastic film at the interface,[68] such as around lipid droplets. Their thickness may only range a little over 1 nm.[68] By doing so, they can effectively alter droplet size distribution by decreasing interfacial tension, and can stabilize emulsions by steric action and electrostatic forces.[69] This is especially the case when the pH is not too close to the p*I* of the protein, due to the higher net charges and repulsive electrical potential (zeta-potential) at the emulsion interface,[70] while mechanical effects and a pH close to the p*I* can result in increased interactions between the proteins and thus produce coalescence. However, following binding to the interface, the films may thin out, resulting in the exposure of reactive protein groups, which could then lead to increased flocculation and coalescence of the lipid droplets. Such unfolding upon adsorption at the interfaces together with aggregation has clearly been shown for BSA and β-lactoglobulin.[70]

Depending on the type and nature of the protein, the protein-coated lipid droplets may have different structure and rheological properties, which would imply a difference in the characteristics of the interfacial film implicated in the stability of the emulsions.[98] For instance, flexible proteins such as β-casein, which is strongly amphiphilic and constitutes up to 35% of the caseins in bovine milk,[99] have been reported to adsorb to lipid droplet surfaces and form a highly viscoelastic interfacial film, resulting in stable emulsions,[100] though this may also be partly related to steric stabilization due to the thick film adsorbed to the surface of lipid droplets.[101] In contrast, monomeric globular proteins such as α-lactalbumin (14.7 kDa) and BSA (66.5 kDa) have been described to form a thin monolayer interfacial film

with high elasticity, but the resulting emulsions were found susceptible to coalescence, especially when shear stress was applied,[102,103] which could also occur during GI peristalsis. However, almost no flocculation was observed when the amount of protein was sufficient to completely cover the lipid droplets.[104] In the case of globulins, such as β-conglycinin (a trimer, with a molecular mass of 180 kDa, comprising 30%–50% of the total SPI[105]) and β-lactoglobulin (at a native dimeric form, 18.5 kDa), a dense and viscoelastic interfacial film could be formed at the surface of lipid droplets, exhibiting a high coalescence stability as a result of high viscoelasticity of the interfacial film and a relatively low tendency to aggregate, due to their highly hydrophilic nature.[98] Finally, some proteins, such as oligomeric glycinin (a hexamer with a molecular mass of 300–400 kDa, constituting ca. 30% of the total SPI[105]), have been reported to form very thick and inhomogeneous multilayer interfacial films with a high tendency to aggregate, leading to entrapped lipid droplets within a network consisting of aggregated particles, especially at high concentrations of these proteins.[98] This would prevent digestive enzymes to adsorb at lipid droplet surfaces, and could also implicate potential negative influences of proteins on the micellization of codigested lipophilic compounds, such as carotenoids.

It is generally recognized that a good balance between hydrophobicity and hydrophilicity within a protein is an important factor for its emulsifying ability.[106] Given their high amphiphilic nature, such proteins exhibit a good ability to diffuse and/or adsorb to lipid droplets, while folding at o/w interfaces, by exposing rather hydrophobic groups to the lipid core of the droplets and hydrophilic groups on their surface,[107] thus acting as effective emulsifiers to form and stabilize o/w emulsions.[108] For example, the structure of WPI consists of two main fractions, namely α-lactalbumin (approximately 25%) and β-lactoglobulin (approximately 50%), and their conformation (secondary structure, mainly α-helix and β-sheet, respectively) determines their properties at the o/w interface.[109] Similarly, glycinin and β-conglycinin account for about 70% of total SPI.[105] Applying an acid treatment resembling the gastric environment (pH 2–3) to SPI led to a progressive increase in the extent of denaturation, and subsequently, a progressive subunit dissociation, exposure of hydrophobic clusters, and aggregation of unfolded and/or denatured protein fractions,[110,111] due to the loss of solubility of glycinin, while β-conglycinin was much less affected by these structural changes.[112]

4. Interactions of Proteins and Fat-Soluble Vitamins, Carotenoids, and Curcumin

4.1. Liposoluble Vitamins

Liposoluble vitamins include vitamins A, D, E, and K. However, since very little information is available for vitamin K, and as it is also formed in the gut by the microbiota, it will not be discussed in this article. A common similarity that is shared by all vitamins and lipophilic secondary plant compounds is their low water solubility, requiring incorporation/solubilization into lipid droplets, which result in the formation of mixed micelles.[113,114] The size of the mixed micelles (Figure 2) is of approximately 5–8 nm in diameter, and they are composed of a hydrophilic surface made from the polar parts of mono- and diglycerides, phospholipids, bile salts, cholesterol, xanthophylls, vitamin E, etc. and of a hydrophobic inner part with more apolar constituents such as carotenes.[114] These micelles then diffuse to the unstirred water layer from where the liposoluble constituents can then be absorbed, either via passive diffusion (at high concentrations) or via protein carriers (e.g., CRBPII, SR-BI, CD36, and NPC1L1[114–116]), typically at lower (dietary) concentrations. The main absorption area is the small intestine.

4.1.1. Vitamin D

Vitamin D is an essential vitamin, with an RDA of 15 µg for adults,[117] which is fairly low compared to the intake of other lipophilic microconstituents such as carotenoids, which are consumed in the range of several mg per day,[118] possibly not raising issues of volume/concentration overload that can result in low micellization, unless taken in form of supplements. However, the unique feature of this vitamin is that it can also be formed under the skin by the influence of UV light, with cholesterol as its precursor, thus bypassing oral bioavailability. Nevertheless, the majority of the populations living in the high latitudes (about 40°–50° and higher) have been reported to have low circulating vitamin D levels, as assessed by 25-hydroxyvitamin D (25-OH D, the major form of vitamin D circulating in the bloodstream and accepted status marker), in winter.[26] For instance, approximately 40% of the population in the UK shows low vitamin D status (as defined as a concentration of <25 nmol L^{-1} of serum 25-OH D) in winter.[119] As low vitamin D status has been correlated in meta-analyses with many diseases, including cardiovascular disease mortality[120] and also prediabetes conditions,[121] factors influencing its absorption have been investigated and reviewed.[122–124]

The absorption of vitamin D has been reviewed by Reboul[125] to be fairly high, varying from 55% to 99%. However, susceptibility of its absorption in persons with intestinal malabsorption, especially steatorrhea, that is, disturbed lipid digestion, has also been highlighted, pointing out to the importance of emulsification factors present in the intestine for its bioavailability.

Vitamin D3 (cholecalciferol) is quite apolar, with a log*P* (octanol/water partition coefficient) value of 10.2,[126] thus its absorption follows micellization and the lymphatic pathway. Similar considerations apply to vitamin D2 (ergocalciferol), with a log*P* value of 10.4. Because of the presence of double bonds, vitamin D has been shown to be sensitive to isomerization under varying conditions such as pH, oxidation, light, and temperature, all potential sources of degradation, which can be avoided to some extent by finding suitable carriers or encapsulation processes.[127] Indeed, the usefulness of, for example, milk proteins for encapsulating has been reviewed earlier, and aspects such as protection of sensitive molecules against oxidation, photo-degradation, and enhanced release and solubility were highlighted.[128] For instance, microencapsulation of vitamin D with WPI, SPI, or both resulted in good release properties in simulated gastric and intestinal fluids, being highest for SPI (>90%, combined gastric + intestinal phases).[33] Also, it has been reported in a recent review that vitamin D encapsulated in the β-lactoglobulin fraction from WPI is thought to have a high bioavailability.[123]

In line with the positive influence of proteins, an interesting study in mice was conducted by Lindahl et al.[129] Animals were receiving vitamin D3 dissolved in ethanol and then administered either as (1) a complex with WPI, (2) a complex with caseinate, or (3) in aqueous solution. For the purpose of complex formation, vitamin D was merely dissolved in WPI or caseinate-rich solutions. Plasma levels of vitamin D3, 25-OH D3, and 24,25-dihydroxyvitamin D3 over 10 h were measured, and the WPI condition showed the fastest and highest absorption (i.e., about 20% higher than caseinate), though also the caseinate complexed form was absorbed better than free vitamin D3 in water. Area under (concentration vs. time) curve AUC values for vitamin D3 were ca. 525, 400, and 350 ng h mL^{-1}, respectively. The authors explained their results because vitamin D3 complexed with proteins had higher solubility and better protection against degradation during the GI passage.

