ASSESSING THE BIOMOLECULAR INTERACTIONS OF PEA PROTEIN FRACTIONS AND CURCUMIN: IMPACT ON *IN VITRO* PROTEIN DIGESTIBILITY

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Thesis submitted to the University of Ottawa
in partial Fulfillment of the requirements for the
degree of Master of Science Nutrition and Food Biosciences

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DEDICATION PAGE

With all my heart, I dedicate this thesis to God Almighty. To Him alone be all the glory.

…..For by strength shall no man prevail.

1 Samuel 2:9.

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ABSTRACT

Leguminous plant proteins, such as pea proteins, are good alternatives to animal proteins due to their biological and techno-functional properties, which greatly benefit health. Unlike other legumes, they are high in lysine, which improves immunological function. Polyphenols, such as curcumin, have bioactive properties such as anti-hypertensive, antioxidant, and anti-diabetic properties, hereby promoting their effective use in food and nutraceuticals. Despite its significant biological value, the use of curcumin is limited due to water insolubility, rapid cell metabolism, degradation, and instability. Thus, the complexation of proteins and curcumin improves all its limitations and enhances high bioavailability, favoring the development of delivery systems. Furthermore, the protein-polyphenolic complex could facilitate high thermal stability and better techno-functional properties of protein such as foaming, emulsification, gelation, water and oil holding capacity. This study examined the complexation of pea protein fractions (albumin, globulin and glutelin) with curcumin and the influence of this interaction on protein digestibility. Results showed that the complexation was facilitated mostly by non-covalent interactions i.e. hydrophobic interactions. Curcumin tends to form compact structures with proteins which reduces the accessibility of digestive enzymes, thereby resulting in decreased protein digestibility in albumin and globulin and no protein hydrolysis in glutelin - mainly due to the tight sheet-like structures formed. The effect of ionic strength on the water-soluble fraction (pea albumin) and curcumin complexes and the extent of this influence on protein digestibility was investigated. The influence of the physiological condition (ionic strength) of the gastrointestinal tract impacted the characteristics of pea protein albumin-curcumin complexes, however, there were negligible changes in protein digestibility. This was mainly attributed to the formation of large aggregates or unstable complexes with increasing NaCl salt concentration which influenced protein digestibility revealed by dynamic light scattering and fluorescence microscopy. The mode and nature of binding of the curcumin to protein in the presence and absence of ionic strength revealed by fluorescence quenching showed more salt-induced effects despite no significant progress in protein digestion. Binding was more favorable in the absence of ionic strength (0 mM NaCl). These findings offer a novel understanding of the mechanisms underlying the interactions between pea protein and curcumin, their impact on protein digestibility, and provide valuable insights for optimizing curcumin loading in the development of delivery systems, achieving both maximum encapsulation efficiency and curcumin bioavailability, without compromising protein digestibility.

ACKNOWLEDGEMENTS

It’s been two years since I left home in pursuit of better prospects. Time flies. It's so surreal. Just like yesterday when my plane landed at Montreal International Airport. Looking around the airport as I approach the arrivals gate with my heavy luggage. I recall the emotions, or should I say confused sentiments and thoughts that pervaded my mind. "What lies ahead of me?" So, I thought. So, I'm in Canada! Wow! With tears streaming down my cheeks, I realized I wouldn't be seeing my family and familiar faces for a long time. I put on my big girl pants and told myself, "I don't know what's ahead of me, but I'll give it a try. I am going to take a chance and take it one step at a time".

Not to sugarcoat it, but the last two years have been a rollercoaster. Each stage of this voyage had its hue of color. I wholeheartedly agree with the adage, "You won't know until you try." My greatest worry was starting over in a different nation, yet I trudged on. My master's degree could not have been completed successfully without the strategic people God placed in my life. So, with a grateful heart, I express my heartfelt gratitude to God first for everything. I am who I am now because of His grace. For His grace has sustained me thus far. Keeping step with Him wasn't easy, but He held me still. Even when my thoughts are filled with doubts, He never let go. Thank you, God.

To my energetic academic and university anchor, my prestigious supervisor, a man full of wisdom and brilliance, who is meticulous to the core and ever-present. Professor Chibuike C. Udenigwe, your life is a testament. You are my greatest inspiration in this field. Many a time, I aspire to be like you. Thank you for your patience, despite the hassles and heartaches I caused. Thank you for never giving up on me. Thank you for your unwavering support and motivation. Thank you for providing me with the opportunity to explore and learn, as well as for changing my perspective on research and all I do. You taught me that I am capable of more than I believe I am. I got to this point due to your consistent guidance. Thank you again for the constant push when I tend to feel more comfortable or relaxed. "Behind every successful man, there is a great woman". To Dr. Mrs. Ogo Udenigwe, an amazing woman with a big heart. Thank you, Aunty (as I fondly refer to you), for your hospitality, kindness, and care. I do not take this for granted. I will never forget you in life. I also use this medium to thank the rest of your family for taking me in as theirs.

To my family (parents and siblings), I miss you so much. Like my parents would say, “We are all doing this master’s program together” *smiles*. Thank you, Mr. and Mrs. Emmanuel, Ifeanyi, Chuks, and Chinaza Oballa for having my back spiritually, mentally, and emotionally. I remember before coming to Canada, my parents boldly told me never to forget my roots. Distance is not a barrier indeed. Thanks for always connecting with me. I didn’t lose my home training because of you. My family has shaped me into the woman I am now. Thanks for not abandoning me in my world. Thanks for being my constant source of strength. I couldn't have asked for a more perfect family.

To my lab members, thank you Raliat and Joy for your unwavering support in my research works, your peer reviews, motivations, and encouragement. I learned a lot from you girls. Above all, I am most grateful for your friendship. Thank you for being ever-present. I wouldn’t have achieved so much without your guidance. To Seyi, thank you for all you do. I appreciate our friendship. To other members of the lab whom I have met or have gained knowledge in various ways, Dr. Obi, Dr. Matthew, Dr. Ruth, Dr. Ogadinma, Precious, Professor Olabisi, Professor Eze, Leticia, Dr. Ezgi, Dr. Ornella. Thank you.

To George, thank you for holding the fort. (a.k.a My Alarm Clock). Thank you for having my back and showing me so much love. Thanks for your prayers, encouragement, understanding and support. I do not take this for granted. I see you!

To others who have become like family, Dr. Chinonye, thanks big sis for your encouragement and always checking in on me. I sincerely appreciate. To Chika, my amazing big sister, who is always present and loving. Thank you for embracing me as your own.

This piece would not be complete without specially honoring my TAC members, Professors Nicholas Bordenave and Riadh Hammami. Thank you for accepting this position and also for contributing your expertise towards the success of my research works and my program in general.

Also, this research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant, and the University Research Chairs Program, University of Ottawa.

Muchas gracias!

CHAPTER 1 - INTRODUCTION

1.1. Background of the study

The health-promoting functions of proteins and the necessity to reduce the potential risks associated with consuming food products from animal proteins are imperative, hence its increased demands in diets worldwide. Moreover, the production of animal proteins through livestock farming has detrimental effects on the environment, including losses to biodiversity, depletion of freshwater supplies, and climate change.[1] Leguminous plant proteins possess significant nutritional and functional qualities and the environmental and economic sustainability associated with their production and distribution.[2] Furthermore, several research studies have shown that consuming plant-based protein is linked to a substantial reduction in the likelihood of overweight and obesity, type 2 diabetes mellitus, coronary heart disease, and low-density lipoprotein (LDL) cholesterol; in contrast, animal-based protein consumption is generally associated with an increased risk of these health problems given to its lipidic profile.[3] Currently, there exists a diversity of legume proteins, including chickpea, soybean, lentil, lupin, bean, and pea; all of which are intensively studied due to their rich compositions and enhanced nutritional attributes. They are also incorporated into food systems as dietary supplements and functional foods.[4]

Polyphenols are a diverse collection of secondary plant metabolites. Plants produce them to defend themselves against external stressors such as pathogenic aggression and ultraviolet (UV) radiation. They are abundant in vegetables, fruits, legumes, chocolate, coffee, wine, and tea.[5] As components of food, these compounds affect the color, taste, odor, astringency and oxidative stability of food.[6] The phenolic structural characteristics of this class of phytochemicals are typified by the existence of various hydroxyl bonds on a few rings of aromatic compounds with six carbons.[7] The number of aromatic rings and additional structural components (such as hydroxyl and carboxylic groups) that connect the rings allow for the classification of polyphenols into lignins, flavonoids, phenolic acids, tannins, stilbenes, and curcumin.[8,9] Their benefits like antioxidant, anti-hypertension, anti-microbial; on health give them an edge in food applications.

Proteins and polyphenols in particular offer nutrients and biologically active compounds that are advantageous to individual well-being and nutrition, nevertheless their interactions also have an impact on the overall stability and quality of foods.[10] Protein-polyphenol interactions are mediated by non-covalent interactions and covalent bonding to form either soluble or insoluble complexes.

The binding of polyphenols to proteins alters the physicochemical and functional characteristics of proteins including, solubility, temperature stability, and digestibility of protein.[11] In some cases, proteins mask the bioavailability and antioxidant potential of polyphenols.[12] Additionally, the type, structures, and concentration ratios of protein and polyphenols as well as environmental factors like temperature, pH, and salt concentrations also influence the interactions tailored to produce stable protein-polyphenol complexes.[12,13] The effect of the above factors is not consistent among all protein-polyphenol systems.[14] For instance: Tightly twisted, globular proteins exhibit a reduced propensity for polyphenols compared to random coiled proteins.[15] Furthermore, these modifications are investigated and characterized with the use of analytical methods such as spectroscopy metrics, electrophoretic, microscopic, thermodynamic, chromatographic and bioinformatics analysis.[16] Protein-polyphenol interactions have been shown to improve the integrity of food and serve as very effective delivery systems for bioactive compounds, which encourages their incorporation into foods to generate functional foods containing substantial protein and polyphenols.[17]

Taking into account the relevant functions of proteins and polyphenols, a greater understanding of their interactions is required, particularly their impact on the digestibility of proteins since these changes are linked with the conformation and nutritional value of proteins. The digestibility and bioavailability of plant proteins are significant considerations when attempting to attain human nutritional requirements.[18] The nutritional value of a protein food is determined not only by the amino acid profile but also by its digestibility in the gastrointestinal tract.[19] The digestion kinetics of proteins are also impacted by various parameters including temperature, ionic strength, pH, ionic strength, protein source, food processing, and food matrix interactions.

In this regard, the composition, functionalities, relevance, health benefits of pea protein and its fractions, and curcumin's structure and health relevance were described. The underlying mechanisms of protein-polyphenol interactions and the factors influencing these interactions, complexation of plant protein and curcumin, nanoencapsulation of curcumin with plant proteins, changes resulting from protein-polyphenol interactions on the digestibility of plant proteins, some determinants of plant protein digestibility, and the effects of protein-polyphenol food matrix interactions on plant protein digestibility were further discussed.

1.2. Pea protein and its fractions

Pea (*Pisum sativum L.*), a member of the Fabaceae family, is among the earliest commercial crops, grown for human consumption or as a feed for livestock.[20] Peas are predominantly cultivated in Canada, Russia, United States of America, France, China, and Australia, with varying growth and soil conditions.[21] Pea plants can withstand frigid temperatures during the process of germination and growth, making them a viable cool-season option for places unsuitable for bean or soybean development.[20] Pea protein is considerably more sustainable compared to various plant proteins used for commercial purposes owing to its superior efficiency in terms of land, water, and energy use.[22] Interestingly, they are high in lysine, which contributes to their distinct advantage over comparable plant-based proteins such as soybeans. Pea proteins derived from pea seeds are commercialized due to their availability, low cost, environmental friendliness, low allergenicity, and high nutritional content, in addition to their good functional properties.[23] Consuming foods rich in pea proteins is related to several health benefits such as antioxidant, anti-inflammatory, anti-diabetic and antihypertensive among others.[24] The structure and composition of pea proteins are further influenced by the extraction technique, which typically involves the use of both physical and chemical procedures. Various techniques have been suggested for obtaining protein from pea flour, including salt and alkaline extraction.[25] They are divided into four groups according to their solubility and method of extraction: globulins, albumins, glutelins, and prolamins.[26]

1.2.1. Albumin fraction

The 2S seed storage pea protein fraction is water soluble and accounts for approximately 18-25% of pea seed proteins.[26] It is abundant in sulfur-containing amino acids such as methionine and cysteine which also makes it nutritionally relevant in food formulations. It is also regarded as a metabolic and enzymatic protein possessing cytosolic functions that play vital functional roles in the development of seedlings.[27] It comprises enzymes, inhibitors of protease, amylase-inhibiting agents, and lectins with molecular weights that range from 5 to 80 kDa.[28] Albumins are divided into two types based on their tiny molecular weights. PA1a (6kDa) has 53 amino acids, while PA1b (4kDa) contains 37 amino acids.[76] PA1a and PA1b possessed very high cysteine levels, at 7.5% and 16.2%, respectively. Furthermore, PA1b has the potential to function as an insecticide in biological treatments.[20] Also, previous research on pea albumin fraction illustrated good emulsion and foam-stabilizing abilities.[29]

1.2.2. Globulin fraction

Characterized by its solubility in a salt solution, this fraction accounts for 55–65% of total proteins in pea seeds.[26] As the major seed storage proteins, globulins are divided into two classes: legumin (11S), which has an isoelectric point of 5-6, and vicilin (7S), which has an isoelectric point of 4-6, as well as a minor class called convicilin.[23] Legumin, a hexameric protein with a molecular weight of 320-400 kDa, is made up of six subunits (60-65 kDa) that contain both acidic (40 kDa) and basic (20 kDa) polypeptides connected by a disulfide bond.[30] The α and β chains of legumin are connected by disulfide bridges, with the hydrophilic α chain situated at the molecular exterior and hydrophobic regions submerged at the interior to minimize water contact.[31] Also, the α chain is primarily composed of glutamic acid and contains leucine as its N-terminal amino group, whereas the β chain consists mainly of valine, alanine, and leucine and has glycine as its N-terminal amino group.[31] Conversely, vicilin is a 150–180 kDa trimeric protein made up of (α, β, and γ) subunits. It is a mixture of polypeptides that are heterogeneous in nature and lack cysteine residues, which prevents them from forming disulfide bonds.[25,32] It contains a greater percentage of acidic (glutamic and aspartic acids) and basic (lysine and arginine) amino acid groups and a lower percentage of sulfur-containing amino acids (methionine, cysteine), in addition to tryptophan.[33] The third seed storage protein, convicilin is minorly categorized and has a molecular weight of 70 kDa. This protein is relatively low in carbohydrates and has a completely distinct amino acid composition compared to legumin and vicilin.[34] Their existence of cysteine, an amino acid containing sulfur, distinguishes them from vicilin.[31] They share the majority of their protein's homology with vicilin. Nevertheless, they are identified by a strongly charged hydrophilic N-terminal extension domain with 122 or 166 residues.[35]

1.2.3. Glutelin fraction

Glutelin is present in trace concentrations in pea seeds. It is a significant component of the protein compound known as gluten. It is commonly referred to as a class of prolamin-like proteins. It is only readily soluble in weak acids or bases, chaotropic or reducing chemicals, as well as surfactants, and it contains many hydrophobic amino acids, including tyrosine, phenylalanine, valine, and proline.[25] It is sparingly used in food formulations.

1.2.4. Prolamin fraction

Prolamin, a different class of seed storage proteins is high in glutamine and proline contents and is similarly found in modest amounts in pea seeds.[26] Typically, it is only soluble in alkaline, mild

acid, and highly concentrated alcoholic solutions about 70–80%. Prolamin hydrolyzes resulting in ammonia and proline as opposed to coagulating when heated.[20]

1.4. Functionality of pea protein and its fractions

The application of proteins in food products highly depends on their functional properties. Both physical and chemical characteristics of pea proteins can exert enormous effects on their behavior during food preparation, preservation, and usage.[36] Functional attributes are classified according to their mechanism of behavior, structure of proteins, rheological traits, and surface qualities.[37]

1.4.1. Solubility

One techno-functional characteristic of food protein that is frequently studied is protein solubility, which also influences other protein qualities.[38] The solubility of protein can be impacted by several variables, such as protein content, pH, ionic strength, temperature, and the type of solvent.[37] The minimal solubility of pea protein isolate ranges from pH 4-6, indicating a considerable dependence on pH, which could potentially reduce its corresponding functional qualities.[26] Several studies have been conducted to assess the solubility and bioavailability of pea protein, among other functional qualities.[30,39] Based on reports from a particular study, the optimum solubility of pea protein ranges from 20% to 90% for commercial pea protein isolate compared to the variety made in the laboratory. Additionally, the values obtained are comparable to soy proteins.[26]

1.4.2. Water and oil holding abilities

The water-holding ability of a protein is described by the quantity of water absorbed per 1 gram of protein. The amino acid content and ionic strength significantly influence this attribute.[23] Few investigations found that pea protein produced through an alkaline method or isoelectric precipitation has a lower water-holding ability (2.7 g/g) than soy protein. The propensity of proteins to bind to water makes them beneficial in food products like doughs, sausages, puddings, and other foods with insufficient water for the dissolution of proteins. Also, it supports the gelling ability, swelling and viscosity of food proteins.[40] Conversely, oil-holding ability refers to the quantity of oil which may be absorbed per 1g protein. Oil-holding ability values of proteins are influenced by the extraction, structure/matrix, and surface hydrophobicity of proteins as well as the kind, spatial distribution, and stability of lipids present.[25] Understanding the variables that affect this attribute is critical for maintaining the quality of products.[41] It corresponds with food

sensory qualities involving texture and flavor stability.[42] The alkali approach yielded a pea protein isolate with an oil-holding ability of 2.8 g/g, which was below that of soy protein isolate. As a result, it was determined that various extraction procedures could have a considerable impact on the oil-holding ability of pea proteins.[40,43]

1.4.3. Emulsifying properties

Emulsifiers are substances that can generate interface layers within liquids that are immiscible. Proteins are commonly employed as emulsifiers in commercial food production due to their amphiphilicity.[42] Proteins are significant in emulsion generation and stability because of their amphiphilic character and ability to form films.[44] Several parameters may impact the emulsifying capacity of pea proteins, including structure and concentration of proteins, homogeneity of pressure or temperature, viscosity, pH values, and the oil-water protein relationship duration.[27] Albumins have excellent emulsification qualities due to their ability to accumulate within the water and oil interfaces. Nevertheless, its low molecular weight did not make a significant difference to the emulsifying behavior. Vicilin of the globulin fraction forms more stable emulsions, whereas legumins possess higher emulsion capacity. Additionally, it was suggested that vicilin is significant in the emulsifying properties of pea protein isolate.[45]

1.4.4. Foaming abilities

This functionality entails both foaming capacity and stability. This feature encompasses both foaming capacity and stability. The level of interfacial region generated by protein is referred to as its forming capacity while foaming stability refers to the capability of a protein to stabilize foams against stress.[23] The capacity of pea protein to produce foam is affected by a variety of parameters, including the concentration of the protein, temperature, ionic strength, pH values, viscosity and extraction process. Some investigations indicated that salt-extracted pea protein isolates have greater foaming properties compared to alkaline processing.[23] Albumin fraction in pea proteins was recorded to exhibit impressive foaming capacity and stability in comparison with other fractions and sources of protein.[46,47]

1.4.5. Gelling property

Gel formation constitutes one of the most significant functional features of food proteins, especially globular proteins from legumes because it is employed to modify food texture.[25] The gelation of globular protein is an intricate process that involves several steps, including protein

degradation, progressive aggregation, and network-forming ability.[23] Protein gel is usually divided into two distinct categories: the cold-set and heat-induced gel formation of a three-dimensional matrix composition containing fats, water, and other dietary components.[23] They are affected by some factors, including concentration, temperature, ionic strength, pH, additives, and enzymes. The gelation characteristics of pea proteins were observed to be influenced by their type, content, and various processing methods.[48,49] The impact of transglutaminase enzyme on pea protein fraction gelation has been reported in recent research. Globulins, either natural or denatured, respond favorably to gel formation induced by enzymes, in contrast to albumins.[50]

1.5. Relevance of pea proteins in the food industry

Due to its nutritional and functional qualities, pea proteins are incorporated into foods and nutraceuticals.