In another study, vitamin D3 was encapsulated into a β-lactoglobulin complex (by mixing), and a coagulum was then created by means of glucono-δ-lactone. This was force-fed to rats. Despite that the coagulum was fairly resistant against proteases in the intestinal phase of digestion (as tested in vitro), the long-term bioavailability (after 3 weeks, as measured in the plasma as 25-OH D3) was about three times higher from the coagulum (coagulated β-lactoglobulin complex) versus free vitamin D3 (in aqueous solution) or vitamin D3 given as a β-lactoglobulin complex alone. This higher bioavailability was attributed to the higher water solubility of the complex, though it may be assumed that also protective aspects, that is, increased stability of vitamin D3 in this form, could have played a role. In another study with rats, vitamin D in a pea protein isolate (PPI) nanoemulsion was most efficient to improve vitamin D status in animals, more so than vitamin D in oil,[37] though it cannot be excluded that the nanoparticles generated by ultrasound treatment contributed mostly to this effect.

Haham et al. nanoencapsulated vitamin D3 into reassembled casein micelles,[130] and showed in human volunteers that this formulation was at least as bioavailable as Tween80 emulsified vitamin D3, as measured by plasma appearance. In an earlier study by Tangpricha et al.,[131] vitamin D2 absorption in humans (measured postprandially during 72 h as plasma appearance of 25-OH D2) was compared in a cross-over trial in 18 adults with either whole milk, skimmed milk, or corn oil added on a toast. Though the AUC of the condition with whole milk appeared slightly smaller, results were not significantly different, and it can be assumed that all three matrices offered comparable bioavailability. However, all three meals contained either lipids or lipids and proteins, so a low lipid and/or low protein source as a control was missing. Such a matrix effect was also seen by Johnson et al.,[132] when studying the bioavailability of vitamin D2 in eight adults in a cross-over trial, delivering vitamin D either in cheese or in water, with peak serum 25-OH D2 concentrations being eight times higher from cheese, though it was not possible to say whether the effect was due to the lipids, the proteins, or both.

In another study by Wagner et al.,[133] vitamin D from different food sources was given for 8 weeks in a study involving 80 adults. Sources were fortified cheddar, fortified low-fat cheese, or a liquid vitamin D supplement, taken either with or without food. 25-OH D responses in plasma were 65.3 (SD: 24.1), 69.4 (21.7), 59.3 (23.3), and 59.36 (19.6) nmol L^{-1}, respectively, thus showing in tendency lower values for the supplement, but due to the design of the study and the high SD (nonpaired design), no significant differences were found.

In summary, considering the results from the human, animal, and in vitro trials together, there is some evidence that proteins can modulate vitamin D absorption positively. However, as often proteins and lipids were administered together, the individual contribution of these components is somewhat unclear in several studies. Furthermore, as the log*P* value of vitamin D is somewhat lower than, for example, that of the very apolar carotenes, it can be hypothesized that the potential effects of proteins or other emulsifiers on boosting vitamin D bioaccessibility are perhaps less potent than those of carotenes.

4.1.2. Vitamin E

Vitamin E has received nutritional interest mostly via its role played as an antioxidant, protecting lipids from (per) oxidation in cellular membranes.[39] It tightly interplays with vitamin C by which it is regenerated following oxidation. Higher vitamin E intake has been shown in meta-analyses to be correlated with reduced risk of stroke[134] and even Alzheimer's disease,[135] though supplementation trials have not clearly shown positive effects.[136] Tocopherols (four types: α, β, γ, δ) and tocotrienols (four types: α, β, γ, δ) have both vitamin E activity, with the latter group having three double bonds in their side chain, making them potentially more prone to lipid peroxidation. Tocotrienols are especially found in vegetable oils.[137] Concentrations in food items may reach up to 100 mg/100 g, such as in wheat germ,[138] though typical amounts are more in the range of 0–50 mg/100 g, with an RDA of 15 mg day^{-1} for adults.[139] Therefore, vitamin E is possibly the most abundant lipophilic micronutrient present in many food items, thus its absorption may be prone to be influenced by digestive factors. The log*P* of 12.2 has been determined for α-tocopherol,[140] and all vitamin-E active compounds are very apolar. The potential clinical relevance of vitamin E has also sparked the interest for aspects influencing vitamin E bioavailability. Reboul[141] and Borel et al.[142] previously reviewed factors impinging on vitamin E bioavailability, with a focus on uptake and transport. These authors highlighted the impact of dietary factors including matrix disruption and the presence of dietary lipids on the micellization and thus absorption of vitamin E from the small intestine, emphasizing that micellization efficiency does appear to play a role regarding vitamin E bioavailability. The importance of dietary lipids for high bioavailability of vitamin E and α-tocopherol acetate as a common food additive (which requires cleavage by bile acid-dependent lipase prior to absorption), has also been reviewed by Schmölz et al.[143] Regarding the influence of proteins, very little is known on their influence on the micellization of vitamin E.

In an in vitro digestion study by Werner and Böhm, pasta with eggs (rich in lipids and proteins) resulted in a decreased bioaccessibility of total vitamin E as opposed to pasta alone, which was unexpected. Similar negative findings in that study were observed for carotenoids. However, the authors reasoned that incomplete lipolysis in the egg pasta matrix could have played a role,[144] possibly emphasizing the importance of complete digestive conditions for predicting physiological results. In another

in vitro study, testing different o/w emulsions including vitamin E in WPI, saponin (triterpene glycosides), and gum arabic.[35] Bioaccessibility was highest from WPI produced emulsions (approximately 85%) following GI digestion, as compared to saponin and gum arabic-emulsions (approximately 60% in both emulsions). The saponin emulsion had the highest absolute zeta-potential (at pH 7), an indicator of repulsive forces preventing agglomeration. However, lower lipid digestion was found in saponin-emulsions, which was explained by the higher surface activity of saponins and more difficult removal of these particles by bile acids from lipid droplets, unlike WPI-based emulsions, which had lowest mean particle diameter (below 100 nm, at pH 7). Similar findings were earlier obtained by Ozturk et al.,[145] investigating vitamin E encapsulated by WPI or gum arabic. These results highlight the importance of factors including small particle size, and rapid displacement from lipid droplets by lipase and bile acids, being most important parameters for high bioaccessibility in the presence of dietary proteins. WPI has earlier been highlighted for its stability at low pH and thus for its properties to incorporate vitamin E into orange-based beverages of high acidity[146]; heat stability of the emulsions was also very high.