1.5.1. As dietary supplements

Pea protein supplements are useful for improving nutrition during physical activity and sports. It has well balanced amounts of leucine, isoleucine, and valine, which can support muscular building.[51] A particular study compared the effects of taking oral supplements with pea protein compared to whey protein and placebo on both the strength and thickness of muscles following a 12-week resistance exercise routine. It was shown that the pea protein supplementation led to a greater improvement in muscle thickness than the placebo. On the contrary hand, no significant difference was seen comparing both protein treatments, suggesting that pea protein can serve as a viable substitute for dietary products containing whey.[47]

1.5.2. As encapsulating agents

Proteins are regarded as an appropriate choice for creating various encapsulating systems i.e. nanoparticles due to their unique characteristics, which include their amphiphility, inherent abundance, biocompatibility, biodegradability, and a balanced amino acid profile that encourages protein-nutraceutical interaction.[24] Pea proteins encapsulate lipophilic bioactive substances such as curcumin. Bioactive compounds provide numerous health benefits, including antioxidant, anticancer, and anti-inflammatory effects. However, they are susceptible to pH, light, and thermal treatments; also, their hydrophobic or crystalline composition with limited water solubility could restrict their use in food formulations.[52]

1.5.3. As food emulsifiers

To be an efficient emulsifier, protein needs to be adsorbed to the oil-water barrier and unfolded therein to create a cohesive film surrounding oil droplets via intermolecular associations.[53] Pea protein is utilized as an emulsifier in both spray-dried and liquid emulsions to microencapsulate oil.[54] Some studies suggest that pea protein is a more effective emulsifying constituent compared to soy protein at a neutral pH level.[20] Although proteins have a strong emulsifying ability, the stabilized emulsions by proteins are particularly susceptible to external factors like pH, ionic strength, and thermal treatment.[55]

1.5.4. As edible films

Pea protein, a biodegradable and biocompatible biopolymer, has been intensively explored for utilization in the fabrication of edible or biodegradable films. Generally, the required film exhibits excellent water and gas barrier qualities such as strong mechanical qualities including modulus, tensile strength, reduced oxygen and water vapor permeability. The variety of plasticizing agents, ratio of protein and plasticizer, pH and heat processing affect the film-forming capabilities of pea protein isolate.[56] According to certain research, sorbitol and pea protein together can create films with excellent tensile strength and transparency.[57]

1.6. Potential health benefits of pea proteins

Sufficient intake of diets rich in pea proteins is linked to various health benefits, including antioxidant, anti-fatigue, anti-microbe, anti-hyperlipidemia, anti-obesity, anti-diabetes, anti-hypertension, anti-osteoporosis, anti-renal fibrosis, anti-cancer, immunomodulation, and anti-inflammatory properties.[58] Pea proteins have been shown to lower pre- and post-meal blood glucose levels and to inhibit postprandial glycemia in adults.[59] In humans, hypertension is closely linked to the onset of cardiovascular disease. Given the critical function of the renin-angiotensin system in controlling blood pressure, antihypertensive peptides derived from pea protein were typically described as inhibitors of the angiotensin I-converting enzyme.[40] The lysine content in it boosts the immune system.

1.7. Curcumin (*Curcuma longa*)

Derived from the turmeric rhizomes, curcumin is a naturally existing polyphenolic compound having its orange color due to the presence of organic chemical molecules known as curcuminoids. Demethoxycurcumin of about 10–20%, bis-demethoxycurcumin (3%), and curcumin (75–85%)

which is the most significant bioactive component of turmeric, are derivatives of curcuminoids.[60] Curcumin (1,7-bis (4-hydroxy-3- methoxyphenyl)-1,6-heptadiene3,5-dione) has a molar mass of 368.38 g/mol and a molecular formula of $C_{21}H_{20}O_6$.[61] Two aromatic ring units comprising o-methoxy phenolic groups and one alpha beta-unsaturated beta-diketone moiety make up its structural composition (Fig. 1.1). The pH of aqueous solutions influences the structure of curcumin, which undergoes keto-enol tautomerism.[62] When the pH is neutral or acidic, the keto form predominates; when the pH is alkaline, the enolate form is predominant. Notably, the enolate form is known to be more chemically liable compared to the keto form, resulting in the low chemical stability of curcumin in aqueous solutions.[63] The United States Food and Drug Administration has evaluated available evidence from research and concluded that curcumin is generally regarded as safe (GRAS).[64] The United Nations, the European Food Safety Authority, and the Expert Committee on Food Additives of the World Health Organization permit a comparatively high daily consumption of curcumin: 0 to 3 mg/kg body weight/day.[65] Curcumin can be taken orally at levels up to 8 g daily without risk, according to research on its chronic toxicity.[66] The human body appears to tolerate curcumin effectively, even at quite high doses—up to 12 grams per day, based on other investigations and clinical trials.[67] It is utilized in cosmetics, as a coloring agent, and as a food supplement.[22]

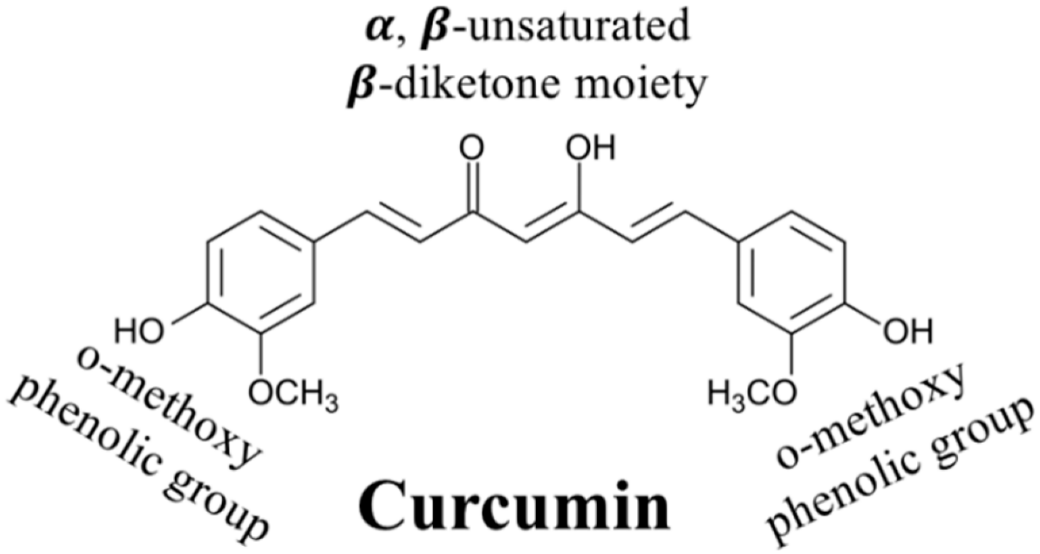

Fig. 1.1. Chemical structural representation of Curcumin with essential functional groups (Adapted[62])

1.7.1. Health relevance of curcumin

In addition to its medicinal properties, curcumin's comparatively high antioxidant activity is a major factor in its incorporation into food systems. This property is thought to extend the shelf life of food products and shield cells from harm caused by free radicals.[62] Additionally, curcumin's antibacterial activity indicates that it can prevent food deterioration, extend shelf life, and deactivate harmful organisms, enhancing food safety.[68] Besides its antioxidant and anti-microbial properties, curcumin has been demonstrated to have anti-inflammatory, antiviral and antibacterial properties. It has also been shown to promote wound healing and has the potential to combat several carcinoma diseases, including diabetes, allergies, arthritis, Alzheimer's disease, and other chronic illnesses.[69] Additionally, curcumin can reduce the proliferation of cancer cells by causing apoptosis and suppressing angiogenesis. Research conducted both in vitro and in vivo indicates that curcumin has the potential to inhibit the growth and multiplication of many cancer cell types, such as those found in the pancreas, prostate, breast, and colon.[70] Its weak solubility and poor intestinal permeability, to mention a few, are obstacles that limit its full potential despite its safety and efficacy.

1.8. Chemistry of plant protein-polyphenolic interactions

The interactions between proteins and polyphenols occur spontaneously via non-covalent interactions and covalent bonding. These contribute to the nutritional and functional changes of both proteins and polyphenols in food systems.

1.8.1. Non-covalent interactions

This mechanism involves four binding interactions: hydrogen bonding, hydrophobic interactions, electrostatic or ionic bonding, and Van der Waals forces.[12] The vast majority of protein-polyphenolic complexes found in nature are formed through non-covalent interactions (Fig. 1.2). Thus, despite its unstable nature, the non-covalent protein-polyphenolic interaction is critical in food industries, influencing the creation and improvement of associated food systems.[71] Polyphenols are prominent hydrogen donors, capable of forming hydrogen bonds with the carboxyl groups of protein. These hydrogen bonds are also produced via interactions between the hydroxyl (-OH) groups of polyphenols and nitrogen or oxygen, notably -OH and amino groups of proteins, and they help stabilize the protein-polyphenol complexes.[12] Furthermore, hydrophobic interactions are generated via interactions between the polyphenols' non-polar aromatic rings

and hydrophobic regions of protein molecules.[72] This modifies the protein structure given that only weak hydrophobic domains can remain within the surface of the protein; consequently, protein structure and functionality change.[73] Ionic bonding and electrostatic interactions play a minor role in polyphenol-protein interactions.[74] Proteins contain either a net negative or positive charge at neutral pH, contingent upon the ratio of negatively to positively charged residues. The charged components of proteins are dispersed on their surface.[12] Ionic or electrostatic interactions are initiated by charged molecules on the protein surface (for example, Lys e-amino groups) and polyphenolic -OH groups.[75] Van der Waals interactions are intermolecular interactions among atoms and are influenced by the environment.[12] Several studies have identified various driving mechanisms for non-covalent interactions between proteins and polyphenols. Hydrophobic interactions and hydrogen bonding enhanced the binding of quercitrin, quercetin and rutin to β-lactoglobulin.[76] In another study, the primary driving force between food proteins and flavones was observed to be the Van der Waals force.[75] Meanwhile, electrostatic interaction was found to facilitate the interaction between phytic acid and bovine serum albumin.[77] Notably, β-casein interacted with cinnamic and benzoic acid derivatives mostly through hydrophobic and electrostatic interactions.[78]

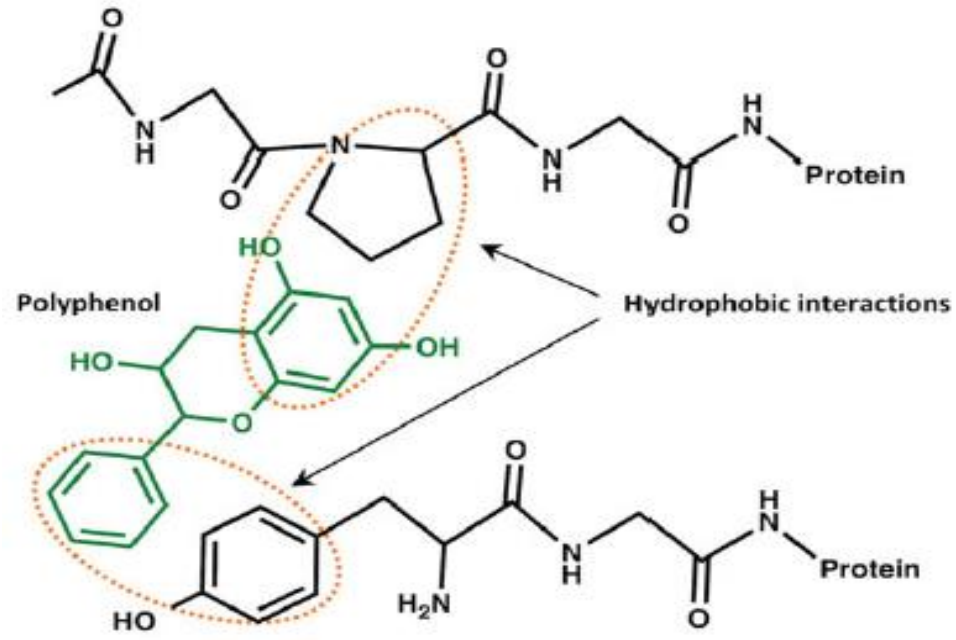

Fig. 1.2. Schematic representation of non-covalent interactions of proteins and polyphenols via hydrophobic interactions (Adapted[71])

1.8.2. Covalent bonding

Proteins and polyphenols interact covalently through two different reaction systems: non-enzymatic and enzymatic (Fig. 1.3). The non-enzymatic technique combines alkaline reaction and free radical grafting. Under an alkaline environment (pH 9) along with access to exposure to air, polyphenolic compounds are susceptible to oxidation and can be easily oxidized to generate semiquinones, which then undergo modification into quinones. These extremely reactive intermediates combine with nucleophilic residues of amino acids such as methionine, lysine, cysteine, and tryptophan in polypeptide chains to create extremely stable protein-polyphenol complexes (cross-linking).[79] In the free-radical grafting approach, ascorbic acid and hydrogen peroxide are frequently used as a redox combination to form radicals containing hydroxyl groups, and these can oxidize amino acids in protein side chains, followed by a chemical reaction with polyphenols to generate cross-linked conjugates.[80] The enzymatic reaction processes essentially employ polyphenol oxidases including laccase and tyrosinase. In this mechanism, non-enzymatic methods convert polyphenol compounds to semiquinone and then quinone intermediates. These semiquinones and quinone intermediates are subsequently engaged by nucleophilic side chains, resulting in C-S or C-N covalent bonds within the molecules. A particular study demonstrated the formation of covalent bonds between coffee storage proteins and caffeoylquinic acid.[81] Protein-polyphenolic covalent bonding with food or plant proteins has been extensively studied, including interactions between β-lactoglobulin and tea polyphenols, soy protein and anthocyanin, chlorogenic acid, epigallocatechin gallate, gallic acid and lactoferrin.[82–84]

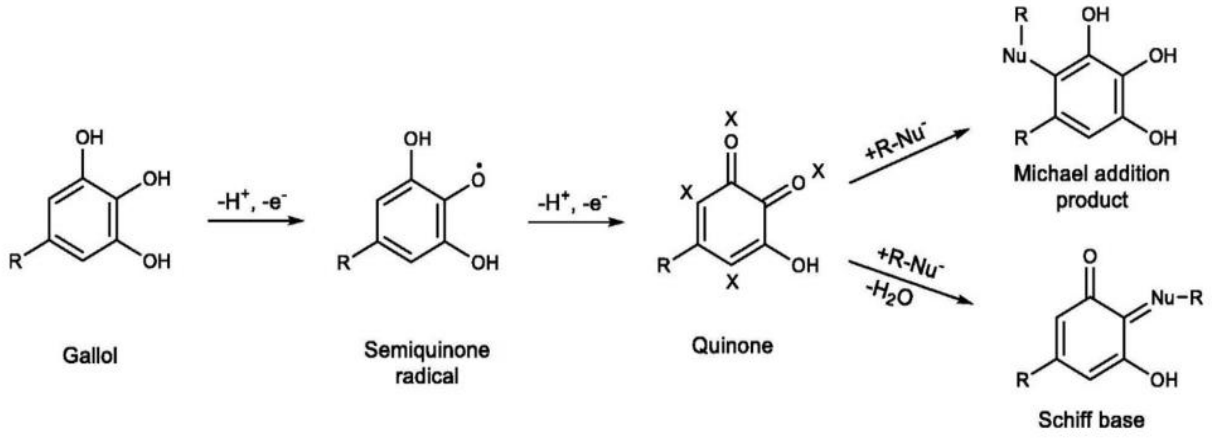

Fig. 1.3. Schematic representation of covalent bonding of proteins and polyphenols (Adapted[85])

1.9. Plant protein-curcumin interactions

Complexation between proteins and polyphenols has become an efficient strategy for improving the stability, water-based solubility, bioaccessibility, as well as bioactivities of several lipophilic compounds, including insoluble water bioactive substances like curcumin. It is important that to achieve optimal complexity, both the hydrophobic bioactive and protein molecules need to be adequately soluble.[86] In order to make certain that these insoluble water ligands can be completely combined with proteins, the majority of studies reported an approach of initial solubilization of these compounds in ethanol or other kinds of organic solvents in compatibility with water, such as dimethyl sulfoxide (DMSO), following gradually incorporation of the organic solvent mixture into the solution containing the protein.[86] To minimize the impact of organic solvents on protein-bioactive complexation, the final mixture should be at least less than 3% (v/v) of organic solvent concentration. One innovative technique to avoid the use of organic solvents that are particular to curcumin is to employ the pH-shifting solubilization approach.[87] Curcumin in its diketone state is extremely insoluble at neutral pH levels, but it is very soluble at an alkaline pH, such as pH 12, due to hydroxyl group deprotonation. The majority of proteins appear unfolded and denatured at alkali pH levels, making them ideal for complexation with curcumin as a result of existing easily accessible sites for binding to curcumin at this stage.[86] Moreso, pH-sensitive injectable curcumin-loaded effective polymeric micelles were utilized to obtain over 90% release efficiency and decreased curcumin photodegradation.[88] In most interactions, a single curcumin molecule binds to one molecule of protein to form the complex. Generally, the formation of protein-curcumin complexes is mediated mostly by hydrophobic interactions, hydrogen bonds and Van der Waals forces might be involved in some cases. Hitherto, the complexation of plant proteins of various origins and curcumin as well as interactions, has been extensively studied.[89,90] It has also recently been reported that new plant proteins, such as those from walnut and sunflower seeds, can be utilized to complex curcumin to improve its solubility and functional properties, including its anti-inflammatory, antioxidant, and antihypertensive properties.[91,92] Soy protein is a well-known plant protein used to complex with curcumin in its native state or following enzymatic modification with glutaminase.[93,94] Studies have reported the use of pea protein isolate to complex with curcumin incorporating polysaccharides such as high methoxylpectin and Ca^{2+} ions; and as an efficient wall material for microencapsulation.[95,96]

1.10. Factors impacting plant protein-polyphenol interactions

Several investigations on plant protein-polyphenol interactions have reported that formation of complexes from both reacting molecules either covalently or non-covalently are driven by two categories. The extrinsic or external factors such as pH, ionic strength and temperature and intrinsic or external factors such as characteristics of protein and polyphenols, protein/polyphenol concentration, type of protein and polyphenol, etc. They are discussed in detail below.