In another in vitro study,[147] vitamin E microcapsules were produced by spray-drying, freeze drying, or spray-freeze drying, employing saponin as a surfactant together with WPI as an outer wall material, using a homogenizer and a microfluidizer. Experiments in rats demonstrated good bioavailability of vitamin E when combined with WPI, as determined by plasma AUC appearance, especially for the spray-freeze dried formulations, which was attributed to the better dissolution behavior, which likely aided in the micellization process.

In an earlier human postprandial study by Lodge et al., vitamin E supplements (α-tocopherol) were given together with a toast and butter or a cereal with full fat milk, among other, both containing the same amount of fat. While both formulations resulted in better absorption of vitamin E, as compared to the supplement with water, the toast/butter combination (being lower in proteins than the full fat milk meal), led to higher vitamin E absorption, perhaps due to the negative effect of dietary fiber from the cereals.[148] Contrarily, in an even earlier human study by Hayes,[39] consuming vitamin E within milk, independent of the fat percentage (though this was limited to 1%), resulted in an increased vitamin E plasma levels after 4 weeks of consumption, compared to vitamin E intake from capsules (α-tocopherol acetate), also suggesting that proteins appeared to have beneficial effects. The authors reasoned indeed that dietary proteins or peptides produced during digestion could have had positive effects on the absorption of vitamin E, such as via improving micellization in vivo. Of note, fat-free milk alone may have limited effects on vitamin E bioavailability from supplements. Indeed, vitamin E absorption drastically increased only when the supplement was consumed together with breakfast cereals.[149] This suggests that stomach emptying plays a role, as too rapid transport through the gastric phase (and perhaps intestinal phase also) would limit the time of food processing during digestion, including any potential positive influence that proteins or peptides could have on vitamin E micellization.

In summary, there are indications that proteins, such as obtained from milk, can aid in the absorption of vitamin E by improving micellization. However, factors such as gastric retention time or transit time through the GI are also expected to play a role.

4.1.3. Vitamin A

Vitamin A active compounds include retinol, retinal, retinoic acid and retinyl esters. Also, provitamin A carotenoids are a source of vitamin A, following their cleavage by β-carotene oxygenases, but the later compounds will be considered in the chapter dealing with carotenoids. Food matrices containing preformed vitamin A include only animal-derived food items, the normally predominant form being retinol and its esters such as palmitate.[150] However, supplements can also contain retinyl acetate.

The log$P$ value of retinol (5.68,[151]) is somewhat lower than that of the other fat-soluble constituents, indicating that the compound is more polar. Absorption via the lymphatic system predominates over uptake via the portal system when log$P$ values are above approximately >5.[150] Preformed vitamin A has been shown to be highly absorbed, at a level around 90%.[152] Thus, there may not be much additional room for further enhancing bioavailability. However, improving gastric stability could play a role, as losses of approximately 25%–35% have been reported for vitamin A after incubation in gastric juices during 2 h (reflecting the transit time of more heavy meals), with losses being higher for the alcohol form compared to retinyl acetate.[153]

It is known for some time that vitamin A can form complexes with proteins. For instance, retinol is incorporated in casein,[154] fixed via binding to hydrophobic amino acids (tryptophan, phenylalanine).[155] The same has been emphasized for retinyl palmitate and casein micelles, which were acting as nanovehicles rich in cavities and channels, whereas BSA, β-lactoglobulin, and α-lactalbumin showed less binding affinity.[156] Similar positive effects have been reported for other lipophilic constituents, such as vitamin D2 and curcumin, though whether this is related to higher bioavailability is not known. Casein micelles were typically degraded in the gastric phase of digestion and the small intestine, liberation in the small intestine is to be assumed.[157] Four main types of proteins (α_{s1}, α_{s2}, β, and κ) belong to casein family, all possessing hydrophobic and hydrophilic parts in their molecule.[158] These proteins are highly surfactant, aiding to stabilize o/w emulsions, especially when heated, they appear to show high binding affinity to lipophilic dietary constituents. Depicturing the use of this property, the review by Sadiq et al. described the use of casein micelles in earlier studies for the encapsulation of resveratrol, curcumin, β-carotene, and vitamin D2/3, among others.[158]

Rana et al.[34] investigated various casein complexes with vitamin A for their cellular uptake by a Caco-2 cellular model following GI digestion with milk. These included caseinate-vitamin A complexes, modified succinylated sodium-caseinate (SC) complexes, reassembled SC complexes, and reassembled succinylated SC complexes with vitamin A. It was found that the total cellular uptake was highest from the latter complex, followed by the reassembled SC complex, control milk, and then the other two complexes, whereas free vitamin A was least well taken up. The authors argued that the more hydrophobic regions exposed during the processing of the reassembled caseinates caused better binding of vitamin A and improved its stability (especially against

the low gastric pH), and thus resulted in higher bioaccessibility and higher cellular uptake into Caco-2 cells.

Encapsulation by more sophisticated means, such as by liposomes together with β-lactoglobulin, has also been carried out, as well as absorption tested in mice.[36] It was shown that β-lactoglobulin improved encapsulation efficiency of vitamin A and improved its digestive stability. However, bioavailability in animals was lower compared to vitamin A dissolved in oil, possibly due to slower release kinetics. For a more in-depth overview on encapsulation techniques for vitamin A, the reader is referred to more comprehensive reviews.[159,160]

Liu et al.[161] studied the absorption of vitamin A from various complexes in a rat gavage study. For this purpose, following washout periods, animals received either vitamin A (retinyl-palmitate form) in oil, complex of vitamin A and β-lactoglobulin, vitamin A in oil + skimmed milk, or complex of vitamin A and β-lactoglobulin combined with skimmed milk for prolonged times (up to 4 weeks). It was shown that the complex form with β-lactoglobulin, prepared by a rather simple mixing technique, had similar bioavailability than the oil-based vitamin A form, as determined by serum and liver retinol measurements. Similar findings were encountered after heat treatment, suggesting high bioavailability of β-lactoglobulin complexes with vitamin A, even in the absence of lipids. Contrarily, negative findings for vitamin A absorption when complexed by β-lactoglobulin were found in an animal study by Mensi et al.[19] In their study, retinol was incorporated into β-lactoglobulin by mixing, and force-fed to mice. When compared to retinol incorporated into emulsions (based on peanut oil and PBS, prepared by sonication), plasma levels were much lower. It is possible that the additional oil aided in the better absorption. Interestingly, β-carotene was also given in both formulations, and here β-carotene showed comparable absorption from the β-lactoglobulin complex versus the emulsion, perhaps indicating that very apolar molecules (as in the study above with retinyl palmitate) may benefit more from protein-complexes than the more polar forms. The authors speculated on lower stability of retinol when bound to β-lactoglobulin than when present in the emulsions, but the reasons for these differences remain unclear.

Already several years ago, β-lactoglobulin was described to complex retinol,[162] in addition to having good stability at low pH and being fairly resistant to proteolysis in the stomach. Also, its potential to increase intestinal uptake has been mentioned over 30 years ago.[163] Said et al. studied the effect of β-lactoglobulin added at a 2:1 molar ratio to retinol, on the intestinal uptake of the latter, by the everted sac technique in suckling rats. Significant higher absorption was found in the small intestine when β-lactoglobulin was added, while other proteins, that is, serum albumin and lactoferrin, had no effect. It was speculated that β-lactoglobulin shares structural and conformational similarities with retinol-binding protein, which may suggest the existence of a protein-ligand receptor that could also recognize β-lactoglobulin at the brush border membrane of the enterocyte, leading to a specific improvement in the intestinal uptake of retinol.

In summary, despite the relatively high polarity and the relatively high absorption of vitamin A compared to other lipophilic constituents, emulsions, and encapsulated forms of vitamin A by means of proteins, especially by β-lactoglobulin and caseinate, improve the bioaccessibility and bioavailability of vitamin A, possibly with differing degrees depending on vitamin A form (palmitate versus nonesterified retinol). These proteins may act by offering protection during the GI passage, though a more limited release in the gut may in part counteract such beneficial effects.

4.2. Carotenoids

Carotenoids belong typically to the category of tetraterpenoids. They are lipophilic isoprenoid compounds synthesized by photosynthetic organisms and some nonphotosynthetic prokaryotes and fungi. Animals (except for certain aphids[164]), including humans, cannot synthesize carotenoids and diet is the only source. Dietary intake or circulating levels of carotenoids are recognized as being related to the prevention of several human chronic diseases,[165] such as lowered risk of coronary heart disease, type 2 diabetes,[166] certain types of cancer,[167] and even total mortality.[168] These correlations may be attributed to their antioxidant and anti-inflammatory activities,[169] as well as their influence on the immune system,[170] both possibly related to the expression of a number of transcription factors and nuclear receptors, such as NF-κB[171] and RAR/RXR,[172] respectively. In addition, many studies have been focusing on provitamin A carotenoids (such as β-carotene, α-carotene and β-cryptoxanthin), in the prevention and control of vitamin A deficiency. Following human digestion and absorption, provitamin A carotenoids can be enzymatically cleaved by β-carotene 15,15′-oxygenase 1 (BCO1) and/or by β-carotene 9′,10′-oxygenase 2 (BCO2) in the enterocytes,[173,174] to yield vitamin A. In addition, nonprovitamin A carotenoids such as lutein and zeaxanthin have been reported to accumulate in the macula of the human retina, and are considered to offer protection against free-radical and blue light induced damage that can cause several eye diseases, such as age-related macular degeneration and cataract.[175]

Despite their nonessentiality, much attention has been dedicated to determine carotenoid dietary intake, which has been estimated around 10–15 mg day^{-1}.[176,177] However, due in part to their poor water-solubility (e.g., the log$P$ for lutein = 7.9, and β-carotene = 17.6[178,179]), the concentration of carotenoids (the most abundant ones being lycopene, β-carotene, α-carotene, β-cryptoxanthin, lutein, and zeaxanthin) in human serum is fairly low and highly variable, estimated between 0.33 and 5.78 μmol L^{-1} in healthy European adults.[180] In fact, several dietary as well as host related factors have been reported to influence carotenoid bioavailability aspects, including their bioaccessibility and absorptive processes. For instance, dietary lipids have been emphasized to foster carotenoid micellization and enhance their absorption,[181,182] while fibers were shown to limit the enzymatic access on lipid droplets and thus decrease the micellization of carotenoids.[183] Similarly, divalent minerals at high concentrations were described to negatively influence carotenoid bioaccessibility, likely by precipitating fatty acids and bile salts in the gut.[184,185]

4.2.1. Studies Based on In Vitro Experiments

Recently, several studies have reported the use of proteins for designing functional foods, with the aim to improve the stability

of carotenoids during the storage of food items and thus shelf-life, as well as to enhance their absorption. For this purpose, emulsification of carotenoids and encapsulation techniques have been employed, as reviewed previously.[27] For instance, corn oil as the dispersed phase (5% or 10%) was homogenized with 2% SC in a microfluidizer to prepare a delivery system of β-carotene (0.1% in corn oil).[186] The results showed that the emulsions were stable to coalescence or flocculation over 30 days, similar to those obtained in another study describing that the encapsulation of carotenoids is a very effective strategy to improve their chemical stability under common processing conditions and also storage.[187] In addition, the nanoemulsion delivery system was postulated to modulate the release kinetics from the protein-carrying system and to enhance the solubility of lipophilic compounds.[186] The incorporation of an in vitro digestion process of different SC-based emulsions, preceded by different homogenization pressures (10–100 mPa) produced samples of various droplet diameters (124–368 nm). The relatively small particle size of these droplets resulted in an improved bioaccessibility of the encapsulated β-carotene at the end of the GI digestion,[186] compared to the conventional emulsions (>200 nm). This showed that SC can be used for the design of effective delivery systems for encapsulating carotenoids and other lipophilic bioactive components, similar to a previous report.[188] In another study, 1% of SC or Tween 80 dissolved in water were homogenized with 10% sunflower oil containing 0.2% β-carotene. The produced emulsions were subjected to in vitro digestion followed by a Caco-2 cellular uptake. The authors reported that SC-stabilized emulsions enhanced the bioaccessibility and facilitated the uptake of β-carotene, compared to Tween 80-stabilized emulsions.[189] Apparently, fat droplets remained small and stable against coalescence throughout the digestion process, and could rapidly be processed further into mixed micelles.

The impact on lipolysis changes and subsequent release of β-carotene were also investigated during in vitro digestion, for example, based on an o/w emulsions stabilized with SPI.[190] The emulsion was prepared by dissolving 0.1% β-carotene in soybean oil, of which 10% were homogenized with a protein solution (1.5% of SPI dispersed in water). Significant interfacial changes were reported as indicated by the increased negative charge (zeta-potential) of the oil droplets (−40 to −70 mV), and a significant increase of lipolysis efficiency was found, resulting in a maximum lipid hydrolysis and a transfer of about 50% of β-carotene to the mixed micelles. Similarly, Zhang et al. have characterized the impact of SPI structure modification on the physicochemical properties of the emulsion and its oxidative stability. Carotenoids from *L. barbarum* (1% from boxthorn) were dissolved in medium-chain triglyceride oil, to which 1%–9% of a protein solution was added (1% of SPI dispersed in PBS). SPI generated short peptide chains, with a better amphiphilic property, as shown by decreased interfacial tension and the size of the emulsion droplets, compared to other small-molecule surfactants (Tween 80).[191]

A commonly investigated protein type, WPI, was used in another study to investigate the influences of interfacial structure on the physical properties, bioaccessibility, and microstructure changes of high pressure homogenized β-carotene emulsions during in vitro digestion.[192] Emulsions were prepared with 10% medium-chain triglyceride oil containing β-carotene (0.15% in the final emulsion), which was further homogenized with different aqueous emulsifier solutions (4% of WPI or decaglycerol monolaurate [ML750]). Size distributions of these emulsions differed from one to another. The authors stated that WPI-stabilized emulsions showed a higher absolute ζ-potential (-36.4 ± 0.65 mV) compared to those stabilized by ML750 (-15.9 ± 0.46 mV) following GI digestion, offering stronger electrostatic repulsion and thus higher stability against coagulation. In addition to these changes, WPI-stabilized emulsions resulted in an increased micellization of β-carotene, reaching approximately 86% versus 30% for ML750-stabilized emulsions. Similar findings were reported by Lu et al. regarding the influence of WPI (1% dispersed in water) on the bioaccessibility of β-carotene (0.2% dissolved in sunflower oil) following in vitro GI digestion.[189] β-Carotene loaded emulsions with WPI resulted in improved bioaccessibility of β-carotene, that is, 60% versus approximately 40% for Tween 80-stabilized emulsions. The effects of WPI on the GI processing of oil droplets were also investigated. Significant differences in lipid droplet size of emulsions stabilized by WPI or Tween 80 were observed, that is, WPI-stabilized emulsions were more negatively charged (−53 mV) versus those stabilized by Tween 80 (−25 mV). This was mainly attributed to the protein, being negatively charged at pH (7.0), which is higher than its p*I* (pH 4.0–5.0). The authors showed a higher rate and extent of lipid digestion for WPI-stabilized emulsions, likely attributed to an increased lipid surface area exposed to pancreatic lipase. Similarly, in one of our previous studies using simulated GI digestion, the addition of WPI at various concentrations (equivalent to 0%, 25%, and 50% of RDA or up to 15 mg mL^{-1} digesta) positively influenced the bioaccessibility of β-carotene (30 µg dissolved in peanut oil) under more complete GI conditions, such as higher peristalsis energy or when reducing the amount of dietary lipids. However, negative effects on β-carotene bioaccessibility were noticed at lower enzymatic activity of pancreatin, lower concentration of bile, and reduced peristalsis,[31] in line with negative effects of undigested lipids on carotenoid bioaccessibility.