1.10.1. pH

Complexes that arise from interactions between polyphenols and proteins appear to be sensitive at various pH levels.[12] Different pH values can have a significant impact on polyphenolic binding properties as well as the conformational configurations of proteins.[71] Certain globular proteins exhibited a greater propensity to bond with tannic acid at pH 4.9 as opposed to pH 7.8.[75] Proteins dissociate at lower pH values, exposing the binding sites through electrostatic interaction.[12]

1.10.2. Temperature

This is an essential variable in protein-polyphenol interactions given that it modulates hydrogen bonding and promotes the formation of hydrophobic interactions.[12] When proteins undergo heat treatment, their arrangement is altered and hydrophobic regions become exposed, influencing how hydrophobic substances interact. These hydrophobic groups facilitate interactions between the polyphenols and non-polar groups.[97] Temperature can also impact interactions through modifications to protein structures, ligand solubility, and the strength of some non-covalent associations.[71] Furthermore, heating promotes polyphenol oxidation, which leads to the generation of quinone derivatives and subsequently, polyphenol disintegration which eventually results in the loss of its bioactivity.[97] Identifying temperature-dependent interactions of specific protein-polyphenol complexes could be beneficial for the development of related food products with specific functionalities.[71]

1.10.3. Ionic strength

This parameter modulates protein-phenolic interactions. The addition of salt could either diminish or enhance the interaction or binding affinity of the polyphenol. This is linked to the salt type and concentration used. The binding affinity of quercetin to a globular protein was reduced with an increase in ionic strength upon the addition of NaCl. This could be attributed to the greater

hydration hulls of the proteins with dissipated electrostatic interactions.[79] On the other hand, the binding of chlorogenic acid and globular protein improved due to increased ionic strength in the buffering system. Moreso, this interaction was also driven by hydrophobic forces.[98]

1.10.4. Polyphenol type and structure

Protein-polyphenol interaction can be influenced by the kind and structure of polyphenol molecules. The molecular mass, hydroxylation, methylation, hydrogenation, and glycosylation of different polyphenols differ which has a substantial impact on how they interact with proteins.[12] Polyphenols with higher molecular weights typically have higher binding affinities.[99] Most of the time, binding strength increases with molecule weight. A greater degree of hydroxylation could potentially have an impact on binding affinity. The binding efficiency of flavonoids is increased by the hydroxylation of the A and B chains.[97] Methylation decreases the capacity of polyphenols to bind to proteins, whereas hydrogenation of flavonoids' C2 = C3 double bond demonstrated decreased binding affinity for proteins.[100]

1.10.5. Protein characteristics

Protein surface features can influence polyphenol-protein interactions. Protein binding capacity to polyphenol compounds can be modulated by changes in amino acid content, hydrophobicity, and isoelectric value.[12] Unfolded proteins possess a greater affinity for polyphenols than globular (or compacted) proteins given that they have more amino acid residues that can interact with polyphenols.[99] Furthermore, protein characteristics such as surface structure, net charges, and secondary structures have been linked to variable degrees of non-covalent binding of dietary proteins to certain phenolic compounds.[71,97]

1.10.6. Protein-polyphenolic ratio

The protein/phenolic ratio is also a key aspect that can impact the interactions of polyphenols and proteins. At different protein/phenolic ratios, both multidentate and monodentate processes are involved. The multidentate mechanism necessitates a lesser phenolic/protein ratio since phenolic compounds act as multi-site ligands, attaching to multiple protein molecules or sites. To stimulate protein crosslinking resulting in dimers or oligomers, phenolics needs to be substantial in size. In the monodentate approach, multiple phenolics bind to a single protein molecule, requiring a substantially higher phenolic concentration.[72]

1.11. Nanoencapsulation of curcumin with plant proteins

The application of curcumin in both pharmaceuticals and food formulations is safe and reliable. However, some limitations, such as extremely low water solubility, chemical instability, inadequate absorption, quick metabolism, and limited bioavailability, impede the utilization of curcumin as a functional and bioactive substance.[101] To address these issues, many technologies have been developed, including encapsulation in various carriers, formation of complexes with biopolymers, and nanocomplexation.[102] Complexation using food-based biopolymers was developed as an economical, effective and relatively easy technique for improving the chemical stability, water solubility and bioavailability of curcumin.[103] Proteins are widely used as encapsulating materials due to their biodegradability, easily metabolized, and readily regulated.[104] Furthermore, a diverse set of functional groups on the surfaces of proteins enables their ability to interact with various bioactive compounds, making it easier to create carriers (i.e. nanocarriers) that can be used to encapsulate these bioactive compounds.[105] These properties have sparked growing interest in using protein as a delivery system for polyphenols. Nanoencapsulation typically results in particles with nanoscale sizes that are less than 1,000 nanometers.[106] Given its enhanced solubility, stability, and permeability, nanoencapsulation has recently gained popularity as a method of protecting and delivering polyphenols.[104] If the protein complexes are nano-sized, the interaction between proteins and curcumin is referred to as nanocomplexation.[86] Various nanoencapsulation systems can be used as delivery vehicles to release bioactive substances or increase their bioavailability such as nano-emulsions and protein-based emulsions, biopolymer nanoparticles, and hydrogels.[71]

Several studies have revealed that protein-based carriers are excellent mediators for polyphenol retention and specific delivery. Similar advances in the stability of bioactives encapsulated in protein-phenolic complex emulsifiers have also been observed for alga oil produced in zein hydrolysate-tannic acid conjugates and curcumin and resveratrol loaded in zein-EGCG.[102,107] The incorporation of pea protein isolate as a single carrier or in combination with other biopolymers for encapsulation systems emerges to be a potential technique for attaining a controlled release rate and maintained food bioactives.[108] In previous studies, binary complexes as novel curcumin delivery methods were created using pea protein isolate and specific surfactants such as tea saponin, rhamnolipid and ethyl lauroyl arginate hydrochloride via a pH-driven mechanism. The curcumin contained within the pea protein isolate-rhamnolipid complex demonstrated greater

photo- and thermal stability. Furthermore, the pea protein isolate-rhamnolipid complex had a regulated release of curcumin and enhanced acid stability in gastric juice following *In vitro* digestion.[109] Similarly, with the use of a pH-driven technique, pea protein isolate nanoparticles were formulated to encapsulate curcumin. This resulted in pea protein isolate-curcumin nanoparticles with significantly greater loading rates and improved water solubility. Also, the pea protein isolate-curcumin nanoparticles demonstrated significantly increased anticancer efficacy, with the mechanism of action being the ROS-induced mitochondria-mediated caspase cascade apoptosis process.[110] It was also reported that to enhance the functionality of the coated biolayers, protein-polyphenol-saccharide complexes were developed to promote the prevention of the disintegration of bioactive substances, including pea protein-curcumin-pectin.[111] In another study, the influence of succinylated pea protein isolate on the mechanism of interaction with curcumin for a potential role in encapsulation and gastrointestinal delivery was investigated. Complexes improved curcumin binding and encapsulation efficiency as well as enhanced curcumin bioavailability under in vitro conditions.[90] Pea protein isolates and carboxymethyl cornellan gum were utilized to generate corn-shell particles to load curcumin. This presented a high loading rate and improved stability as well as antioxidant activity.[112]

Recently, Pickering emulsions have gained popularity as delivery vehicles for hydrophobic nutraceuticals considering their significant loading capacity, stability, and gel-like qualities. Protein-based nanoparticles have been shown to be an effective emulsifier for stabilizing Pickering emulsions through the formation of a dense film around oil droplets.[113] Creating Pickering emulsions stabilized with nanoparticles using biopolymers can be a viable approach for improving curcumin stability against degradation, improving bioavailability in the gastrointestinal tract, and obtaining its controlled release at different locations owing to changes in environmental factors such as pH and temperature.[114] Curcuminoids encapsulated in Pickering emulsion systems were reported to lead to their controlled release in distinct areas of the digestive tract as a result of environmental changes such as bile salts, pH and digestive enzymes.[115,116] Additionally, nanoparticles used for stabilizing curcumin-loaded Pickering emulsions were formulated from proteins such as kafirin; protein gel nanoparticles from whey protein nano gels or whey protein isolate; zein and ovotransferrin have been also used for improving the stability of curcumin-loaded-emulsions and enhancing the bioavailability of curcumin.[117–125]

Protein-curcumin nanocomplexes are easily incorporated into a wide range of food compositions, including bread, beverages, cake, yogurt, and protein powders.[86] However, there are certain constraints when employing nanocomplexes containing dietary proteins as nanocarriers for curcumin. For instance, the majority of curcumin molecules encapsulated in nanocomplexes bind to sites near protein surface areas. In numerous instances, the binding capacity of proteins to curcumin is limited, therefore the amount of curcumin loaded in nanocomplexes may be lower than that in nanoparticles generated from the desolvation method.[86] Curcumin binding to protein surfaces may impact the colloidal stability of proteins in dispersions, as well as the water dispersibility of dry protein products. The integration of curcumin into protein nanocomplexes can also result in modifications to the appearance or color of dispersions or products comprising these nanocomplexes.[86]

1.12. Influence of protein-polyphenol interactions on plant protein digestibility

The effects of polyphenolic substances on protein digestibility are facilitated by their association with endogenous proteins, specifically digestive enzymes such as proteases and protein substrates. Phenolic compounds have an impact on the activity of enzymes and the accessibility of protein substrates, both of which are crucial for protein digestibility.[126] Understanding how polyphenols impact the digestibility of related proteins is critical for nutrition. Studies have reviewed the roles of polyphenols on plant protein digestibility. The collective body of knowledge suggests that the protein complexation with polyphenols seems to have a positive or negative effect on protein digestibility. However, inconsistencies in research outcomes may be due to differences in the individual proteins and polyphenols used in the investigations.[127] Many studies have revealed that polyphenols reduce or accelerate protein digestibility *In vitro* as a result of both covalent and noncovalent interactions.[126] On the one hand, noncovalent polyphenol binding can reduce the steric accessibility of proteins to enzymes. Conversely, covalent interactions can trigger the partial unfolding of proteins and enhance the exposure of cleavage sites, giving rise to improved protein digestibility.[126] Polyphenols are recognized to bind with digestive enzymes, modulating their functions. Polyphenol binding to digestive enzymes causes the development of insoluble aggregates and denaturation of the enzymatic polypeptide chain of proteins.[14] Additionally, the physiological conditions of the gastrointestinal tract can modulate the behavior of the protein substrate and polyphenol, thereby impacting protein digestion. For example, pH influences the protein-polyphenol complexes. During the process of digestion, these

complexes exhibit varying solubility and stability characteristics under highly acidic (gastric phase) or alkaline conditions (intestinal phase). Some studies conducted under simulated gastrointestinal conditions evaluated the influence of curcumin complexation on the digestion qualities of dietary proteins. The release of trichloroacetic acid (TCA)-soluble nitrogen, a protein digestibility index, was assessed to investigate the influence of nanocomplexation with curcumin on soy protein isolates *In vitro*. Under simulated gastrointestinal conditions, protein-curcumin had greater digestibility relative to the control group.[128] A particular study reported the effects of the pea protein isolate-curcumin complexes on In vitro protein digestibility and concluded that the presence of curcumin impacted the physicochemical characteristics of proteins which contributed to the inhibition of enzyme hydrolysis.[129] In contrast, soybean milk enhanced with green coffee showed marginally improved protein digestibility when juxtaposed with unfortified soymilk.[130] Similarly, the non-covalent complexation of chlorogenic acid with pea protein isolate was investigated. Electrostatic interactions were identified as the primary mode of interaction between the protein sample and phenolic acid. It was discovered that there was a 5% rise in the degree of hydrolysis, which serves as an indicator of in vitro digestibility of pea protein.[131]

1.13. Food matrix effects on plant protein digestibility

Food matrices are intricate systems made up of numerous food components that can be either solid or liquid. The interaction of compounds with one another are complicated due to a variety of factors and technical procedures such as the use of heat, high temperatures, pressure, etc.[132] Within the food matrix, polyphenol compounds disrupt the intra- or intermolecular relationships of proteins and initiate novel molecular interactions. The nutritional and biological qualities of proteins and polyphenols in foods undergo modifications as a result of these interactions.[133] Protein digestibility is considerably dependent on the food matrix, which alludes to the physical and chemical composition of food.[134] Studies on the effects of plant protein–polyphenolic interaction on protein digestibility in foods are limited. This reason could be that a complex system such as a food matrix undergoes extensive processes and therefore results might be contradicting. An in vitro investigation found that the addition of onion powder to bean paste did not significantly alter albumin digestibility. However, there was a decrease in globulin digestibility from 70% to 55% following the gastric phase and from 93% to 80% after intestinal digestion, however, there was an increase in the bioaccessibility of the entire or individual polyphenolic

content.[135] As a result, careful consideration should go into designing functional food products enriched with polyphenol compounds. Adequate analysis should also be done on the impact of the added polyphenol compounds on the In vitro protein digestibility, in addition to examining other characteristics of the products.[126]

1.14. Some determinants of plant protein digestibility

Food protein digestibility measures the vulnerability of proteins to enzyme breakdown.[136] It can be improved or reduced depending on several conditions, which are classified as exogenous or endogenous. Endogenous factors include pH, ionic strength, temperature conditions, and secondary molecules found in the protein environment, whereas endogenous factors are those that contribute to the inherent properties and further modulate its characteristics. These include protein amino acid composition and structural properties.[137] Most significantly, the presence of particular substances in plants is detrimental to plant protein digestibility. These substances are referred to as antinutrients.

1.14.1. Antinutrients

One of the main factors affecting plant protein digestibility is the presence of anti-nutritional factors. Antinutrients are chemical substances generated by plants to defend themselves from environmental stresses like light, pathogenic attacks and also to carry out other biological functions.[138] Antinutrients have been reported to reduce protein and amino acid bioavailability and modulate metabolic processes, hence, reducing their digestibility and having a deleterious impact on gastrointestinal tract function.[139] In some situations, they induce allergic reactions in humans.[140] Plant proteins typically contain a variety of antinutritional components, including protease inhibitors, lectins, tannins, phytic acids, and saponins.

1.14.1.1. Protease inhibitors

They are alternatively referred to as trypsin inhibitors. They are low molecular weight proteins that can bind to and inactivate the digestive enzyme (trypsin), lowering protein digestibility, amino acid absorption, and mineral availability. The presence of trypsin in diet inhibits the active region of trypsin and chymotrypsin (the principal proteases in the upper part of the gastrointestinal lumen) via the N- or C-terminus and the exposed loop, successfully hindering these digestive enzymes from acting on the protein substrate.[141] As a result, whenever plant protein crops are consumed raw or improperly cooked, they disrupt digestive systems, causing diarrhea or bloating.[138] Even

with a substantial protein diet, the complete absorption of amino acids is hindered.[137] Furthermore, they are divided into two groups: Kunitz (KTIs) and Bowman-Birk (BBTIs), having molecular weights of around 20 and 8 kDa, respectively.[142] Some plant proteins, such as soybeans, include both of these components.[143]

1.14.1.2. Lectins

They attach to proteins, forming complexes that impede food absorption. Protein digestion is disrupted, resulting in loss of nitrogen. Proteins that are undigested and unabsorbed in the small intestines enter the colon, where fermentation to short-chain fatty acids occurs and the release of gases, triggering gastrointestinal problems. The altered intestinal permeability enables bacteria and their endotoxins to enter the bloodstream, resulting in a toxic effect.[137]

1.14.1.3. Tannins

Tannin intake can promote gastrointestinal mucosal stiffening, which reduces nutrition absorption. Tannins influence protein digestibility by generating reversible and irreversible complexes involving protein carbonyl groups and the hydroxyl groups of tannins, resulting in a decline in the availability of essential amino acids.[144,145] These complexes are quite substantial and hydrophobic. The breakdown products include a vast range of chemicals that could potentially be hazardous.[146]

1.14.1.4. Phytic acids

Phytic acid is commonly present in tissues of plants as mono- and divalent cation salts identified as phytate, with substantial amounts of it linked with proteins in globoid particles.[147] Phytic acid ($C_6H_{18}O_{24}P_6$) is composed of a cyclic ring with each oxygen bonded to a phosphate group. Its peculiar structure contributes to its chelating ability. This allows it to interact with metal ions such as iron, magnesium, and calcium, lowering their bioavailability and causing mineral ion deficits in human nutrition.[148] Phytates have interactions with proteins to generate phytate-protein complexes, which influence protein shape, solubility, and digestibility while also inhibiting digestive enzymes.[149] It also binds to trypsin, affecting its activity on proteins.[150] Although mineral chelation by phytate is widely recognized as a nutritionally detrimental interaction, it has also been proposed to give some shielding against carcinoma of the colon and to justify the putative antioxidant capacity of phytates.[151] Saponins, another type of antinutrient, can hinder protein digestion by developing complexes with enzymes like trypsin and chymotrypsin.[152]

1.15. Conclusion

The prevalence of the utilization of plant-based proteins in food formulations has gained momentum due to their significance to health. Their excellent nutritional and functional qualities make them potential candidates in novel food development. In the same light, polyphenolic compounds have gained global recognition due to their health-promoting attributes such as antioxidants, anti-diabetic and anti-hypertensive are a hallmark of their incorporation in pharmaceuticals. Both proteins and polyphenols can co-interact either via non-covalent interactions or covalent bonding forming soluble or insoluble complexes which can be influenced by several factors such as the type and structure of proteins and polyphenols, concentration, surface characteristics, temperature, ionic strength, pH. All these facilitate the physicochemical changes in proteins as well as the bioavailability of polyphenols. Among other leguminous plant proteins, pea proteins are more economically viable for commercial production due to their low cost, availability, high-quality protein yield, low allergenicity, good nutritional and functional properties, and health benefits. Besides, pea protein fractions including albumins and globulins possess significant techno-functional properties that promote their incorporation into the food system. The wide range of beneficial biological effects that curcumin possesses reflects its unique characteristics. The interaction between plant-derived proteins and curcumin is frequently applied in the formulation of delivery systems. Existing studies predominantly underscore the importance of optimizing encapsulation efficiency to mitigate curcumin's inherent challenges, including low solubility in water, limited chemical stability, and reduced bioavailability within the gastrointestinal tract. However, there remains a paucity of data on the influence of these molecular interactions on the digestibility of proteins. Hence, this innovative investigation into the interaction between pea protein fractions and curcumin aims to advance the understanding of protein-curcumin complexation, facilitating the development of delivery systems optimized for high encapsulation efficiency and enhanced curcumin bioavailability, while preserving or improving the digestibility of the proteins.