Differences between various proteins have been observed on their influence on processing carotenoids from lipid droplets into mixed micelles. In a previous study, we have reported that various proteins (WPI, SPI, SC, and gelatin), added at different concentrations to the digesta (0, 3, 7.5, or 15 mg mL^{-1} of digesta, equivalent to 0%, 10%, 25%, and 50% of RDA), had both positive and negative effects on the bioaccessibility of isolated carotenoids (β-carotene, lycopene, and lutein).[22] β-Carotene bioaccessibility was greatly enhanced in the presence of SC, compared to WPI and gelatin, while SPI strongly decreased carotenoid bioaccessibility versus the control (no protein added). On the one hand, bioaccessibility depended on the type of carotenoids, with a higher polarity of carotenoids being related to stronger negative effects on their bioaccessibility, that is, while the bioaccessibility of β-carotene was enhanced by up to 189%, the bioaccessibility of lutein was decreased by up to 50%. On the other hand, bioaccessibility also depended on the type and concentration of proteins, where their digestibility and hydrophobicity were related to their emulsifying properties. Experiments on proteolysis revealed that all protein types employed in the study were completely digested after complete GI digestion, except for SPI, for which some low-size polypeptide fragments remained, while lipid digestion did not differ significantly between the GI digesta of various protein

types. Other physicochemical parameters were also compared. The absolute changes in surface tension remained rather small, while the presence of proteins in the simulated GI digestion remarkably reduced the size of the emulsions, as well as the average of the absolute ζ-potential in a dose-dependent manner for WPI and SPI, while a significant difference for SC and gelatin was found only at higher concentrations equivalent to 25% and 50% protein RDA. In general, it was found that higher protein concentration correlated positively with carotenoid bioaccessibility, smaller micelle size, decreased repulsive forces, and higher surface tension. Overall, the addition of proteins during GI digestion influenced lipolysis, and impacted digesta characteristics such as surface tension and macroviscosity, which in turn could influence emulsion stability, repulsive forces, mixed micelle size, all of which could also impinge on carotenoid bioaccessibility, constituting an important stage in their bioavailability.[22]

Further interactions of carotenoid-rich food matrices with proteins were investigated, employing mixed diets containing various types of proteins (including WPI, SPI, SC, and gelatin), as well as proteins within turkey and cod, at protein concentrations equivalent to 0%, 10%, 25%, and 50% of RDA.[13] Overall, the effects of proteins from regular food matrices (turkey, cod) were more limited regarding altering carotenoid bioaccessibility, while matrix-free proteins improved the bioaccessibility in matrices rich in nonpolar carotenes (such as tomato juice), compared to matrices rich in more polar xanthophylls (carrot juice, spinach). Negative interactions apparently occurred at the o/w interface between xanthophylls and proteins with a high surface hydrophobic nature such as SPI, while improved β-carotene micellization (close to three-fold) suggested a positive interaction with carotenes, fostering their incorporation into mixed micelles.[13] It was speculated that xanthophylls, residing at the outer layer of the micelles, would be more prone to interact with surface-active proteins, resulting in reduced concentrations in the mixed micelles (Figure 2).

Similar to what was encountered for bioaccessibility, the presence of proteins improved the cellular uptake of carotenes (β-carotene, up to 33%) as well as xanthophylls (lutein/zeaxanthin, up to 12%). Interestingly, this was also observed from matrices with an initially poor carotenoid micellization (i.e., tomato juice), especially in the presence of soluble WPI and SC. Though bioaccessibility may have been compromised for xanthophylls as compared to carotenes, it is possible that improved transfer of xanthophylls from the outer layers of the mixed micelles to the cellular surface counterbalanced this negative impact, and/or that peptides facilitated the interactions of the mixed micelles with the cellular surface of Caco-2 cells.[13]

4.2.2. Studies Based on In Vivo Trials

Although carotenoid micellization is assumed to be a key step for carotenoid bioavailability, and is a frequently measured parameter when using in vitro models, it needs to be proven that similar interactions occur in vivo, that is, that the presence of proteins can influence the efficiency of carotenoid absorption. In our recent human study, we reported the effect of WPI and SPI at a concentration of 50% of the RDA (about 30 g), on postprandial absorption of carotenoids from a carrot–tomato juice mixture, as determined by their plasma triacylglycerol-rich lipoprotein (TRL) levels.[14] The absorption kinetics (i.e.AUC) of carotenoids and triacylglycerols (TAGs) were measured in 24 adults at timed intervals up to 10 h after test meal intake, and WPI-supplemented meal increased carotenoid/TAGs AUC significantly by 45% compared to control, and by 57% compared to SPI-supplemented meal, while the latter did not differ significantly compared to control meal. AUC for total carotenoids (nmol h L^{-1} ±SEM) was significantly higher for the WPI-supplemented meal (249 ± 7.0) versus the SPI meal (124 ± 7.0). Similarly, significant differences on the postprandial plasma AUC of TRL-carotenoid concentrations (nmol h L^{-1}) between WPI and SPI for individual carotenoids, that is, phytoene (160 ± 3.3 versus 82 ± 3.3), phytofluene (55 ± 0.9 versus 31 ± 0.9), and α-carotene (14 ± 0.6 versus 3 ± 0.6) were observed.[14] This was the first study reporting an effect of added proteins on carotenoid absorption in humans, confirming the previously reported in vitro results, pointing out that a well digestible protein such as WPI could be beneficial for carotenoid bioavailability, whereas the less digestible SPI resulted in hampered bioavailability of carotenoids. The lower digestibility of SPI versus WPI has been earlier emphasized, for instance in studies focusing on sport supplements, with SPI showing significantly slower breakdown into amino acids.[2]

Human trials studying the influence of proteins on the bioavailability of carotenoids are rare. However, proteins have been studied on their impact on TAGs, which could also be interesting and relevant. In fact, as TAGs are fostering carotenoid sequestration into chylomicrons, and also later lipoprotein sequestration by the liver, it can be speculated that affecting plasma TAG levels also influence circulating carotenoid concentrations to some extent.[193]

It is noteworthy that in our recent study stated above, the lipemic response corresponded with carotenoid absorption. Indeed, AUC values for TAGs (mg h dL^{-1} ±SEM) were also significantly higher for the WPI-supplemented meal (332.5 ± 9.6) than for the control meal (196.3 ± 9.6), while SPI-supplemented meals did not differ significantly from the control (276.2 ± 9.3). In another study, Wang et al. examined the effect of different protein sources on TAGs in hypercholesterolemic subjects undergoing a 6-week randomized cross-over trial.[194] Diets contained either SPI or common sources of animal protein (25 g/1000 kcal), adjusted for their fatty acid profile to be similar across diets. SPI reduced TAGs by 12.4%, total cholesterol by 4.4%, and LDL cholesterol by 5.7%, compared to animal protein diet, demonstrating that dietary protein type may modulate circulating TAG and cholesterol levels in hypercholesterolemic individuals. Such effects were explained either by the protein fraction itself or also by co-occurring soy-isoflavonoids. Other studies have reported the influence of reducing protein intake on the level of plasma TAGs. In a randomized controlled clinical trial, Treviño-Villarreal et al. have shown that patients receiving 4–6 weeks of protein restricted diets (7%–9% of energy from protein) significantly decreased plasma TAGs, compared to controls continuing their usual diet (n = 19 per group).[195] The authors speculated that protein restriction mediated the increase in *APOA5* expression, which plays a role in facilitating TAG hydrolysis from lipoprotein particles by stimulating lipoprotein lipase activity in the periphery.[196] The findings suggest that absorption of both carotenoids and TAGs can be significantly impacted by the

presence of proteins.[14] However, care should be taken to extend results from long-term trials on TAGs to short, that is, postprandial bioavailability results of carotenoids. Nevertheless, such long-term protein intakes may influence also circulating carotenoid concentrations, as well as other lipophilic concentrations such as those of vitamins, also associated with circulating lipoproteins.

In summary, due to the relatively poor water-solubility of carotenoids, the use of proteins as emulsifiers and encapsulants has proven to be an effective target for their incorporation in o/w emulsions, and for enhancing several carotenoid bioavailability aspects, such as micellization and cellular uptake. However, considering the wide polarity range of carotenoids (polar xanthophylls versus nonpolar carotenes), a special attention should be given to the structural differences between proteins and their effect on carotenoid micellization. Although in theory smaller sizes, higher flexibility, high amphiphilicity, and solubility over a wider pH-range warrant good emulsifying properties of a protein, their final effectiveness to enhance bioavailability should generally be confirmed in vivo. Thus, further investigations on the complex interactions of coingestion of proteins and carotenoids are necessary in sight of the potential health-associated benefits of this group of bioactive plant metabolites.

4.3. Curcumin

Curcumin (diferuloylmethane), an active component of turmeric (belonging to the ginger family), has been associated with anti-inflammatory, antioxidant, anticancer, and antiviral properties, as well as improved epithelial function.[197] It has been used as a spice (e.g., within curry) for centuries in the Indian subcontinent and Southeast Asia, also for the purpose of treating respiratory-, liver- and GI diseases, as well as sinusitis, wound healing and pain relief.[198] The properties of curcumin have been investigated in many studies, and findings have suggested that curcumin suppresses the NF-kB activity and thus proinflammatory downstream genes.[199] Curcumins may also quench free radicals, initiate the activity of intracellular enzymes such as catalase, superoxide dismutase, and glutathione peroxidase,[199] and most notably has been shown to induce apoptosis and inhibit the proliferation of cancer cells.[200,201]

In recent years, a large volume of research has been carried out on curcumin due to its functional mechanisms concerning health. In addition to studying its dietary intake, curcumin supplements have been designed and recommended in some cases. However, its absorption and bioavailability are typically low. Its log*P* value of approximately 3.3 makes it poorly water soluble,[202] and absorption at least in part via mixed micelles is likely.[203,204] However, compared to liposoluble vitamins and other secondary plant metabolites such as carotenoids, its distribution in the aqueous phase is higher. Curcumin, when administered orally is to a good extent excreted in the feces (approximately 90% according to rat study,[205]). Earlier studies in rats based on stable isotopes have indicated an absorption of 60%–66% (regardless of the dose administered),[206] though the relative low bioavailability is also due to extensive metabolism, following absorption and re-excretion into the gut via bile.[207]

Curcumin is also sensitive to the acid milieu in the stomach. For instance, in free form at pH 3.6, about 80% was degraded after 20 min,[208] though losses in food matrices are possibly much lower, for example, 11% from a yogurt-based food items.[209] Indeed, proteins such as albumin are known to increase the stability of curcumin and prevent autoxidation,[210] which could also stabilize curcumin during digestion.

Various dietary factors such as fats, proteins, and polysaccharides have been reported to affect curcumin absorption and availability.[202] An interesting study was conducted by Fu et al.,[209] incorporating curcuminoids in a buttermilk-based yogurt (300 mg/100 g), which improved bioaccessibility 15-fold compared to that of curcuminoids in aqueous buffer, though bioaccessibility was still low with 6.2%. In another study, Qazi et al. examined the influence of two dairy gel matrices (i.e., containing oil and dairy protein) prepared by either coagulation with rennet or acid, on lipid digestion and the bioaccessibility of a fortified curcumin nanoemulsion during GI digestion.[211] The emulsions were prepared by mixing for 2 h the lipid fraction that included 0.03% curcumin, and the aqueous phase containing 1% SC. Both gels had the same composition and rheological properties, but the acid gel underwent faster disintegration under gastric conditions compared to the rennet gel, which slowed the release of oil droplets and protein in the digesta, and the content of curcumin-enriched oil in the digesta of the acid gel was higher than that in the digesta of the rennet gel. This resulted in greater digestion of lipids and higher bioaccessibility of curcumin in the acid gel, emphasizing the importance of the presence of proteins toward the protection of curcumin and its role in solubilization of food bioactive compounds. Similarly, Zou et al. investigated the impact of SC on curcumin bioaccessibility and its stability in nanoemulsion delivery systems.[212] Adding casein to these systems did not significantly improve particle size, proposing that caseinate did not prevent inevitable droplet aggregation. However, SC did influence the zeta-potential in the systems, being highly negative (i.e., −23 versus 6 mV for the control), which would be in line with more stable droplets. In addition, the presence of SC in the nanoemulsion prevented the chemical degradation of curcumin under simulated GI tract conditions, and increased curcumin bioaccessibility (78.3% ± 1.1% versus the control 61.8% ± 9.3%).[212]

Vijayan et al. examined complexes of 0.5 mg mL^{-1} of PPI as well as WPI formed with curcumin (1% w/v added to protein solution), to overcome the low aqueous solubility and limited bioaccessibility of curcumin.[213] The WPI-curcumin complex showed better aqueous solubility for curcumin than the PPI-curcumin complex at pH 7, all of which would be relevant for digestion in the GI tract. Despite this difference, both proteins showed a similar high bioaccessibility of curcumin from their respective complexes (approximately 78% for WPI and 72% for PPI) compared to control (approximately 18%). The PPI complex was regarded as a good nondairy protein alternative for curcumin delivery, especially at higher temperature of 80°C.[213]

As another nondairy alternative, Tapal and Tiku investigated the complexation of SPI with curcumin (5% w/v of protein solution) and its implications on the solubility and stability of curcumin,[214] stating that SPI can form a water-soluble complex with curcumin, which increased solubility of curcumin by almost 812-fold.[214] Fluorescence spectroscopy of the SPI–curcumin

complex revealed that the complex was formed through hydrophobic interactions, for which SPI seems especially suitable due to its hydrophobic nature.[214,215] In addition, stability studies by UV-spectroscopy showed that over 80% of the curcumin was stable in the SPI-curcumin complex in gastric and intestinal fluids for 12 h, which would provide sufficient time for GI passage. The SPI–curcumin complex, using various protein concentrations (5, 10, 15, 20 mg mL^{-1}), exhibited further enhanced antioxidant activity and could form foams and emulsions, indicating its possible utilization in food products.[214] Another study characterized SPI–curcumin complexes, by adding curcumin at a ratio of 1:50 to SPI solution (5% w/v). The complexation remarkably increased the solubility of curcumin in the aqueous phase, and greatly enhanced curcumin bioaccessibility in vitro, compared to free curcumin in water (60% versus 90%), following digestion.[216] However, a recent study involving spray-drying microencapsulation of curcumin in SPI[217] showed that spray-drying remarkably decreased the bioaccessibility of encapsulated curcumin, possibly associated with the aggregation of the nanocomplexes.[217]