1.16. Study hypothesis and objectives

1.16.1. Hypothesis

Modifications occur within proteins and polyphenols as a result of their interactions. Most of the changes in proteins that have been identified are associated with their structure, nutritional value,

and digestibility. However, the total polyphenolic contents, antioxidant efficacy, and bioavailability of polyphenols are altered due to these interactions. It is therefore hypothesized that the complexation of pea protein fractions and curcumin leads to the formation of soluble or insoluble complexes. Proteins can unfold upon binding to curcumin or form more compact structures with curcumin which impact the extent of digestive enzymes accessibility in the gastrointestinal tract.

1.16.2. Objectives

This study aims to assess the biomolecular interactions of pea protein fractions and curcumin and evaluate the effect of this interaction on protein digestibility. **The specific objectives of this study include** a) Examining the effects of the formation of pea albumin, globulin, and glutelin-curcumin complexation on *In vitro* gastric protein digestion phase and b) Investigating the impact of ionic strength on pea albumin-curcumin complexes and its influence on protein digestibility.

1.17. References

(1) Sun, M.; Mu, T.; Zhang, M.; Arogundade, L. A. Nutritional Assessment and Effects of Heat Processing on Digestibility of Chinese Sweet Potato Protein. *Journal of Food Composition and Analysis* **2012**, *26* (1–2), 104–110. https://doi.org/10.1016/J.JFCA.2012.03.008.

(2) González, A. D.; Frostell, B.; Carlsson-Kanyama, A. Protein Efficiency per Unit Energy and per Unit Greenhouse Gas Emissions: Potential Contribution of Diet Choices to Climate Change Mitigation. *Food Policy* **2011**, *36* (5), 562–570. https://doi.org/10.1016/J.FOODPOL.2011.07.003.

(3) Guasch-Ferré, M.; Zong, G.; Willett, W. C.; Zock, P. L.; Wanders, A. J.; Hu, F. B.; Sun, Q. Associations of Monounsaturated Fatty Acids from Plant and Animal Sources with Total and Cause-Specific Mortality in Two Us Prospective Cohort Studies. *Circ Res* **2019**, *124* (8), 1266–1275. https://doi.org/10.1161/CIRCRESAHA.118.313996/SUPPL_FILE/CIRCRES_CIRCRES-2018-313996_SUPP1.PDF.

(4) Opazo-Navarrete, M.; Schutyser, M. A. I.; Boom, R. M.; Janssen, A. E. M. Effect of Pre-Treatment on in Vitro Gastric Digestion of Quinoa Protein (Chenopodium Quinoa Willd.) Obtained by Wet and Dry Fractionation. *Int J Food Sci Nutr* **2018**, *69* (1), 1–11. https://doi.org/10.1080/09637486.2017.1332171.

(5) Tsao, R. Chemistry and Biochemistry of Dietary Polyphenols. *Nutrients 2010, Vol. 2, Pages 1231-1246* **2010**, *2* (12), 1231–1246. https://doi.org/10.3390/NU2121231.

(6) Vauzour, D.; Rodriguez-Mateos, A.; Corona, G.; Oruna-Concha, M. J.; Spencer, J. P. E. Polyphenols and Human Health: Prevention of Disease and Mechanisms of Action. *Nutrients 2010, Vol. 2, Pages 1106-1131* **2010**, *2* (11), 1106–1131. https://doi.org/10.3390/NU2111106.

(7) D'Archivio, M.; Filesi, C.; Varì, R.; Scazzocchio, B.; Masella, R. Bioavailability of the Polyphenols: Status and Controversies. *International Journal of Molecular Sciences 2010, Vol. 11, Pages 1321-1342* **2010**, *11* (4), 1321–1342. https://doi.org/10.3390/IJMS11041321.

(8) Seczyk, L.; Swieca, M.; Kapusta, I.; Gawlik-Dziki, U. Protein–Phenolic Interactions as a Factor Affecting the Physicochemical Properties of White Bean Proteins. *Molecules 2019, Vol. 24, Page 408* **2019**, *24* (3), 408. https://doi.org/10.3390/MOLECULES24030408.

(9) Wang, Y.; Xie, Y.; Wang, A.; Wang, J.; Wu, X.; Wu, Y.; Fu, Y.; Sun, H. Insights into Interactions between Food Polyphenols and Proteins: An Updated Overview. *J Food Process Preserv* **2022**, *46* (5), e16597. https://doi.org/10.1111/JFPP.16597.

(10) Foegeding, E. A. Food Protein Functionality—A New Model. *J Food Sci* **2015**, *80* (12), C2670–C2677. https://doi.org/10.1111/1750-3841.13116.

(11) Precupas, A.; Leonties, A. R.; Neacsu, A.; Sandu, R.; Popa, V. T. Gallic Acid Influence on Bovine Serum Albumin Thermal Stability. *New Journal of Chemistry* **2019**, *43* (9), 3891–3898. https://doi.org/10.1039/C9NJ00115H.

(12) Ozdal, T.; Capanoglu, E.; Altay, F. A Review on Protein–Phenolic Interactions and Associated Changes. *Food Research International* **2013**, *51* (2), 954–970. https://doi.org/10.1016/J.FOODRES.2013.02.009.

(13) Foegeding, E. A.; Plundrich, N.; Schneider, M.; Campbell, C.; Lila, M. A. Protein-Polyphenol Particles for Delivering Structural and Health Functionality. *Food Hydrocoll* **2017**, *72*, 163–173. https://doi.org/10.1016/J.FOODHYD.2017.05.024.

(14) Bandyopadhyay, P.; Ghosh, A. K.; Ghosh, C. Recent Developments on Polyphenol–Protein Interactions: Effects on Tea and Coffee Taste, Antioxidant Properties and the Digestive System. *Food Funct* **2012**, *3* (6), 592. https://doi.org/10.1039/c2fo00006g.

(15) Schefer, S.; Oest, M.; Rohn, S. Interactions between Phenolic Acids, Proteins, and Carbohydrates—Influence on Dough and Bread Properties. *Foods 2021, Vol. 10, Page 2798* **2021**, *10* (11), 2798. https://doi.org/10.3390/FOODS10112798.

(16) Czubinski, J.; Dwiecki, K. A Review of Methods Used for Investigation of Protein–Phenolic Compound Interactions. *Int J Food Sci Technol* **2017**, *52* (3), 573–585. https://doi.org/10.1111/IJFS.13339.

(17) Schneider, M.; Esposito, D.; Lila, M. A.; Foegeding, E. A. Formation of Whey Protein–Polyphenol Meso-Structures as a Natural Means of Creating Functional Particles. *Food Funct* **2016**, *7* (3), 1306–1318. https://doi.org/10.1039/C5FO01499A.

(18) Sá, A. G. A.; Moreno, Y. M. F.; Carciofi, B. A. M. Food Processing for the Improvement of Plant Proteins Digestibility. *Crit Rev Food Sci Nutr* **2020**, *60* (20), 3367–3386. https://doi.org/10.1080/10408398.2019.1688249.

(19) Dallas, D. C.; Sanctuary, M. R.; Qu, Y.; Khajavi, S. H.; Van Zandt, A. E.; Dyandra, M.; Frese, S. A.; Barile, D.; German, J. B. Personalizing Protein Nourishment. *Crit Rev Food Sci Nutr* **2017**, *57* (15), 3313–3331. https://doi.org/10.1080/10408398.2015.1117412.

(20) Lu, Z. X.; He, J. F.; Zhang, Y. C.; Bing, D. J. Composition, Physicochemical Properties of Pea Protein and Its Application in Functional Foods. *Crit Rev Food Sci Nutr* **2020**, *60* (15), 2593–2605. https://doi.org/10.1080/10408398.2019.1651248.

(21) Tulbek, M. C.; Wang, Y. (Lindsay); Hounjet, M. Pea—A Sustainable Vegetable Protein Crop. *Sustainable Protein Sources* **2024**, 143–162. https://doi.org/10.1016/B978-0-323-91652-3.00027-7.

(22) Kannamangalam Vijayan, U.; Shah, N. N.; Muley, A. B.; Singhal, R. S. Complexation of Curcumin Using Proteins to Enhance Aqueous Solubility and Bioaccessibility: Pea Protein Vis-à-Vis Whey Protein. *J Food Eng* **2021**, *292*, 110258. https://doi.org/10.1016/J.JFOODENG.2020.110258.

(23) Shanthakumar, P.; Klepacka, J.; Bains, A.; Chawla, P.; Dhull, S. B.; Najda, A. The Current Situation of Pea Protein and Its Application in the Food Industry. *Molecules* **2022**, *27* (16), 5354. https://doi.org/10.3390/molecules27165354.

(24) Li, M.; Zou, L.; Zhang, L.; Ren, G.; Liu, Y.; Zhao, X.; Qin, P. Plant-Based Proteins: Advances in Their Sources, Digestive Profiles in Vitro and Potential Health Benefits. *Crit Rev Food Sci Nutr* **2024**. https://doi.org/10.1080/10408398.2024.2315448.

(25) Lam, A. C. Y.; Can Karaca, A.; Tyler, R. T.; Nickerson, M. T. Pea Protein Isolates: Structure, Extraction, and Functionality. *Food Reviews International* **2018**, *34* (2), 126–147. https://doi.org/10.1080/87559129.2016.1242135.

(26) Adebiyi, A. P.; Aluko, R. E. Functional Properties of Protein Fractions Obtained from Commercial Yellow Field Pea (Pisum Sativum L.) Seed Protein Isolate. *Food Chem* **2011**, *128* (4), 902–908. https://doi.org/10.1016/j.foodchem.2011.03.116.

(27) McCarthy, N. A.; Kennedy, D.; Hogan, S. A.; Kelly, P. M.; Thapa, K.; Murphy, K. M.; Fenelon, M. A. Emulsification Properties of Pea Protein Isolate Using Homogenization, Microfluidization and Ultrasonication. *Food Research International* **2016**, *89*, 415–421. https://doi.org/10.1016/J.FOODRES.2016.07.024.

(28) Lan, Y.; Chen, B.; Rao, J. Pea Protein Isolate–High Methoxyl Pectin Soluble Complexes for Improving Pea Protein Functionality: Effect of PH, Biopolymer Ratio and Concentrations. *Food Hydrocoll* **2018**, *80*, 245–253. https://doi.org/10.1016/J.FOODHYD.2018.02.021.

(29) Lu, B. Y.; Quillien, L.; Popineau, Y. Foaming and Emulsifying Properties of Pea Albumin Fractions and Partial Characterisation of Surface-active Components. *J Sci Food Agric* **2000**, *80* (13), 1964–1972.

(30) Barac, M.; Cabrilo, S.; Pesic, M.; Stanojevic, S.; Zilic, S.; Macej, O.; Ristic, N. Profile and Functional Properties of Seed Proteins from Six Pea (Pisum Sativum) Genotypes. *International Journal of Molecular Sciences 2010, Vol. 11, Pages 4973-4990* **2010**, *11* (12), 4973–4990. https://doi.org/10.3390/IJMS11124973.

(31) Reinkensmeier, A.; Bußler, S.; Schlüter, O.; Rohn, S.; Rawel, H. M. Characterization of Individual Proteins in Pea Protein Isolates and Air Classified Samples. *Food Research International* **2015**, *76* (P1), 160–167. https://doi.org/10.1016/J.FOODRES.2015.05.009.

(32) Acquah, C.; Zhang, Y.; Dubé, M. A.; Udenigwe, C. C. Formation and Characterization of Protein-Based Films from Yellow Pea (Pisum Sativum) Protein Isolate and Concentrate for Edible Applications. *Curr Res Food Sci* **2020**, *2*, 61–69. https://doi.org/10.1016/J.CRFS.2019.11.008.

(33) Robinson, G. H. J.; Domoney, C. Perspectives on the Genetic Improvement of Health- and Nutrition-Related Traits in Pea. *Plant Physiology and Biochemistry* **2021**, *158*, 353–362. https://doi.org/10.1016/J.PLAPHY.2020.11.020.

(34) Perera, Thushanthi. Grain Legume Consumption Inhibits Colorectal Tumorigenesis a Meta-Analysis of Human and Animal Studies. **2016**.

(35) Geerts, M. E. J.; Mienis, E.; Nikiforidis, C. V.; van der Padt, A.; van der Goot, A. J. Mildly Refined Fractions of Yellow Peas Show Rich Behaviour in Thickened Oil-in-Water Emulsions. *Innovative Food Science & Emerging Technologies* **2017**, *41*, 251–258. https://doi.org/10.1016/J.IFSET.2017.03.009.

(36) Shevkani, K.; Singh, N.; Kaur, A.; Rana, J. C. Structural and Functional Characterization of Kidney Bean and Field Pea Protein Isolates: A Comparative Study. *Food Hydrocoll* **2015**, *43*, 679–689. https://doi.org/10.1016/J.FOODHYD.2014.07.024.

(37) Klupšaitė, D.; Juodeikienė, G. Legume: Composition, Protein Extraction and Functional Properties. A Review. *Chemical Technology* **2015**, *66* (1), 5–12. https://doi.org/10.5755/j01.ct.66.1.12355.

(38) Bogahawaththa, D.; Bao Chau, N. H.; Trivedi, J.; Dissanayake, M.; Vasiljevic, T. Impact of Selected Process Parameters on Solubility and Heat Stability of Pea Protein Isolate. *LWT* **2019**, *102*, 246–253. https://doi.org/10.1016/J.LWT.2018.12.034.

(39) Barac, M.; Cabrilo, S.; Stanojevic, S.; Pesic, M.; Pavlicevic, M.; Zlatkovic, B.; Jankovic, M. Functional Properties of Protein Hydrolysates from Pea (Pisum Sativum, L) Seeds. *Int J Food Sci Technol* **2012**, *47* (7), 1457–1467. https://doi.org/10.1111/J.1365-2621.2012.02993.X.

(40) Ge, J.; Sun, C.; Corke, H.; Gul, K.; Gan, R.; Fang, Y. The Health Benefits, Functional Properties, Modifications, and Applications of Pea (Pisum Sativum L.) Protein: Current Status, Challenges, and Perspectives. *Compr Rev Food Sci Food Saf* **2020**, *19* (4), 1835–1876. https://doi.org/10.1111/1541-4337.12573.

(41) Yang, M.; Yang, J.; Zhang, Y.; Zhang, W. Influence of Succinylation on Physicochemical Property of Yak Casein Micelles. *Food Chem* **2016**, *190*, 836–842. https://doi.org/10.1016/J.FOODCHEM.2015.06.030.

(42) Bessada, S. M. F.; Barreira, J. C. M.; Oliveira, M. B. P. P. Pulses and Food Security: Dietary Protein, Digestibility, Bioactive and Functional Properties. *Trends Food Sci Technol* **2019**, *93*, 53–68. https://doi.org/10.1016/J.TIFS.2019.08.022.

(43) Stone, A. K.; Avarmenko, N. A.; Warkentin, T. D.; Nickerson, M. T. Functional Properties of Protein Isolates from Different Pea Cultivars. *Food Sci Biotechnol* **2015**, *24* (3), 827–833. https://doi.org/10.1007/S10068-015-0107-Y/METRICS.

(44) Jarzębski, M.; Fathordoobady, F.; Guo, Y.; Xu, M.; Singh, A.; Kitts, D. D.; Kowalczewski, P. Ł.; Jeżowski, P.; Pratap Singh, A. Pea Protein for Hempseed Oil Nanoemulsion Stabilization.

Molecules 2019, Vol. 24, Page 4288 **2019**, *24* (23), 4288. https://doi.org/10.3390/MOLECULES24234288.

(45) Burger, T. G.; Zhang, Y. Recent Progress in the Utilization of Pea Protein as an Emulsifier for Food Applications. *Trends Food Sci Technol* **2019**, *86*, 25–33. https://doi.org/10.1016/j.tifs.2019.02.007.

(46) Wong, A.; Pitts, K.; Jayasena, V.; Johnson, S. Isolation and Foaming Functionality of Acid-Soluble Protein from Lupin (Lupinus Angustifolius) Kernels. *J Sci Food Agric* **2013**, *93* (15), 3755–3762. https://doi.org/10.1002/JSFA.6249.

(47) Babault, N.; Christos Païzis; Deley, G.; Laetitia Guérin-Deremaux; Marie-Hélène Saniez; Lefranc-Millot, C.; Allaert, F. A. Pea Proteins Oral Supplementation Promotes Muscle Thickness Gains during Resistance Training: A Double-Blind, Randomized, Placebo-Controlled Clinical Trial vs. Whey Protein. *J Int Soc Sports Nutr* **2015**, *12* (1), 1–9. https://doi.org/10.1186/S12970-014-0064-5/FIGURES/4.

(48) Sun, X. D.; Arntfield, S. D. Gelation Properties of Salt-Extracted Pea Protein Isolate Induced by Heat Treatment: Effect of Heating and Cooling Rate. *Food Chem* **2011**, *124* (3), 1011–1016. https://doi.org/10.1016/J.FOODCHEM.2010.07.063.

(49) Sun, X. D.; Arntfield, S. D. Gelation Properties of Salt-Extracted Pea Protein Induced by Heat Treatment. *Food Research International* **2010**, *43* (2), 509–515. https://doi.org/10.1016/J.FOODRES.2009.09.039.

(50) Djoullah, A.; Husson, F.; Saurel, R. Gelation Behaviors of Denaturated Pea Albumin and Globulin Fractions during Transglutaminase Treatment. *Food Hydrocoll* **2018**, *77*, 636–645. https://doi.org/10.1016/J.FOODHYD.2017.11.005.

(51) Shimomura, Y.; Murakami, T.; Nakai, N.; Nagasaki, M.; Harris, R. A. Exercise Promotes BCAA Catabolism: Effects of BCAA Supplementation on Skeletal Muscle during Exercise. *J Nutr* **2004**, *134* (6), 1583S-1587S. https://doi.org/10.1093/JN/134.6.1583S.

(52) Tamnak, S.; Mirhosseini, H.; Tan, C. P.; Tabatabaee Amid, B.; Kazemi, M.; Hedayatnia, S. Encapsulation Properties, Release Behavior and Physicochemical Characteristics of Water-in-Oil-in-Water (W/O/W) Emulsion Stabilized with Pectin–Pea Protein Isolate Conjugate and Tween 80. *Food Hydrocoll* **2016**, *61*, 599–608. https://doi.org/10.1016/J.FOODHYD.2016.06.023.

(53) Damodaran, S. Protein Stabilization of Emulsions and Foams. *J Food Sci* **2005**, *70* (3), R54–R66. https://doi.org/10.1111/J.1365-2621.2005.TB07150.X.

(54) McClements, D. J.; Jafari, S. M. Improving Emulsion Formation, Stability and Performance Using Mixed Emulsifiers: A Review. *Adv Colloid Interface Sci* **2018**, *251*, 55–79. https://doi.org/10.1016/J.CIS.2017.12.001.

(55) Lam, R. S. H.; Nickerson, M. T. Food Proteins: A Review on Their Emulsifying Properties Using a Structure–Function Approach. *Food Chem* **2013**, *141* (2), 975–984. https://doi.org/10.1016/J.FOODCHEM.2013.04.038.

(56) Can Karaca, A. Encapsulation of Black Pepper Seed Oil Using Maltodextrin and Pea Protein. *Food Science and Technology International* **2020**, *26* (5), 369–378.

https://doi.org/10.1177/1082013219896429/ASSET/IMAGES/LARGE/10.1177_1082013219896429-FIG3.JPEG.

(57) Perez, V.; Felix, M.; Romero, A.; Guerrero, A. Characterization of Pea Protein-Based Bioplastics Processed by Injection Moulding. *Food and Bioproducts Processing* **2016**, *97*, 100–108. https://doi.org/10.1016/J.FBP.2015.12.004.

(58) Wu, D.-T.; Li, W.-X.; Wan, J.-J.; Hu, Y.-C.; Gan, R.-Y.; Zou, L. A. A Comprehensive Review of Pea (Pisum Sativum L.): Chemical Composition, Processing, Health Benefits, and Food Applications. *Foods 2023, Vol. 12, Page 2527* **2023**, *12* (13), 2527. https://doi.org/10.3390/FOODS12132527.

(59) Krefting, J. The Appeal of Pea Protein. *Journal of Renal Nutrition* **2017**, *27* (5), e31–e33. https://doi.org/10.1053/j.jrn.2017.06.009.

(60) Jiang, T.; Liao, W.; Charcosset, C. Recent Advances in Encapsulation of Curcumin in Nanoemulsions: A Review of Encapsulation Technologies, Bioaccessibility and Applications. *Food Research International* **2020**, *132*, 109035. https://doi.org/10.1016/J.FOODRES.2020.109035.

(61) Sharifi-Rad, J.; Rayess, Y. El; Rizk, A. A.; Sadaka, C.; Zgheib, R.; Zam, W.; Sestito, S.; Rapposelli, S.; Neffe-Skocińska, K.; Zielińska, D.; Salehi, B.; Setzer, W. N.; Dosoky, N. S.; Taheri, Y.; El Beyrouthy, M.; Martorell, M.; Ostrander, E. A.; Suleria, H. A. R.; Cho, W. C.; Maroyi, A.; Martins, N. Turmeric and Its Major Compound Curcumin on Health: Bioactive Effects and Safety Profiles for Food, Pharmaceutical, Biotechnological and Medicinal Applications. *Front Pharmacol* **2020**, *11*, 550909. https://doi.org/10.3389/FPHAR.2020.01021/BIBTEX.

(62) Zheng, B.; McClements, D. J. Formulation of More Efficacious Curcumin Delivery Systems Using Colloid Science: Enhanced Solubility, Stability, and Bioavailability. *Molecules 2020, Vol. 25, Page 2791* **2020**, *25* (12), 2791. https://doi.org/10.3390/MOLECULES25122791.

(63) Manolova, Y.; Deneva, V.; Antonov, L.; Drakalska, E.; Momekova, D.; Lambov, N. The Effect of the Water on the Curcumin Tautomerism: A Quantitative Approach. *Spectrochim Acta A Mol Biomol Spectrosc* **2014**, *132*, 815–820. https://doi.org/10.1016/J.SAA.2014.05.096.

(64) European Food Safety Authority. Refined Exposure Assessment for Curcumin (E 100). *EFSA Journal* **2014**, *12* (10), 3876. https://doi.org/https://doi.org/10.2903/j.efsa.2014.3876.

(65) Hewlings, S. J.; Kalman, D. S. Curcumin: A Review of Its Effects on Human Health. *Foods 2017, Vol. 6, Page 92* **2017**, *6* (10), 92. https://doi.org/10.3390/FOODS6100092.

(66) Sharma, R. A.; Euden, S. A.; Platton, S. L.; Cooke, D. N.; Shafayat, A.; Hewitt, H. R.; Marczylo, T. H.; Morgan, B.; Hemingway, D.; Plummer, S. M.; Pirmohamed, M.; Gescher, A. J.; Steward, W. P. Phase I Clinical Trial of Oral Curcumin Biomarkers of Systemic Activity and Compliance. *Clinical Cancer Research* **2004**, *10* (20), 6847–6854. https://doi.org/10.1158/1078-0432.CCR-04-0744.

(67) Lao, C. D.; Ruffin IV, M. T.; Normolle, D.; Heath, D. D.; Murray, S. I.; Bailey, J. M.; Boggs, M. E.; Crowell, J.; Rock, C. L.; Brenner, D. E. Dose Escalation of a Curcuminoid Formulation. *BMC Complement Altern Med* **2006**, *6*, 10. https://doi.org/10.1186/1472-6882-6-10.

(68) Kunnumakkara, A. B.; Bordoloi, D.; Harsha, C.; Banik, K.; Gupta, S. C.; Aggarwal, B. B. Curcumin Mediates Anticancer Effects by Modulating Multiple Cell Signaling Pathways. *Clin Sci* **2017**, *131* (15), 1781–1799. https://doi.org/10.1042/CS20160935.

(69) Nabavi, S. F.; Daglia, M.; Moghaddam, A. H.; Habtemariam, S.; Nabavi, S. M. Curcumin and Liver Disease: From Chemistry to Medicine. *Compr Rev Food Sci Food Saf* **2014**, *13* (1), 62–77. https://doi.org/10.1111/1541-4337.12047.

(70) Tomeh, M. A.; Hadianamrei, R.; Zhao, X. A Review of Curcumin and Its Derivatives as Anticancer Agents. *International Journal of Molecular Sciences 2019, Vol. 20, Page 1033* **2019**, *20* (5), 1033. https://doi.org/10.3390/IJMS20051033.

(71) Zhang, Q.; Cheng, Z.; Wang, Y.; Fu, L. Dietary Protein-Phenolic Interactions: Characterization, Biochemical-Physiological Consequences, and Potential Food Applications. *Crit Rev Food Sci Nutr* **2021**, *61* (21), 3589–3615. https://doi.org/10.1080/10408398.2020.1803199.

(72) Chu, Q.; Bao, B.; Wu, W.; Chu, Q.; Bao, B.; Wu, W. Mechanism of Interaction between Phenolic Compounds and Proteins Based on Non-Covalent and Covalent Interactions. *Medicine Research* **2018**, *2* (3), 180014. https://doi.org/10.21127/YAOYIMR20180014.

(73) Zhao, H.; Zhu, M.; Wang, K.; Yang, E.; Su, J.; Wang, Q.; Cheng, N.; Xue, X.; Wu, L.; Cao, W. Identification and Quantitation of Bioactive Components from Honeycomb (Nidus Vespae). *Food Chem* **2020**, *314*, 126052. https://doi.org/10.1016/J.FOODCHEM.2019.126052.

(74) Nassarawa, S. S.; Nayik, G. A.; Gupta, S. D.; Areche, F. O.; Jagdale, Y. D.; Ansari, M. J.; Hemeg, H. A.; AL-Farga, A.; Alotaibi, S. S. Chemical Aspects of Polyphenol-Protein Interactions and Their Antibacterial Activity. *Crit Rev Food Sci Nutr* **2023**, *63* (28), 9482–9505. https://doi.org/10.1080/10408398.2022.2067830.

(75) Le Bourvellec, C.; Renard, C. M. G. C. Interactions between Polyphenols and Macromolecules: Quantification Methods and Mechanisms. *Crit Rev Food Sci Nutr* **2012**, *52* (3), 213–248. https://doi.org/10.1080/10408398.2010.499808.

(76) Sahihi, M.; Heidari-Koholi, Z.; Bordbar, A. K. The Interaction of Polyphenol Flavonoids with β-Lactoglobulin: Molecular Docking and Molecular Dynamics Simulation Studies. *Journal of Macromolecular Science, Part B* **2012**, *51* (12), 2311–2323. https://doi.org/10.1080/00222348.2012.672854.

(77) Kaspchak, E.; Mafra, L. I.; Mafra, M. R. Effect of Heating and Ionic Strength on the Interaction of Bovine Serum Albumin and the Antinutrients Tannic and Phytic Acids, and Its Influence on in Vitro Protein Digestibility. *Food Chem* **2018**, *252*, 1–8. https://doi.org/10.1016/J.FOODCHEM.2018.01.089.

(78) Li, T.; Li, X.; Dai, T.; Hu, P.; Niu, X.; Liu, C.; Chen, J. Binding Mechanism and Antioxidant Capacity of Selected Phenolic Acid - β-Casein Complexes. *Food Research International* **2020**, *129*, 108802. https://doi.org/10.1016/J.FOODRES.2019.108802.

(79) Rawel, H. M.; Meidtner, K.; Kroll, J. Binding of Selected Phenolic Compounds to Proteins. *J Agric Food Chem* **2005**, *53* (10), 4228–4235. https://doi.org/10.1021/JF0480290/SUPPL_FILE/JF0480290SI20050322_101120.PDF.

(80) Zhang, Y.; Chen, S.; Qi, B.; Sui, X.; Jiang, L. Complexation of Thermally-Denatured Soybean Protein Isolate with Anthocyanins and Its Effect on the Protein Structure and in Vitro Digestibility. *Food Research International* **2018**, *106*, 619–625. https://doi.org/10.1016/J.FOODRES.2018.01.040.

(81) Ali, M.; Homann, T.; Kreisel, J.; Khalil, M.; Puhlmann, R.; Kruse, H. P.; Rawel, H. Characterization and Modeling of the Interactions between Coffee Storage Proteins and Phenolic Compounds. *J Agric Food Chem* **2012**, *60* (46), 11601–11608. https://doi.org/10.1021/JF303372A/SUPPL_FILE/JF303372A_SI_001.PDF.

(82) Liu, F.; Sun, C.; Yang, W.; Yuan, F.; Gao, Y. Structural Characterization and Functional Evaluation of Lactoferrin–Polyphenol Conjugates Formed by Free-Radical Graft Copolymerization. *RSC Adv* **2015**, *5* (20), 15641–15651. https://doi.org/10.1039/C4RA10802G.

(83) Sui, X.; Sun, H.; Qi, B.; Zhang, M.; Li, Y.; Jiang, L. Functional and Conformational Changes to Soy Proteins Accompanying Anthocyanins: Focus on Covalent and Non-Covalent Interactions. *Food Chem* **2018**, *245*, 871–878. https://doi.org/10.1016/J.FOODCHEM.2017.11.090.

(84) Kanakis, C. D.; Hasni, I.; Bourassa, P.; Tarantilis, P. A.; Polissiou, M. G.; Tajmir-Riahi, H. A. Milk β-Lactoglobulin Complexes with Tea Polyphenols. *Food Chem* **2011**, *127* (3), 1046–1055. https://doi.org/10.1016/J.FOODCHEM.2011.01.079.

(85) Nassarawa, S. S.; Nayik, G. A.; Gupta, S. D.; Areche, F. O.; Jagdale, Y. D.; Ansari, M. J.; Hemeg, H. A.; AL-Farga, A.; Alotaibi, S. S. Chemical Aspects of Polyphenol-Protein Interactions and Their Antibacterial Activity. *Crit Rev Food Sci Nutr* **2023**, *63* (28), 9482–9505. https://doi.org/10.1080/10408398.2022.2067830.

(86) Tang, C.-H. Nanocomplexation of Proteins with Curcumin: From Interaction to Nanoencapsulation (A Review). *Food Hydrocoll* **2020**, *109*. https://doi.org/10.1016/j.foodhyd.2020.106106.

(87) Taghavi Kevij, H.; Mohammadian, M.; Salami, M. Complexation of Curcumin with Whey Protein Isolate for Enhancing Its Aqueous Solubility through a Solvent-Free PH-Driven Approach. *J Food Process Preserv* **2019**, *43* (12), e14227. https://doi.org/10.1111/JFPP.14227.

(88) Iurciuc-Tincu, C.-E.; Cretan, M. S.; Purcar, V.; Popa, M.; Daraba, O. M.; Atanase, L. I.; Ochiuz, L. Drug Delivery System Based on PH-Sensitive Biocompatible Poly (2-Vinyl Pyridine)-b-Poly (Ethylene Oxide) Nanomicelles Loaded with Curcumin and 5-Fluorouracil. *mdpi.comCE Iurciuc-Tincu, MS Cretan, V Purcar, M Popa, OM Daraba, LI Atanase, L OchiuzPolymers, 2020•mdpi.com*. https://doi.org/10.3390/polym12071450.

(89) Okagu, O. D.; Udenigwe, C. C. Molecular Interactions of Pea Globulin, Albumin and Glutelin With Curcumin: Formation and Gastric Release Mechanisms of Curcumin-Loaded Bio-Nanocomplexes. *Food Biophys* **2022**, *17* (1), 10–25. https://doi.org/10.1007/s11483-021-09697-5.

(90) Okagu, O. D.; Jin, J.; Udenigwe, C. C. Impact of Succinylation on Pea Protein-Curcumin Interaction, Polyelectrolyte Complexation with Chitosan, and Gastrointestinal Release of Curcumin in Loaded-Biopolymer Nano-Complexes. *J Mol Liq* **2021**, *325*. https://doi.org/10.1016/j.molliq.2020.115248.

(91) Moghadam, M.; Salami, M.; Mohammadian, M.; Delphi, L.; Sepehri, H.; Emam-Djomeh, Z.; Moosavi-Movahedi, A. A. Walnut Protein–Curcumin Complexes: Fabrication, Structural Characterization, Antioxidant Properties, and in Vitro Anticancer Activity. *Journal of Food Measurement and Characterization* **2020**, *14* (2), 876–885. https://doi.org/10.1007/s11694-019-00336-9.

(92) Sneharani, A. H. Curcumin–Sunflower Protein Nanoparticles—A Potential Antiinflammatory Agent. *J Food Biochem* **2019**, *43* (8). https://doi.org/10.1111/JFBC.12909.

(93) Tapal, A.; Tiku, P. K. Complexation of Curcumin with Soy Protein Isolate and Its Implications on Solubility and Stability of Curcumin. *Food Chem* **2012**, *130* (4), 960–965. https://doi.org/10.1016/J.FOODCHEM.2011.08.025.

(94) Xiang, H.; Sun-waterhouse, D.; Cui, C.; Wang, W.; Dong, K. Modification of Soy Protein Isolate by Glutaminase for Nanocomplexation with Curcumin. *Food Chem* **2018**, *268*, 504–512. https://doi.org/10.1016/J.FOODCHEM.2018.06.059.

(95) Nesterenko, A.; Alric, I.; Silvestre, F.; Durrieu, V. Vegetable Proteins in Microencapsulation: A Review of Recent Interventions and Their Effectiveness. *Ind Crops Prod* **2013**, *42* (1), 469–479. https://doi.org/10.1016/J.INDCROP.2012.06.035.

(96) Guo, Q.; Su, J.; Xie, W.; Tu, X.; Yuan, F.; Mao, L.; Gao, Y. Curcumin-Loaded Pea Protein Isolate-High Methoxyl Pectin Complexes Induced by Calcium Ions: Characterization, Stability and in Vitro Digestibility. *Food Hydrocoll* **2020**, *98*, 105284. https://doi.org/10.1016/J.FOODHYD.2019.105284.

(97) Quan, T. H.; Benjakul, S.; Sae-leaw, T.; Balange, A. K.; Maqsood, S. Protein–Polyphenol Conjugates: Antioxidant Property, Functionalities and Their Applications. *Trends Food Sci Technol* **2019**, *91*, 507–517. https://doi.org/10.1016/J.TIFS.2019.07.049.

(98) Prigent, S. V. E.; Gruppen, H.; Visser, A. J. W. G.; Van Koningsveld, G. A.; De Jong, G. A. H.; Voragen, A. G. J. Effects of Non-Covalent Interactions with 5-O-Caffeoylquinic Acid (Chlorogenic Acid) on the Heat Denaturation and Solubility of Globular Proteins. *J Agric Food Chem* **2003**, *51* (17), 5088–5095. https://doi.org/10.1021/JF021229W.

(99) Yildirim-Elikoglu, S.; Erdem, Y. K. Interactions between Milk Proteins and Polyphenols: Binding Mechanisms, Related Changes, and the Future Trends in the Dairy Industry. *Food Reviews International* **2018**, *34* (7), 665–697. https://doi.org/10.1080/87559129.2017.1377225.

(100) Xiao, J.; Mao, F.; Yang, F.; Zhao, Y.; Zhang, C.; Yamamoto, K. Interaction of Dietary Polyphenols with Bovine Milk Proteins: Molecular Structure–Affinity Relationship and Influencing Bioactivity Aspects. *Mol Nutr Food Res* **2011**, *55* (11), 1637–1645. https://doi.org/10.1002/MNFR.201100280.