Kadam et al. examined the effect of *Lepidium sativum* (garden cress) protein hydrolysate (LSPH) on curcumin in vitro stability and bioaccessibility.[218] The complex generated by stirring and a high-speed homogenizer improved the aqueous solubility of curcumin by almost 850 times. In addition, in vitro GI digestion showed that the bioaccessibility of curcumin increased from 67% to 95% post complexation. It was proposed that the LSPH–curcumin complex had a competent foam-forming capacity and emulsion stability and could act as a superior lipophilic bioactive delivery vehicle in food formulations.[218]

Many studies have focused on generating more sophisticated encapsulation vehicles for curcumin. Dai et al. examined the stability and bioaccessibility of curcumin encapsulated by a protein-polysaccharide-surfactant ternary system.[219] The authors examined the bioavailability aspect of curcumin (Cur, containing various amounts between 25 and 250 mg) complexed with either zein (Z, 1% dissolved in 70% ethanol–water solution w/v), Z and propylene glycol alginate (P, 0.2% dissolved in 70% ethanol–water solution w/v v), Z-P and rhamnolipid (R, 1% added to the mixture w/v), or Z-P and lecithin (L, mass ratio of Z to L 5:1).[219] Several methods of characterization were employed post in vitro GI digestion, as a measure of curcumin bioaccessibility.[219] Ternary protein–polysaccharide–surfactant complexes had a higher encapsulation efficiency than simple zein nanoparticles or binary zein–polysaccharide complexes, that is, Z-Cur (21%), Z-P-Cur (67%), Z-P-R-Cur (92%), and Z-P-L-Cur (94%). The ternary complexes also enhanced the photo-stability and bioaccessibility of curcumin, that is, Z-Cur (29.1%), Z-P-Cur (48%), Z-P-R-Cur (82.5%), and Z-P-L-Cur (87.6%), possibly via offering protection against degradation and improving the solubility of the complexes.

Human absorption studies are scan. Li et al. investigated the absorption mechanism of curcumin using Caco-2 cell monolayers.[29] β-Lactoglobulin protein fraction and WPI-stabilized nanoemulsions (1 g of protein dispersed in 100 mL of PBS at pH 7) were used as carriers of curcumin. The effect of various carriers on the cellular permeability of curcumin was determined. The pH stability of curcumin was significantly enhanced and water solubility improved by binding to β-lactoglobulin. As shown by SDS-PAGE, the β-lactoglobulin-curcumin complex and WPI-stabilized nanoemulsion were resistant to gastric pepsin digestion, but sensitive to trypsin as present in the small intestine, suggesting a release in the latter phase of digestion. In the permeability model, the digested WPI-stabilized nanoemulsion and β-lactoglobulin–curcumin complex significantly improved the permeation rate of curcumin through the epithelium, by about 21%.[29]

Similarly, Jayaprakasha et al. investigated curcumin nanoencapsulation with WPI in vitro for colon cancer chemoprevention.[220] Intracellular (SW840, colon cancer cell line) presence of curcumin increased by up to 30% for nanoencapsulated (prepared by a desolvation method) compared to plain curcumin, suggesting that such protein-based formulations may find their way into clinical practice.

In summary, despite the better water solubility compared to, for example, liposoluble vitamins, curcumin administered together with proteins such as caseinates or β-lactoglobulin has shown to enhance bioaccessibility, mostly via increased stability, and in part higher solubility versus the free form. Nevertheless, human bioavailability, determined largely by further metabolism such as glucuronidation and sulfation, has thus far not been shown to be improved by proteins.

5. Summary of the Main Emulsifying Properties of Proteins

Despite the various applications of proteins and the mechanisms by which they are involved in potentially enhancing the bioavailability of fat-soluble vitamins as well as apolar secondary plant metabolites, the findings indicated that positive effects are essentially linked to their emulsifying activity. Several protein properties have been described to play a role in the capacity of proteins to penetrate, unfold, rearrange, and form an interfacial film at lipid droplet surfaces that can foster emulsion stability.[221] These properties include sufficient surface hydrophobicity, low molecular size of the protein, high amphiphilicity, solubility, flexibility/digestibility, and surface charge (pH and ionic strength). Indeed, these protein properties could translate into main effects influencing the bioaccessibility of lipophilic compounds, especially by altering aspects of lipid droplet stability and processing in which they are solubilized (Figure 1), and their incorporation into mixed micelles (Figure 2), but could also be linked to direct interactions with apolar constituents via exposed hydrophobic pockets during digestion, related to solubility and prevention of oxidation of these liposoluble compounds.

Although many of these properties can result in a beneficial effect on bioaccessibility, protein properties may be more akin to a double edged sword, with also possible negative consequences. Indeed, surface hydrophobicity should be considered with attention, especially for proteins with low digestibility.[222] For example, the high hydrophobicity of the β-lactoglobulin fraction of WPI,[223] as well as its resistance to pepsinolysis,[224] was reported to allow the adsorption of large peptides to the surface of lipid droplets through the exposition of hydrophobic groups, offering stability and protection to the solubilized carotenoids in the acidic environment of the gastric digestion. However, the adsorption of large molecules at o/w interfaces could turn into

a disadvantage, such as constituting a biological barrier to digestive enzymes,[22] rendering the emulsions stabilized by β-lactoglobulin more resistant to lipolysis,[101] thus interfering with mixed micelle formation and hampering the bioaccessibility of lipophilic compounds.[59]

Similarly, protein solubility is essential to ensure a rapid adsorption to o/w interfaces.[222] Therefore, proteins with high solubility such as SC and gelatin were shown to be involved in the formation of emulsions with more dispersed oil droplets, due to an increased charge repulsion at a wider pH range (away from the protein's p*I* and/or under low ionic conditions),[225] enhancing the bioaccessibility of, for example, carotenoids.[22] However, the often low solubility of plant-based proteins, such as SPI, appears decisive for their emulsification performance,[98] as this could also lead to high surface hydrophobicity, especially during the structural rearrangement occurring in the acidic environment of the gastric digestion. Results from in vitro protein digestion experiments and analyses performed on SPI-stabilized emulsions indicated that repulsive forces between lipid droplets were reduced, favoring hydrophobic interactions between the adsorbed proteins to lipid droplet surfaces, leading to the formation of a bridged lipid droplet network.[225,226] This negatively impacted the processing of lipids and the incorporation of carotenoids into mixed micelles, and reduced their bioaccessibility.