(101) Li, H.; Zhang, X.; Zhao, C.; Zhang, H.; Chi, Y.; Wang, L.; Zhang, H.; Bai, S.; Zhang, X. Entrapment of Curcumin in Soy Protein Isolate Using the PH-Driven Method: Nanoencapsulation and Formation Mechanism. *LWT* **2022**, *153*, 112480. https://doi.org/10.1016/J.LWT.2021.112480.

(102) Guo, Q.; Bayram, I.; Zhang, W.; Su, J.; Shu, X.; Yuan, F.; Mao, L.; Gao, Y. Fabrication and Characterization of Curcumin-Loaded Pea Protein Isolate-Surfactant Complexes at Neutral PH. *Food Hydrocoll* **2021**, *111*, 106214. https://doi.org/10.1016/J.FOODHYD.2020.106214.

(103) Mohammadian, M.; Salami, M.; Assadpour, E.; Jafari, S. M. Curcumin-Protein Complexes: Technological and Biological Functionalities. *Trends Food Sci Technol* **2024**, *145*, 104372. https://doi.org/10.1016/J.TIFS.2024.104372.

(104) Gharehbeglou, P.; Jafari, S. M.; Hamishekar, H.; Homayouni, A.; Mirzaei, H. Pectin-Whey Protein Complexes vs. Small Molecule Surfactants for Stabilization of Double Nano-Emulsions as Novel Bioactive Delivery Systems. *J Food Eng* **2019**, *245*, 139–148. https://doi.org/10.1016/J.JFOODENG.2018.10.016.

(105) Fathi, M.; Donsi, F.; McClements, D. J. Protein-Based Delivery Systems for the Nanoencapsulation of Food Ingredients. *Compr Rev Food Sci Food Saf* **2018**, *17* (4), 920–936. https://doi.org/10.1111/1541-4337.12360.

(106) Rehman, A.; Ahmad, T.; Aadil, R. M.; Spotti, M. J.; Bakry, A. M.; Khan, I. M.; Zhao, L.; Riaz, T.; Tong, Q. Pectin Polymers as Wall Materials for the Nano-Encapsulation of Bioactive Compounds. *Trends Food Sci Technol* **2019**, *90*, 35–46. https://doi.org/10.1016/J.TIFS.2019.05.015.

(107) Wang, Y. H.; Wan, Z. L.; Yang, X. Q.; Wang, J. M.; Guo, J.; Lin, Y. Colloidal Complexation of Zein Hydrolysate with Tannic Acid: Constructing Peptides-Based Nanoemulsions for Alga Oil Delivery. *Food Hydrocoll* **2016**, *54*, 40–48. https://doi.org/10.1016/J.FOODHYD.2015.09.020.

(108) Hadidi, M.; Boostani, S.; Jafari, S. M. Pea Proteins as Emerging Biopolymers for the Emulsification and Encapsulation of Food Bioactives. *Food Hydrocoll* **2022**, *126*, 107474. https://doi.org/10.1016/J.FOODHYD.2021.107474.

(109) Guo, Q.; Shu, X.; Hu, Y.; Su, J.; Chen, S.; Decker, E. A.; Gao, Y. Formulated Protein-Polysaccharide-Surfactant Ternary Complexes for Co-Encapsulation of Curcumin and Resveratrol: Characterization, Stability and in Vitro Digestibility. *Food Hydrocoll* **2021**, *111*, 106265. https://doi.org/10.1016/J.FOODHYD.2020.106265.

(110) Ren, J.; Wu, H.; Lu, Z.; Qin, Q.; Jiao, X.; Meng, G.; Liu, W.; Li, G. PH-Driven Preparation of Pea Protein Isolate-Curcumin Nanoparticles Effectively Enhances Antitumor Activity. *Int J Biol Macromol* **2024**, *256*, 128383. https://doi.org/10.1016/J.IJBIOMAC.2023.128383.

(111) Yi, J.; Huang, H.; Liu, Y.; Lu, Y.; Fan, Y.; Zhang, Y. Fabrication of Curcumin-Loaded Pea Protein-Pectin Ternary Complex for the Stabilization and Delivery of B-carotene Emulsions. *Food Chem* **2020**, *313*, 126118. https://doi.org/10.1016/J.FOODCHEM.2019.126118.

(112) Wei, Y.; Cai, Z.; Wu, M.; Guo, Y.; Wang, P.; Li, R.; Ma, A.; Zhang, H. Core-Shell Pea Protein-Carboxymethylated Corn Fiber Gum Composite Nanoparticles as Delivery Vehicles for Curcumin. *Carbohydr Polym* **2020**, *240*, 116273. https://doi.org/10.1016/J.CARBPOL.2020.116273.

(113) Kong, Z.; Li, Z.; Zhang, L.; Dai, L.; Wang, Y.; Sun, Q.; McClements, D. J.; Cheng, Y.; Zhang, Z.; Wang, C.; Xu, X. Development of Pea Protein Nanoparticle/Hydrolyzed Rice Glutelin Fibril Emulsion Gels for Encapsulation of Curcumin. *Int J Biol Macromol* **2024**, *276*, 133640. https://doi.org/10.1016/J.IJBIOMAC.2024.133640.

(114) Saffarionpour, S.; Diosady, L. L. Curcumin, a Potent Therapeutic Nutraceutical and Its Enhanced Delivery and Bioaccessibility by Pickering Emulsions. *Drug Delivery and Translational Research 2021 12:1* **2021**, *12* (1), 124–157. https://doi.org/10.1007/S13346-021-00936-3.

(115) Wei, Z.; Wang, C.; Zou, S.; Liu, H.; Tong, Z. Chitosan Nanoparticles as Particular Emulsifier for Preparation of Novel PH-Responsive Pickering Emulsions and PLGA Microcapsules. *Polymer (Guildf)* **2012**, *53* (6), 1229–1235. https://doi.org/10.1016/J.POLYMER.2012.02.015.

(116) Liu, H.; Wang, C.; Zou, S.; Wei, Z.; Tong, Z. Simple, Reversible Emulsion System Switched by PH on the Basis of Chitosan without Any Hydrophobic Modification. *Langmuir* **2012**, *28* (30), 11017–11024. https://doi.org/10.1021/LA3021113/SUPPL_FILE/LA3021113_SI_001.PDF.

(117) Wei, Z.; Zhu, J.; Cheng, Y.; Huang, Q. Ovotransferrin Fibril–Stabilized Pickering Emulsions Improve Protection and Bioaccessibility of Curcumin. *Food Research International* **2019**, *125*, 108602. https://doi.org/10.1016/J.FOODRES.2019.108602.

(118) Wei, Z.; Cheng, J.; Huang, Q. Food-Grade Pickering Emulsions Stabilized by Ovotransferrin Fibrils. *Food Hydrocoll* **2019**, *94*, 592–602. https://doi.org/10.1016/J.FOODHYD.2019.04.005.

(119) Zou, Y.; Zhong, J.; Pan, R.; Wan, Z.; Guo, J.; Wang, J.; Yin, S.; Yang, X. Zein/Tannic Acid Complex Nanoparticles-Stabilised Emulsion as a Novel Delivery System for Controlled Release of Curcumin. *Int J Food Sci Technol* **2017**, *52* (5), 1221–1228. https://doi.org/10.1111/IJFS.13380.

(120) Lv, P.; Wang, D.; Dai, L.; Wu, X.; Gao, Y.; Yuan, F. Pickering Emulsion Gels Stabilized by High Hydrostatic Pressure-Induced Whey Protein Isolate Gel Particles: Characterization and Encapsulation of Curcumin. *Food Research International* **2020**, *132*, 109032. https://doi.org/10.1016/J.FOODRES.2020.109032.

(121) Liu, G.; Li, W.; Qin, X.; Zhong, Q. Pickering Emulsions Stabilized by Amphiphilic Anisotropic Nanofibrils of Glycated Whey Proteins. *Food Hydrocoll* **2020**, *101*, 105503. https://doi.org/10.1016/J.FOODHYD.2019.105503.

(122) Araiza-Calahorra, A.; Sarkar, A. Pickering Emulsion Stabilized by Protein Nanogel Particles for Delivery of Curcumin: Effects of PH and Ionic Strength on Curcumin Retention. *Food Structure* **2019**, *21*, 100113. https://doi.org/10.1016/J.FOOSTR.2019.100113.

(123) Araiza-Calahorra, A.; Wang, Y.; Boesch, C.; Zhao, Y.; Sarkar, A. Pickering Emulsions Stabilized by Colloidal Gel Particles Complexed or Conjugated with Biopolymers to Enhance Bioaccessibility and Cellular Uptake of Curcumin. *Curr Res Food Sci* **2020**, *3*, 178–188. https://doi.org/10.1016/J.CRFS.2020.05.001.

(124) Xiao, J.; Nian, S.; Huang, Q. Assembly of Kafirin/Carboxymethyl Chitosan Nanoparticles to Enhance the Cellular Uptake of Curcumin. *Food Hydrocoll* **2015**, *51*, 166–175. https://doi.org/10.1016/J.FOODHYD.2015.05.012.

(125) Xiao, J.; Shi, C.; Li, Y.; Pan, Y.; Huang, Q. Pickering Emulsions Immobilized within Hydrogel Matrix with Enhanced Resistance against Harsh Processing Conditions and Sequential Digestion. *Food Hydrocoll* **2017**, *62*, 35–42. https://doi.org/10.1016/J.FOODHYD.2016.07.025.

(126) Cirkovic Velickovic, T. D.; Stanic-Vucinic, D. J. The Role of Dietary Phenolic Compounds in Protein Digestion and Processing Technologies to Improve Their Antinutritive Properties. *Compr Rev Food Sci Food Saf* **2018**, *17* (1), 82–103. https://doi.org/10.1111/1541-4337.12320.

(127) Zhang, K.; Huang, J.; Wang, D.; Wan, X.; Wang, Y. Covalent Polyphenols-Proteins Interactions in Food Processing: Formation Mechanisms, Quantification Methods, Bioactive Effects, and Applications. *Front Nutr* **2024**, *11*, 1371401. https://doi.org/10.3389/FNUT.2024.1371401/BIBTEX.

(128) Chen, F. P.; Li, B. S.; Tang, C. H. Nanocomplexation between Curcumin and Soy Protein Isolate: Influence on Curcumin Stability/Bioaccessibility and in Vitro Protein Digestibility. *J Agric Food Chem* **2015**, *63* (13), 3559–3569. https://doi.org/10.1021/ACS.JAFC.5B00448/SUPPL_FILE/JF5B00448_SI_001.PDF.

(129) Abioye, R. O.; Obeme-Nmom, J. I.; Udenigwe, C. C. Pea Protein–Curcumin Interactions and Their Effects on In Vitro Protein Digestibility. *ACS Food Science & Technology* **2024**, *4* (3), 711–718. https://doi.org/10.1021/acsfoodscitech.3c00614.

(130) Sęczyk, Ł.; Świeca, M.; Gawlik-Dziki, U. Soymilk Enriched with Green Coffee Phenolics – Antioxidant and Nutritional Properties in the Light of Phenolics-Food Matrix Interactions. *Food Chem* **2017**, *223*, 1–7. https://doi.org/10.1016/J.FOODCHEM.2016.12.020.

(131) Hao, L.; Sun, J.; Pei, M.; Zhang, G.; Li, C.; Li, C.; Ma, X.; He, S.; Liu, L. Impact of Non-Covalent Bound Polyphenols on Conformational, Functional Properties and in Vitro Digestibility of Pea Protein. *Food Chem* **2022**, *383*. https://doi.org/10.1016/j.foodchem.2022.132623.

(132) Günal-Köroğlu, D.; Lorenzo, J. M.; Capanoglu, E. Plant-Based Protein-Phenolic Interactions: Effect on Different Matrices and in Vitro Gastrointestinal Digestion. *Food Research International* **2023**, *173*, 113269. https://doi.org/10.1016/J.FOODRES.2023.113269.

(133) Guan, H.; Zhang, W.; Sun-Waterhouse, D.; Jiang, Y.; Li, F.; Waterhouse, G. I. N.; Li, D. Phenolic-Protein Interactions in Foods and Post Ingestion: Switches Empowering Health Outcomes. *Trends Food Sci Technol* **2021**, *118*, 71–86. https://doi.org/10.1016/J.TIFS.2021.08.033.

(134) Kamiloglu, S.; Tomas, M.; Ozkan, G.; Ozdal, T.; Capanoglu, E. In Vitro Digestibility of Plant Proteins: Strategies for Improvement and Health Implications. *Curr Opin Food Sci* **2024**, *57*, 101148. https://doi.org/10.1016/J.COFS.2024.101148.

(135) Swieca M; Gawlik-Dziki, U. Nutritional and Health-Promoting Properties of Bean Paste Fortified with Onion Skin in the Light of Phenolic–Food Matrix Interactions. *Food Funct* **2015**, *6* (11), 3560–3566. https://doi.org/10.1039/C5FO00805K.

(136) Duodu, K. G.; Taylor, J. R. N.; Belton, P. S.; Hamaker, B. R. Factors Affecting Sorghum Protein Digestibility. *J Cereal Sci* **2003**, *38* (2), 117–131. https://doi.org/10.1016/S0733-5210(03)00016-X.

(137) Misquitta, S. A.; Kshirsagar, D. N.; Dange, P. R.; Choudhari, V. G.; Kabra, M. M. Digestibility of Proteins in Legumes. *Production and Utilization of Legumes - Progress and Prospects* **2023**. https://doi.org/10.5772/INTECHOPEN.110372.

(138) Thakur, A.; Sharma, V.; Thakur, A.; Vishal Sharma, C. An Overview of Anti-Nutritional Factors in Food. *~ 2472 ~ International Journal of Chemical Studies* **2019**, *7* (1).

(139) Gilani, G. S.; Xiao, C. W.; Cockell, K. A. Impact of Antinutritional Factors in Food Proteins on the Digestibility of Protein and the Bioavailability of Amino Acids and on Protein Quality. *British Journal of Nutrition* **2012**, *108* (S2), S315–S332. https://doi.org/10.1017/S0007114512002371.

(140) Mookerjee, A.; Tanaka, T. Influence of Enzymatic Treatments on Legume Proteins for Improved Functional and Nutritional Properties: Expansion of Legume Protein Utilization as Food Ingredients. *Curr Opin Food Sci* **2023**, *49*, 100974. https://doi.org/10.1016/J.COFS.2022.100974.

(141) Vergnolle, N. Protease Inhibition as New Therapeutic Strategy for GI Diseases. *Gut* **2016**, *65* (7), 1215–1224. https://doi.org/10.1136/GUTJNL-2015-309147.

(142) Avilés-Gaxiola, S.; Chuck-Hernández, C.; Serna Saldívar, S. O. Inactivation Methods of Trypsin Inhibitor in Legumes: A Review. *J Food Sci* **2018**, *83* (1), 17–29. https://doi.org/10.1111/1750-3841.13985.

(143) Guillamón, E.; Pedrosa, M. M.; Burbano, C.; Cuadrado, C.; Sánchez, M. de C.; Muzquiz, M. The Trypsin Inhibitors Present in Seed of Different Grain Legume Species and Cultivar. *Food Chem* **2008**, *107* (1), 68–74. https://doi.org/10.1016/J.FOODCHEM.2007.07.029.

(144) Joye, I. Protein Digestibility of Cereal Products. *Foods 2019, Vol. 8, Page 199* **2019**, *8* (6), 199. https://doi.org/10.3390/FOODS8060199.

(145) Raes, K.; Knockaert, D.; Struijs, K.; Van Camp, J. Role of Processing on Bioaccessibility of Minerals: Influence of Localization of Minerals and Anti-Nutritional Factors in the Plant. *Trends Food Sci Technol* **2014**, *37* (1), 32–41. https://doi.org/10.1016/J.TIFS.2014.02.002.

(146) Frutos Fernández, P. de; Ruiz Mantecón, Á.; Hervás Angulo, G.; Giráldez García, F. J. Tannins and Ruminant Nutrition: Review. *Spanish journal of agricultural research* **2004**, *2* (2), 191–202.

(147) Reddy, N. R. Occurrence, Distribution, Content, and Dietary Intake of Phytate. **2001**, 41–68. https://doi.org/10.1201/9781420014419-5.

(148) Bora, P. Anti-Nutritional Factors in Foods and Their Effects. *Journal of Academia and Industrial Research (JAIR)* **2014**, *3* (6).

(149) Kumar, S.; Verma, A. K.; Das, M.; Jain, S. K.; Dwivedi, P. D. Clinical Complications of Kidney Bean (Phaseolus Vulgaris L.) Consumption. *Nutrition* **2013**, *29* (6), 821–827. https://doi.org/10.1016/J.NUT.2012.11.010.

(150) Nissar, J.; Ahad, T.; Naik, H. R.; Hussain, S. Z. A Review Phytic Acid: As Antinutrient or Nutraceutical. *J Pharmacogn Phytochem* **2017**, *6* (6), 1554–1560.

(151) Burgess, J. R.; Gao, F. The Antioxidant Effects of Inositol Phosphates. **2001**, 205–214. https://doi.org/10.1201/9781420014419-13.

(152) Gemede, H. F.; Ratta, N. Habtamu Fekadu Gemede, Negussie Ratta. Antinutritional Factors in Plant Foods: Potential Health Benefits and Adverse Effects. *International Journal of Nutrition and Food Sciences* **2014**, *3* (4), 284–289. https://doi.org/10.11648/j.ijnfs.20140304.18.

CHAPTER 2 - PEA ALBUMIN, GLUTELIN, AND GLOBULIN INTERACTIONS WITH CURCUMIN AND THEIR EFFECTS ON *IN VITRO* GASTRIC DIGESTIBILITY

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Abstract

Yellow pea (*Pisum sativum*) is considered a rich source of high-quality protein. In this study, three pea protein fractions, albumin, globulin, and glutelin, were investigated for their interaction with curcumin, and the effect on *In vitro* gastric protein digestibility. A combination of protein fractions (0.25 mg/mL) with curcumin (2-100 μM) decreased the average particle size based on dynamic light scattering analysis. Ultraviolet-visible spectroscopy, which showed decreased transmittance, and surface hydrophobicity analysis revealed the formation of protein-curcumin nanocomplexes via noncovalent interactions. Transmission electron microscopy revealed the formation of spherical nano-complexes for albumin and globulin fractions and ordered sheet-like structures for glutelin fraction in the presence of curcumin. Curcumin binding did not alter the protein profiles, as demonstrated by reducing SDS-PAGE. Furthermore, curcumin binding at high concentration significantly decreased the degree of hydrolysis of albumin, glutelin and globulin by 76.64%, 58.67%, and 100% respectively, indicating that the resulting protein aggregation may have hindered pepsin accessibility to the proteins for hydrolysis. The results reflect the possible effect of polyphenol interactions on protein digestibility at the intestinal phase and undigested peptide fractions in the colon. Taken together, the study enhances the understanding of how interactions between food biomolecules influence the nutritional value of protein in food products and dietary supplements.

Keywords: Pea protein fractions, curcumin, biomolecular interactions, aggregation, food matrix interactions, *In vitro* gastric protein digestibility.

2.1. Introduction

Common pea seed (*Pisum sativum* L) is a well-known and widely consumed bean variety in the Fabaceae family. Due to its commercial availability, low cost, high yield, improved digestibility, low allergenic potential, and many health benefits, including anti-inflammatory, antioxidant, antimicrobial, anti-renal fibrosis, anti-diabetic, anti-hypertensive, and regulation of intestinal microbial composition as well as metabolism syndrome, it is considered a high-quality protein and a beneficial ingredient in the global food system.[1] Pea protein has a balanced amino acid profile and a great deal of lysine which contributes to immune system health.[2] It is comprised of four distinct proteins, albumin, globulin, glutelin, and prolamin, each of which can be isolated in separate fractions depending on the extraction solvent used and its solubility in aqueous solutions.[3] These protein fractions are important components of pea seeds that can be extracted individually or in combination to produce a heterogeneous product known as pea protein isolate (PPI).[3] Pea albumin, an enzymatic and metabolic protein with cytosolic function, is made up of components essential to the development of seedlings and accounts for about 18-25% of the entire protein content in pea seeds.[4,5] This water-soluble protein is a member of the 2S storage protein family and contains lectins, enzymes, and inhibitors of amylase and protease.[4,6] Pea globulin is the primary protein used for storing seeds and, is soluble in salt solutions. This fraction also accounts for 65–80% of the total protein content of peas and is a member of the seed storage protein classes 7S and 11S. It contains three parts: two significant portions, legumin and vicilin and a small quantity of a third kind called convicilin.[7–9] Pea glutelin contains a high concentration of hydrophobic amino acids, including proline, phenylalanine, valine, and tyrosine, and it is soluble in diluted alkaline solution.[9] Pea prolamin, another class of plant storage proteins, is found in modest amounts in pea seeds and is distinguished by high glutamine and proline levels. It is often only soluble in strong alcohol solutions of roughly 70–80%.[10] In general, albumins and globulins are the primary protein fractions that contribute to the functionality of pea proteins in food applications, such as drinks, sauces, and custards.[11]

Pea proteins have gained recognition as a sustainable alternative for functional food formulation and food applications owing to their extensive nutritional value and effective functional properties. These properties can be categorized based on their system such as hydrating capacity (solubility, water and oil holding capacity), structural and rheological attributes of proteins (gel formation and viscosity), and surface morphology (foaming and emulsification).[12] However, interactions

between proteins and dietary ingredients in complex food matrices can result in structural and functionality changes.[13] Bioactive substances, such as curcumin, which possess medicinal and nutraceutical properties, are incorporated into functional food formulations considering their many health-promoting and therapeutic attributes, including antibacterial, hypoglycemic, anticancer, anti-inflammatory, and antiparasitic properties.[14] In order to achieve these attributes, the bioactive compound has to reach the absorptive region unaffected, avoiding enzymatic hydrolysis or oxidative degradation. In this context, protein-polyphenol complexes are employed as a delivery system in functional food formulations.[15]

Turmeric (*Curcuma longa*), a member of the Zingiberaceae family with medicinal tuberous roots known as rhizomes, contains the phenolic compound curcumin which is a water-insoluble bioactive molecule.[16] It is produced and used for food coloring, spices, herbs, and nutritional supplements in tropical Asian countries like India.[16] Despite its safety and value indications, this hydrophobic ingredient has properties that restrict its use in food composition, including low bioavailability, physiological pH instability, insolubility in water, delayed cell absorption, and rapid metabolism inside cells.[14] Several delivery approaches, including emulsions, nanoparticles, and nano-emulsions—also referred to as encapsulating systems have been identified as a means of overcoming these obstacles and are utilized to enhance its solubility, stability, bioaccessibility, and bioavailability.[17,18] Currently, a lot of work is being done to determine and employ the natural sources of dietary proteins as transporters for these substances.[19] As naturally occurring biopolymers, proteins offer a safe, affordable, and potentially effective delivery method for highly nutritious, water-insoluble bioactive substances.[20,21] In addition to other uses including biodegradable/edible films, pea protein appears to be a viable option to develop a protein-based controlled-release delivery technique for bioactive components like curcumin, commonly known as nanoencapsulation.[17,21–23] Additionally, curcumin has been encapsulated or complexed utilizing anti-solvent processes using a variety of food-grade proteins, such as β-lactoglobulin, soy protein isolate, and rice bran albumin.[24,25] In a particular study, the walnut protein was used as a novel medium for the encapsulation of curcumin utilizing the solvent-free basic pH-shifting approach. The process of encapsulating curcumin into walnut proteins using a pH-driven method was found to enhance water solubility, antioxidant activity, and sustained release behavior in a simulated gastrointestinal environment. Additionally, this was found to be beneficial for suppressing the growth of breast cancer cell lines.[21]

Protein-curcumin interactions lead to the spontaneous development of nanocomplexes through hydrophobic interactions between the hydrophobic domains on the surface of proteins and curcumin molecules. Van der Waals interactions and hydrogen bonding may also be involved in specific situations.[26] This formation has been recognized to improve the solubility of curcumin-loaded native pea proteins and has demonstrated that the binding of curcumin and pea protein isolate with or without succinylation resulted in adequate encapsulation efficiency and release.[23] In earlier work, how variations in the structural properties of individual pea protein fractions affect their interaction with curcumin, the physicochemical properties and biostability of their nanocomplexes against pepsin in the gastric stage, and the importance in the gastrointestinal delivery of curcumin was investigated.[17] Although previous studies examined the interaction, structural difference, and *in vitro* protein digestibility of pea protein isolate-curcumin complexes,[27] little is known on how the pea protein fractions-curcumin complexation influences the physicochemical properties and *in vitro* gastric protein digestibility. Thus, this study examines the biomolecular interactions of pea albumin, glutelin, and globulin with their diverse properties in the presence of varying concentrations of curcumin and their effects on the *in vitro* gastric protein digestion phase.

2.2. Materials and Methods

2.2.1. Materials

Pea protein fractions from pea protein isolate of yellow pea seeds donated by Pulse Canada (Winnipeg, MB, Canada). Curcumin, ethanol, 8-anilino-1-naphthalenesulfonic acid (ANS), tris (hydroxymethyl) aminomethane (Tris-base), glycine, glycerol, hydrochloric acid (HCl), sodium hydroxide (NaOH), ammonium persulfate, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), Tris-HCl, bromophenol blue, disodium tetraborate decahydrate, *O*-phthaldialdehyde (OPA), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), L-serine, acrylamide/bis-acrylamide (30% solution), β-mercaptoethanol, acetic acid, methanol, and pepsin (≥250 units/mg solid) from porcine gastric mucosa were purchased from Millipore Sigma (Oakville, ON, Canada). Precision Plus Protein™ Dual Xtra Prestained Protein Standard (2-250 kDa) and Coomassie Brilliant Blue R-250 staining solution were purchased from BioRad (Mississauga, Ontario, Canada), UranyLess counterstain was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). The reagents utilized were of analytical grade (≥95%) without further purification.

2.2.2. Preparation of pea protein isolate fractions-curcumin complexes

Pea protein isolate (PPI) from yellow pea seeds were obtained using a previously described method.[3] Subsequent protein fractions (albumin from water (WSF), glutelin from alkaline (ASF) and globulin from salt (SSF) soluble fractions) were isolated as described previously.[17] Curcumin solution was prepared based on previous method.[23] A stock solution of 2.7 mM curcumin in ethanol was prepared then diluted to 1 mM and 0.5 mM in 60% and 30% ethanol, respectively, and protected from light. Subsequent dilutions were prepared in water to achieve curcumin concentrations of 0-200 μM. Protein fractions stock solution with a concentration of 0.25 mg/mL was prepared in water and mixed with an equal volume (1:1) of different curcumin concentrations to obtain a final concentration of 0.125 mg/mL of protein and 0, 2, 5, 10, 25, 50, 80 and 100 μM curcumin. All experiments were performed at room temperature (25 °C) in triplicates.

2.2.3. Dynamic light scattering

Particle and surface properties of the protein-curcumin complexes were characterized using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The average particle size, polydispersity index (PDI), and zeta potential of the protein fractions and their curcumin-loaded complexes were ascertained by preparing samples in disposable polystyrene cuvettes in Milli Q water (pH 7.0). Cuvettes were equilibrated for 10 s at 25 °C with a backscattered angle set as 173°, refractive index as 1.330, viscosity as 0.8872 cP, and dielectric constant as 78.5. An equal concentration of each protein fraction in water was used as the control.

2.2.4. Surface hydrophobicity

Surface hydrophobicity of the pea protein fractions-curcumin complexes was assessed using the ANS fluorescent probe. Mixtures of each protein fraction with curcumin in a final volume of 200 μL were combined with 5 μL of ANS in black 96-well microplates with clear bottoms and then incubated in the dark for 15 minutes. The fluorescence intensity was measured using a Spark multimode microplate reader (Tecan, Stockholm, Sweden), at the excitation (λ_{ex}) 390 nm and emission (λ_{em}) 470 nm.

2.2.5. Turbidity analysis

Each prepared protein fraction–curcumin mixtures were assessed for the complexation and absorbance spectral analysis from 280–600 nm wavelengths in a clear 96-well microplate using a

microplate reader (Tecan, Stockholm, Switzerland). Interactions between the protein fractions and curcumin were reported as percent transmittance using equation (1):

$$absorbance = -\log_{10}(\%\ transmittance) \quad (1)$$

2.2.6. Reducing SDS PAGE

The SDS-PAGE profile of the protein fractions under reducing conditions was analyzed on a 12% polyacrylamide gel (w/v, pH 8.8) and a 3% stacking gel (w/v, pH 6.8). A sample buffer, comprising 1.25 mL of 0.5 M Tris-HCl (pH 6.8), 2.5 mL of 50% glycerol, 2.0 mL of 10% (w/v) SDS, 0.2 mL of 0.5% (w/v) bromophenol blue, and 0.5 mL of β-mercaptoethanol (≥99.0%) - 14.3 M (pure liquid) in 6.45 mL deionized water, was prepared and mixed with an equal volume of the protein fraction-curcumin samples. The mixture was incubated at 95 ºC for 4 min with shaking at a speed of 300 rpm (Thermomixer, Eppendorf AG, Hamburg, Germany). In each well, 10 µL of the samples or 7 µL of the standard protein molecular weight marker were loaded and electrophoresis was performed at 120 V for 1.4h using BioRad Mini- PROTEAN Tetra System (BioRad Inc., Mississauga, ON, Canada). Afterward, the gel was stained with the Coomassie Brilliant Blue R-250 staining solution (Bio-Rad Inc., Mississauga, ON, Canada), destained with a destaining reagent (40% (v/v) methanol and 10% (v/v) acetic acid in water), and imaged using the ChemiDoc MP imaging system (Bio-Rad Inc., Canada).

2.2.7. Transmission electron microscopy

The morphology of the complexes formed by the mixtures of each protein fraction and curcumin was analyzed with a transmission electron microscope (JEM-1400Flash Electron Microscope, JEOL, Tokyo, Japan) with an accelerating voltage of 120 kV. Samples of each protein fraction with 100 µM curcumin (High) were assessed. On a 300-mesh Formvar-carbon-coated copper grid, 10 µL of each sample was added and incubated for 2 minutes. The unabsorbed sample was blotted off each grid using a Kim wipe and afterward, the grids were stained with UranyLess counterstain in the dark for 1 minute. Images of each sample nanocomplexes were processed using the ImageJ software (NIH, Bethesda, MD, USA).[28]

2.2.8. In vitro gastric digestion

Solutions of protein fractions-curcumin complexes (5 mL) were adjusted to pH 3.0 using 1 M HCl followed by the addition of 30 µL of pepsin solution (25,000 U/mL in Milli Q water). Thereafter, digestion was initiated by incubating at 37 ºC for 2 h with constant shaking at 90 rpm. To halt the

enzymatic activity, the digesta was adjusted to pH 7.0 with 1 M NaOH with vigorous shaking followed by freezing at -20 ºC.

2.2.9. Degree of hydrolysis

The degree of hydrolysis was determined by OPA assay as previously reported.[29] Briefly, 30 µL of serine standard, blank (Milli-Q water), or sample solutions were mixed with 225 µL of OPA reagent in a black 96-well microplate with clear bottoms. Fluorescence intensity was measured at λ_{ex} = 360 nm and λ_{em} = 460 nm using a Spark multimode microplate reader (Tecan, Stockholm, Sweden). Serine equivalents, represented as NH_2/g protein, were determined using a serine (0.5–5 mM) standard curve. The number of hydrolyzed bonds, *h*, was calculated using Equation 2:

$$h = \frac{serine{-}NH_2{-}\beta}{\alpha} \text{megq/g protein} \qquad (2)$$

Serine-NH_2 is the serine equivalent determined from the standard curve, and α and β are constants (α = 1.00, β = 0.4, theoretical general values for unexamined raw materials).[29,30] The degree of hydrolysis (%) was calculated using *h* and the total number of peptide bonds per protein equivalent, h_{tot}, as follows (Equation 3):

$$\text{Degree of hydrolysis (\%)} = \frac{h}{h_{tot}} \times 100 \qquad (3)$$

2.2.10. Statistical analysis

All data were provided as means of experimental triplicates ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) with GraphPad Prism version 10.0.2 for Windows (GraphPad Software, La Jolla, CA, USA). The significant difference between the mean values was defined at $p < 0.05$ using the Šídák multiple comparisons tests.

2.3. Results and discussion

2.3.1. Surface properties of pea protein fractions-curcumin complexes

Dynamic light scattering was used to observe the possible complexation between the protein fractions and curcumin. The particle characteristics of these complexes encompass the average size, size distribution and surface charge variation (Table 2.1) of the interactions formed between the three pea protein fractions and their curcumin-loaded complexes. As shown in (Table 2.1), with an increasing curcumin concentration, the average particle size of protein fractions-curcumin complexes was smaller relative to pea protein fractions. This suggests that the protein fraction-

curcumin interaction resulted in the formation of compact structures with increased monodispersity as indicated by the decrease in particle size and polydispersity index, respectively (Table 2.1). Results show fraction-dependent differences in the behavior of the surface characteristics of each protein-curcumin complex. At lower curcumin concentrations (≤10 μM), complexes with larger particles are formed with the WSF and curcumin (Table 2.1). On the other hand, interactions between curcumin and ASF and SSF significantly decreased the average particle size at all curcumin concentrations (Table 2.1). Similarly, the particle size of PPI-polyphenol complexes with increasing concentrations of epigallocatechin-3-gallate, chlorogenic acid, and resveratrol were smaller compared to PPI.[31] Meanwhile, in another study, the PPI-curcumin complexes were reported to have increased particle size with an increasing concentration of curcumin indicating the presence of agglomerate polydisperse species and curcumin encapsulation.[27] Increased curcumin concentrations (≥25 μM) resulted in increasing homogenous samples, characterized by a low polydispersity index (PDI ≤0.4), particularly with the WSF and SSF fractions (Table 2.1). Protein-curcumin complexes of all fractions at the highest concentration of curcumin showed PDI values mostly at 0.2, coupled with the decreased average particle size suggesting the formation of unified, tightly packed nanoparticles. This is also depicted with the unimodal particle size distribution with an exception to the behavior of ASF compared to WSF and SSF, which showed more heterogenicity initially and a value of 0.5 at the highest curcumin concentration (Fig. 2.1). ASF-curcumin-loaded complex recorded the same behavior.[17] It was observed that increasing the concentration of curcumin from 0 to 100 μM also reduced the zeta potential of the protein fractions. The high absolute value of the zeta potential generates a repulsive electrostatic force between particles, which is a key property of an agglomeration-resistant suspension. Thus, the higher the zeta potential, the more stable the dispersion and vice versa.[32] Also, high negative or positive zeta potential greater than ±30 mV leads to monodispersed particles.[33] WSF and SSF showed almost similar trends ranging from -46.4 to -27.7 mV with increased curcumin concentration (Table 2.1), indicating incipient particle stability in suspension. This further implies the possibility of forming nanoparticle aggregates. Of the three fractions, ASF showed the most negative surface charge however, the trend was similar to the other fractions with a significant increase in zeta potential to -40.8 mV at the highest curcumin concentration, implying protein-curcumin complexes with moderate stability. Previous studies also observed a decrease in

negative zeta potential for CWSF and CSSF, signifying poor stability, low binding with curcumin as well as low encapsulation efficiency.[17]

Table 2.1. Particle characteristics of pea albumin (WSF), glutelin (ASF) and globulin (SSF) protein fractions-curcumin complexes at increasing curcumin concentrations.