In addition to solubility and surface hydrophobicity, protein flexibility is believed to be an important dynamic factor governing emulsification.[227] The flexibility of a protein reflects its ability to realign itself at the o/w interface when subjected to proteases such as during digestion (i.e., conformational changes).[221] This ability has been reported to foster the interactions between the lipid and aqueous phases, via hydrophobic and hydrophilic groups, respectively, decreasing the surface tension and assuring the stability of the emulsions throughout the digestion process.[69] α-Lactalbumin, β-lactoglobulin, caseins, and BSA are some examples of flexible proteins, and a good correlation was obtained between the emulsifying activity and the digestion susceptibility by pepsin and α-chymotrypsin.[227] The same findings were reported in our previous work, that is, producing peptides of amphiphilic structure have been especially noticed in more digestible proteins, such as in SC and WPI, following GI digestion.[13] The resulting peptides were beneficial for carotenoids, as the bioaccessibility and the cellular uptake of these lipophilic compounds improved, probably by facilitating the interactions at the surface of the mixed micelles[189] and making them potentially more available for Caco-2 cells.[13]

Finally, proteins of small molecular size, such as those from animal sources, were described to have higher diffusion rates to the o/w interface, compared to larger protein fractions from plant sources (e.g., casein proteins 20 kDa versus soy glycinin 350 kDa),[228] which should offer kinetic advantages that may go along with enhanced bioaccessibility of emulsified lipophilic food compounds. Taking into account all these properties, that is, the tendency of animal-derived proteins to be soluble over a wider pH range, being of relatively smaller size and more flexible than plant-based proteins, appear to make animal proteins a better choice for improving the bioavailability of liposoluble vitamins as well as other apolar food constituents such as carotenoids, as their properties allow a more rapid diffusion to the interface in order to stabilize the lipid phase within an emulsion,[221] resulting in smaller droplet size, eventually facilitating the transition of lipid droplets and contained liposoluble constituents into mixed micelles.[108]

However, due to consumer concerns about ethical, environmental, and health issues (e.g., allergic reactions) associated to some extent with animal-based proteins, the use of plant proteins has been receiving more interest recently, especially from pharmaceutical and food industries, for encapsulation and delivery of the above mentioned lipophilic compounds. Being cheaper, renewable, biodegradable, and amphiphilic material, legume proteins, for example, are a rich source of proteins such as albumins, globulins, prolamins, and glutelins, though with relatively low aqueous solubility and poorer emulsifying functionality compared to animal-based proteins.[221,229] Physical, chemical, and enzymatic modifications have been mainly used to overcome these obstacles, but most of the produced isolates, such as SPI, have undergone a thermal treatment that could lead to denaturation of these proteins, and are therefore present in an aggregated form, with high hydrophobicity and even being insoluble.[98] Nevertheless, the main effects of the produced SPI (i.e., denaturation and aggregation-insolubilization) have been reported to be highly related to the applied concentrations. At low protein concentration, denaturation increases the surface hydrophobicity of the proteins, without distinctly decreasing their solubility, while at high levels, insolubilization processes markedly increase, promoting the formation of aggregates.[98] In line with these observations, Tzoumaki et al. reported that the intermolecular association between adsorbed proteins to o/w interfaces was more favorable at higher protein concentrations, which led to formation of aggregates, thus negatively impacting the stability of the emulsions.[230] These opposing concentration-related effects have also been reported in our previous studies when using SPI during simulated GI digestion. At lower concentrations (equivalent to 10% of the RDA for proteins), SPI enhanced the bioaccessibility of β-carotene, while higher concentrations of 25% and 50% of RDA negatively influenced β-carotene bioaccessibility, compared to no added proteins.[22] Similar findings were obtained when using carotenoid-rich food matrices.[13]

Finally, the choice of the most suitable protein type could also depend on the digestive conditions and the type of lipophilic food compound to be solubilized (e.g., different polarity), which constitutes an additional challenge given the availability of a limited range of well-studied proteins thus far. For example, as studied previously,[22] and in line with results based on drug–micelle interactions (Figure 2), very apolar constituents located in the core of mixed micelles may be differently influenced compared to less apolar compounds situated at the outside of micelles. Regarding digestive conditions, which also can vary significantly from individual to individual,[41] less complete digestive conditions, such as low concentrations of bile, pancreatic enzymes (or surplus of lipids), and also limited peristalsis, could rather foster low bioaccessibility in the presence of proteins.[31]

6. Conclusion and Perspectives

Literature indicates a growing interest in the use of proteins to improve storage aspects and stability of biologically active lipophilic compounds, such as fat-soluble vitamins and carotenoids, by offering a protection against oxidation and

degradation, and enhancing their solubilization throughout digestion processes, also ensuring a better cellular uptake. An array of studies have been conducted, though typically on a rather limited number of proteins, encompassing studies with animal models such as in mice,[129,36,195] a few human studies,[14,130,131,220] and a large number of in vitro investigations. In general, the latter support results on living beings, that is, that proteins could offer stability and protection of sensitive molecules against oxidation, and enhance the solubility and bioavailability of liposoluble constituents.

However, a number of knowledge gaps remain. One of the important aspects to consider is that limited data exists on the interactions between liposoluble food constituents and proteins. For carotenoids for instance, despite their very high hydrophobicity, they are ubiquitous in aqueous cellular environments, often bound to proteins. The orange carotenoid protein, glutathione-S-transferase pi isoform 1, and crustacyanin are examples of carotenoid-binding proteins that have been well characterized,[231] and protein-carotenoid complexes may well form during GI digestion. An in vitro study by Mensi et al. has shown this type of interaction, such as the ability of β-lactoglobulin to bind β-carotene within its interior cavity,[19] which may act as a transporter of hydrophobic carotenoids in an aqueous environment during digestion.

The use of proteins has also been considered as a potent delivery system for effective intracellular delivery of liposoluble vitamins/secondary plant metabolites. However, only very little is known about how the cellular uptake is affected by the interaction of proteins with such apolar compounds. For carotenoids, Lu et al. proposed that protein emulsifiers such as WPI and SC may improve carotenoid uptake by facilitating the interaction of micelles with Caco-2 cells,[189] but detailed mechanistic studies in this area do not exist.

Finally, the release kinetics of fat-soluble vitamins and other apolar compounds such as carotenoids from the food matrix or encapsulated materials are still poorly understood, but can be expected to be crucial for developing effective delivery systems for such lipophilic compounds. Thus, designing studies to understand their release rate under physiological relevant digestive conditions is paramount, as this will allow a better understanding on the influence of various proteins on the timely release behavior of liposoluble compounds, and their processing from their presence within lipid droplets to mixed micelles in the gut.[232] Therefore, further in vitro studies, as well as clinical trials, are desired to better explore protein structure-function relationships and to overcome the above mentioned limitations and gaps to enable proteins to be used in a wide range of fat-soluble bioactive compounds. Indeed, the use of proteins offers encouraging opportunities to enhance the bioavailability of liposoluble vitamins and vitamin precursors (carotenoids) by natural means, and opens further doors for the creation of functional foods.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Funding acquisition, T.B.; conceptualization, M.I., Y.L., and T.B.; writing–original draft preparation, M.I., F.V., D.M., and T.B.; writing–review and editing, M.I., F.V., D.M., Y.L., and T.B.; supervision, Y.L. and T.B. All authors have read and agreed to the published version of the manuscript.

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Mohammed Iddir is a research scholar. His research project aims to provide indications for better absorption of carotenoids, by studying the interaction between these phytochemicals and proteins. Specifically, it focuses on the influence of various protein types and concentrations on carotenoid digestion and their relevance regarding aspects of carotenoid bioavailability, including matrix release, bioaccessibility, cellular uptake, as well as colonic recovery and fermentation of carotenoids. This would be particularly beneficial for subjects affected by gastrointestinal disorders limiting the metabolism of carotenoids, or people not consuming enough preformed vitamin A from meat, such as vegetarians/vegans, as well as people living in developing countries, where meat consumption is relatively limited.

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