	WSF			***ASF***			***SSF***		
[Curcumin] ***(μM)***	Average particle size ***(nm)***	Polydispersity index	Zeta potential **(mV)**	Average particle size ***(nm)***	Polydispersity index	Zeta potential ***(mV)***	Average particle size ***(nm)***	Polydispersity index	Zeta potential ***(mV)***
0	4214 ± 52.8	0.796 ± 0.354	-46.4± 9.2	8068 ± 1673	0.945 ± 0.095	-56.9±1.9	3210 ± 186.0	1	-33.9± 0.2
2	3337 ± 02.6	0.619 ± 0.113	-33.5± 3.3**	2420±386.5 ***	0.961 ± 0.031	-54.1±3.8	2100±161.8* ***	0.736± 0.122**	-32.5 ± 18
5	3677 ± 02.3	0.842 ± 0.141	-37.3± 3.0*	2567±484.2 ***	0.963 ± 0.035	-50.9±3.5	1875±231.9* ***	0.697± 0.137**	-30.6± 1.4***
10	3314 ± 724.0	0.702 ± 0.056	-32.8± 0.4**	3010±745.2 ***	1.00	-54.3±2.2	812.6±112.2* ***	0.646±0.041** *	-31.8± 0.3*
25	352.3±59.90* ***	0.420 ± 0.019*	-35.8± 0.3*	3361±2768* *	1.00	-44.9±2.8	419.4±174.1* ***	0.426±0.126** **	-30± 0.5***
50	241.5±0.666* ***	0.267±0.018**	-34.3± 0.9**	742.1±322.4 ****	0.759 ± 0.188	-44.1 ± 14	234.7±7.488* ***	0.265±0.033** **	-28.7± 0.6****
80	291.1±24.42* ***	0.195±0.007** *	-32.2± 2.8**	577.0±137.0 ****	0.564±0.065** *	-41.3± 0.4*	283.5±8.823* ***	0.218±0.039** **	-25.7± 0.5****
100	365.7±12.05* ***	0.235±0.043**	-27.7± 0.8****	733.1±88.48 ****	0.579±0.078** *	-40.8± 2.4*	304.3±12.10* ***	0.272±0.023** **	-22.4± 1.1****

The asterisks in a column indicate that the mean values are significantly different compared to WSF, ASF and SSF only (0 μM curcumin); * = significant ($0.01 < p < 0.05$), ** = very significant ($0.001 < p < 0.01$), * = extremely significant ($0.0001 < p < 0.001$), **** = extremely significant ($p < 0.0001$)**

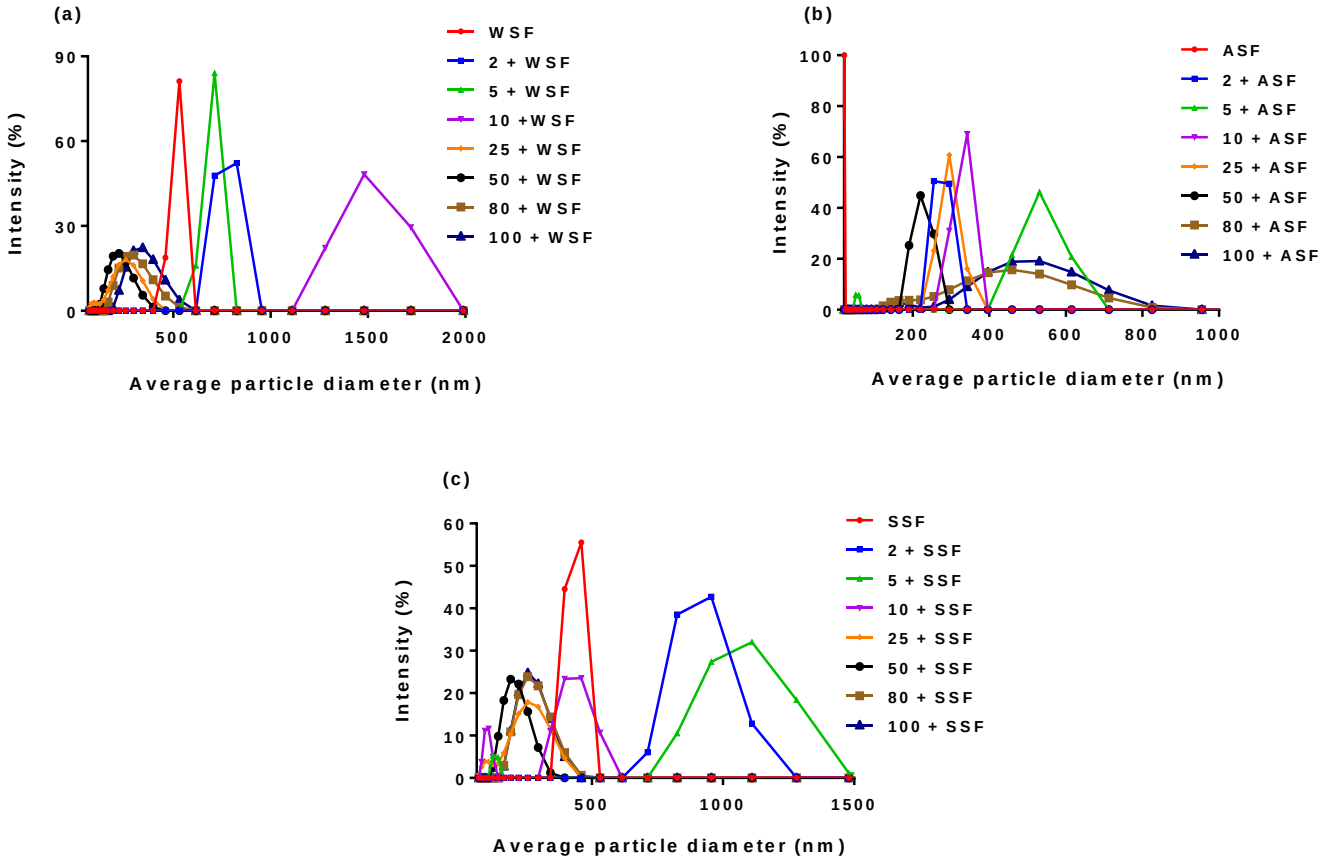

Fig. 2.1. Particle size distribution by intensity of pea (a) albumin (WSF), (b) glutelin (ASF), and (c) globulin (SSF) respectively with each protein fractions-curcumin mixtures at different concentrations in µM.

2.3.2. Surface hydrophobicity

Hydrophobic interactions are one of the prominent driving forces toward the formation of protein-curcumin nanocomplexes. A major approach to quantify the surface hydrophobicity of protein is through fluorescent probes like ANS which preferentially binds hydrophobic protein sites. Upon binding of the probes to accessible hydrophobic regions of proteins, an increase in fluorescence is observed, which is used as a measure of protein surface hydrophobicity.[34] Thus, the effect of protein-curcumin interaction on the surface hydrophobicity of pea protein fractions was explored using this probe. As shown in (Fig. 2.2), increasing curcumin concentration resulted in a significant decrease (p<0.05) in the surface hydrophobicity of the protein fractions, especially at 100 µM curcumin, in order of ASF, WSF and SSF as previously reported.[17] Increased surface

hydrophobicity of ASF was reported to imply its high content of hydrophobic amino acid residues like tryptophan, leucine, and alanine whereas the lower surface hydrophobicity of the latter pea protein fractions (WSF and SSF) were attributed to their high hydrophilic amino acid contents.[17] The binding of curcumin to the hydrophobic regions of the protein fractions reduces the available binding sites for ANS, thus reducing the fluorescence intensity of ANS. It appears that the binding of curcumin is more efficient in WSF and SSF compared to ASF with the highest surface hydrophobicity (Fig. 2.2b). This could imply that there is still the presence of unoccupied hydrophobic pockets of the protein and probably the formation of nanoparticles with increasing curcumin concentration inhibits adequate access to binding with ANS. Since the hydrophobic sites are buried in the nanoparticle core, this decrease can be attributed to the extent of nanoparticle formed or how densely packed the nanoparticle is depending on the protein fraction. This result indicates that the binding affinity of ANS was reduced due to competition for the protein hydrophobic core by curcumin, as observed by previous studies with PPI.[27] Of the three fractions, ASF was observed to have the most similar behavior to PPI with a reduced surface hydrophobicity and a slight increase at the highest curcumin concentrations (>50 µM) (Fig. 2.2b).[27] This may be explained by the possibility that some of the original PPI proteins were retained in the alkaline soluble fraction since the PPI extraction procedure was performed in an alkaline environment.[17]

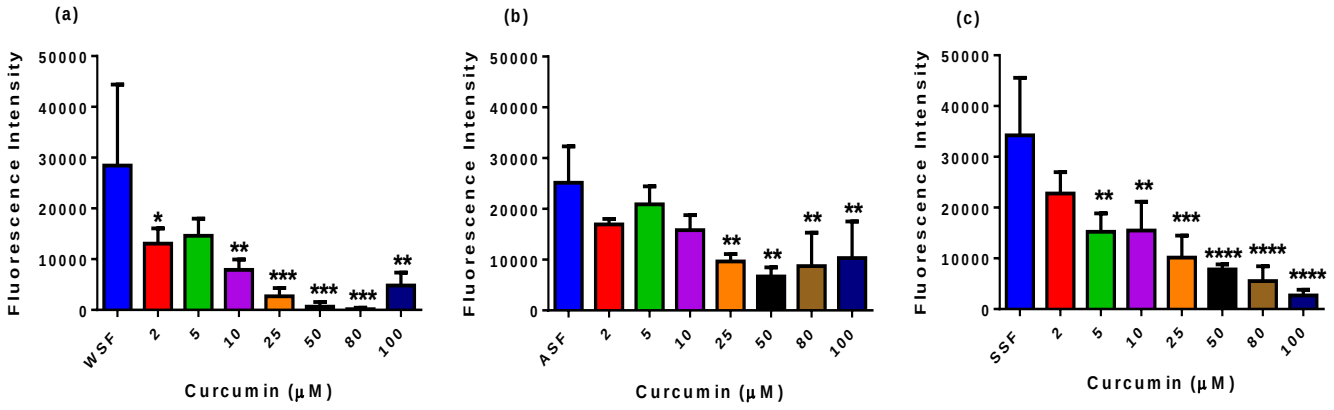

Fig. 2.2. Surface hydrophobicity of pea (a) albumin (WSF), (b) glutelin (ASF), and (c) globulin (SSF) fractions with increasing curcumin concentrations in µM.* = significant (0.01 < p < 0.05), ** = very significant (0.001 < p < 0.01), * = extremely significant (0.0001< p < 0.001), **** = extremely significant (p < 0.0001).**

2.3.3. Turbidity analysis

The absorbance spectra were obtained to understand the nature of the interaction between the protein fractions and curcumin (Fig. 2.3a-c). Results showed a concentration-dependent increase in absorbance with a peak absorbance at 430 nm, corresponding to the absorbance maxima of curcumin. Moreover, there were no further shifts at the absorbance maxima of both protein fractions and their complexes formed with curcumin which could imply the absence of the formation of new covalent bonds as seen in previous studies for PPI.[27] This can be attributed to the fact that hydrogen bonding, Van der Waals forces, and hydrophobic and electrostatic interactions which are characteristic of non-covalent interactions may be the driving force behind the nano-complexation between protein and curcumin.[31] Furthermore, a significant decrease in transmittance with increasing curcumin concentrations was observed for all protein fractions (Fig. 2.3d-f). Results obtained follow the literature that showed similar trends for PPI in the presence of increasing curcumin concentrations.[27] A slight increase in transmittance at the highest curcumin concentration for ASF was also observed (Fig. 2.3e). The deviation in trend highlights the potential instability of the interaction between protein and curcumin, which might result in partial disintegration of some unstable aggregates and an increase in protein and curcumin solubility.

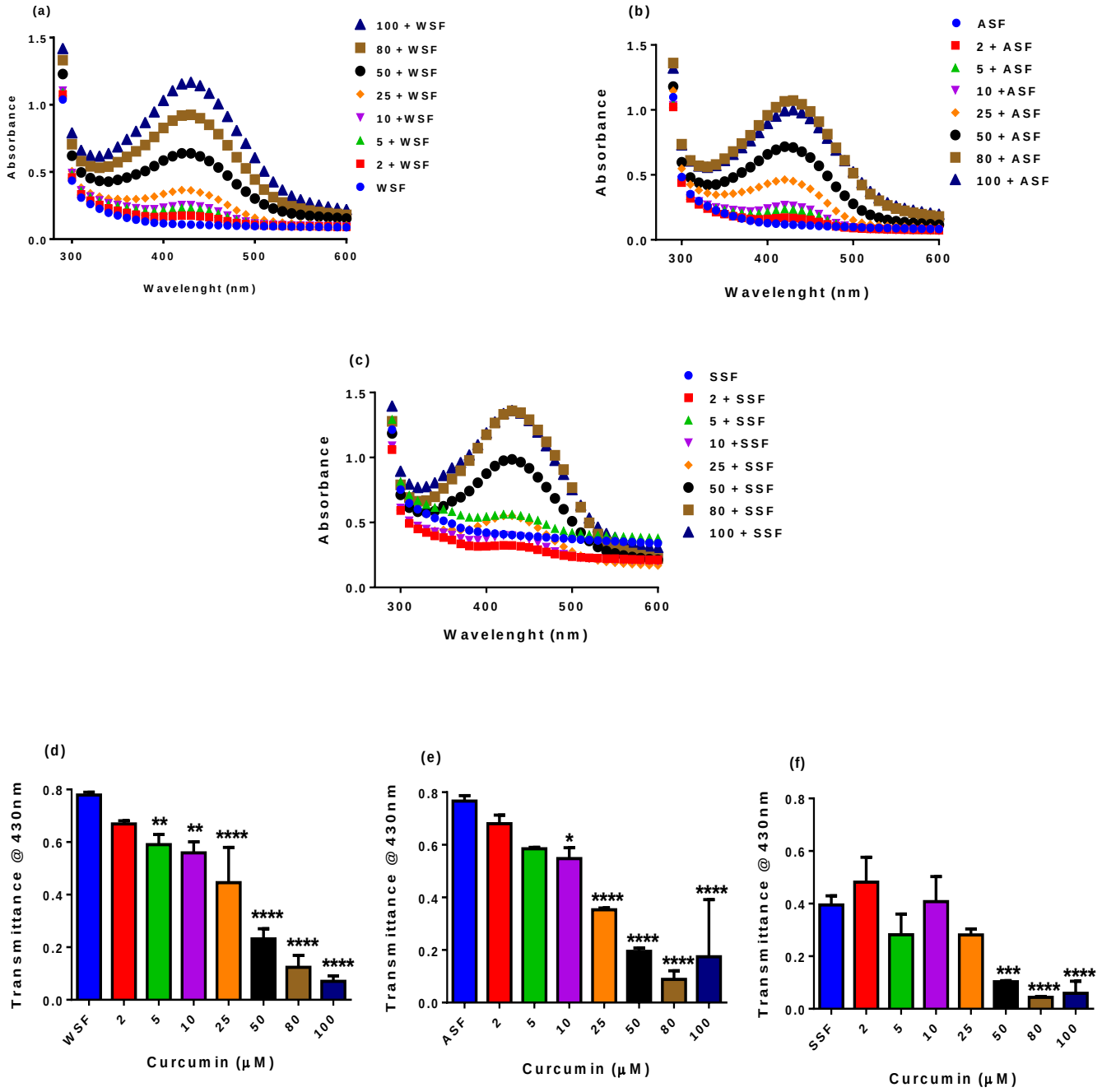

Fig. 2.3. Ultraviolet-visible spectroscopy (absorbance spectra) (a-c) and percent transmittance (d-f) (at curcumin λ_{max} = 430 nm) of pea albumin (WSF), glutelin (ASF), and globulin (SSF) at different curcumin concentrations in µM.

2.3.4. SDS gel electrophoresis

Under reducing gel electrophoresis conditions of the protein disulfide bonds, the effects of the interaction of pea protein fractions and curcumin on the molecular weight protein profile at the highest concentration of curcumin were analyzed. As observed in (Fig. 2.4), WSF, ASF and SSF have different protein composition profiles comparable to results in previous studies.[3,17] Differences in the outcome of the displayed protein profile for each protein fraction alone and in the presence of curcumin mixtures were barely noticeable except for a very slight difference in band intensity. The electrophoretic patterns for WSF showed prominent bands <37 kDa, ASF had several bands between 150 and 20 kDa with 37 kDa having the highest proportion corresponding to the results obtained for PPI.[3,17] Protein bands between 50 and 20 kDa were observed for SSF with an increased intensity at 20 kDa. This indicates that curcumin displayed no effects on the protein composition of the proteins. This is explained by the ultraviolet (UV)-visible spectroscopy results (Fig. 2.3a-c), which suggest that no new covalent bonds emerged during the interaction.

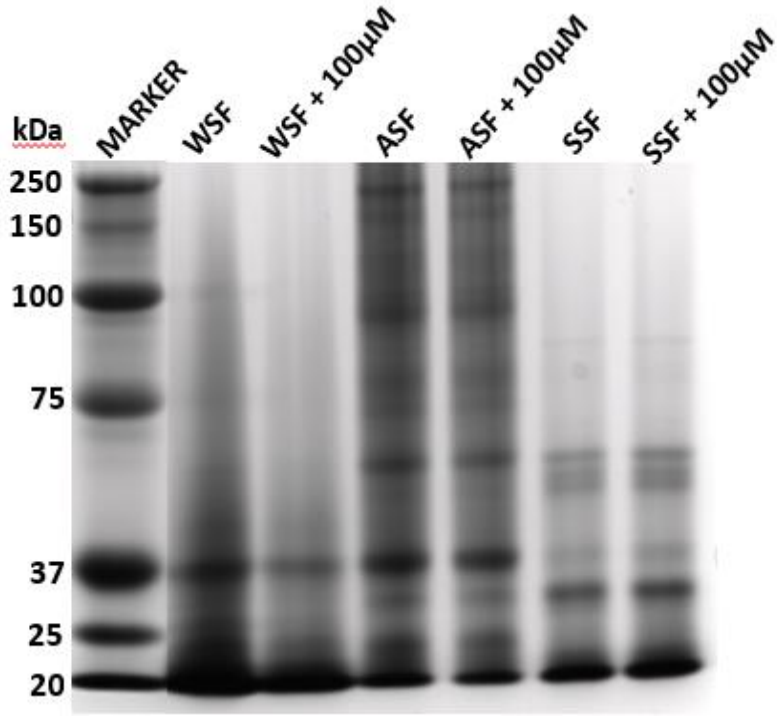

Fig. 2.4. Protein profile from reducing SDS-PAGE of WSF, ASF, and SSF protein fractions alone and in the presence of 100µM curcumin.

2.3.5. Transmission electron microscopy

The morphology of protein fractions alone and with the highest curcumin concentration (100 µM) was investigated via transmission electron microscopy (TEM). In the absence of curcumin, all

protein fractions showed amorphous morphology to varying degrees, with SSF showing the formation of some fibrillar structures (Fig. 2.5). However, clear morphological changes in the protein aggregates are observed in the presence of curcumin. Complexation between WSF and curcumin displayed the formation of hollow nanoparticles. The surface of the nanoparticles has a denser structure indicating the possible presence of precipitates or agglomerates around the nanoparticles, unlike the control which displayed a network of particles connected by weak fibrils. This also supports the findings where curcumin was not effectively encapsulated in all CWSF particles with images showing nanoparticles with empty or partially occupied cores.[17] SSF in the presence of curcumin results in the formation of denser nanoparticles of smaller sizes compared to WSF. Unlike WSF and SSF, ASF with curcumin formed ordered sheet-like structures connected by a network of fibrils compared to the control which showed a more heterogeneous species strongly held together by intermolecular forces. This result can be supported by the DLS analysis and surface hydrophobicity which revealed a reduction in average-size particles hence the formation of nanoparticles with increasing curcumin concentration (Table 2.1, Fig. 2.2b). However, these particles showed the possibility of forming aggregates due to insoluble complexes and precipitation as a result of binding with curcumin (Fig. 2.2b, Fig. 2.3b). Conversely, these modifications could play a crucial role in the digestion of proteins in gastrointestinal conditions.

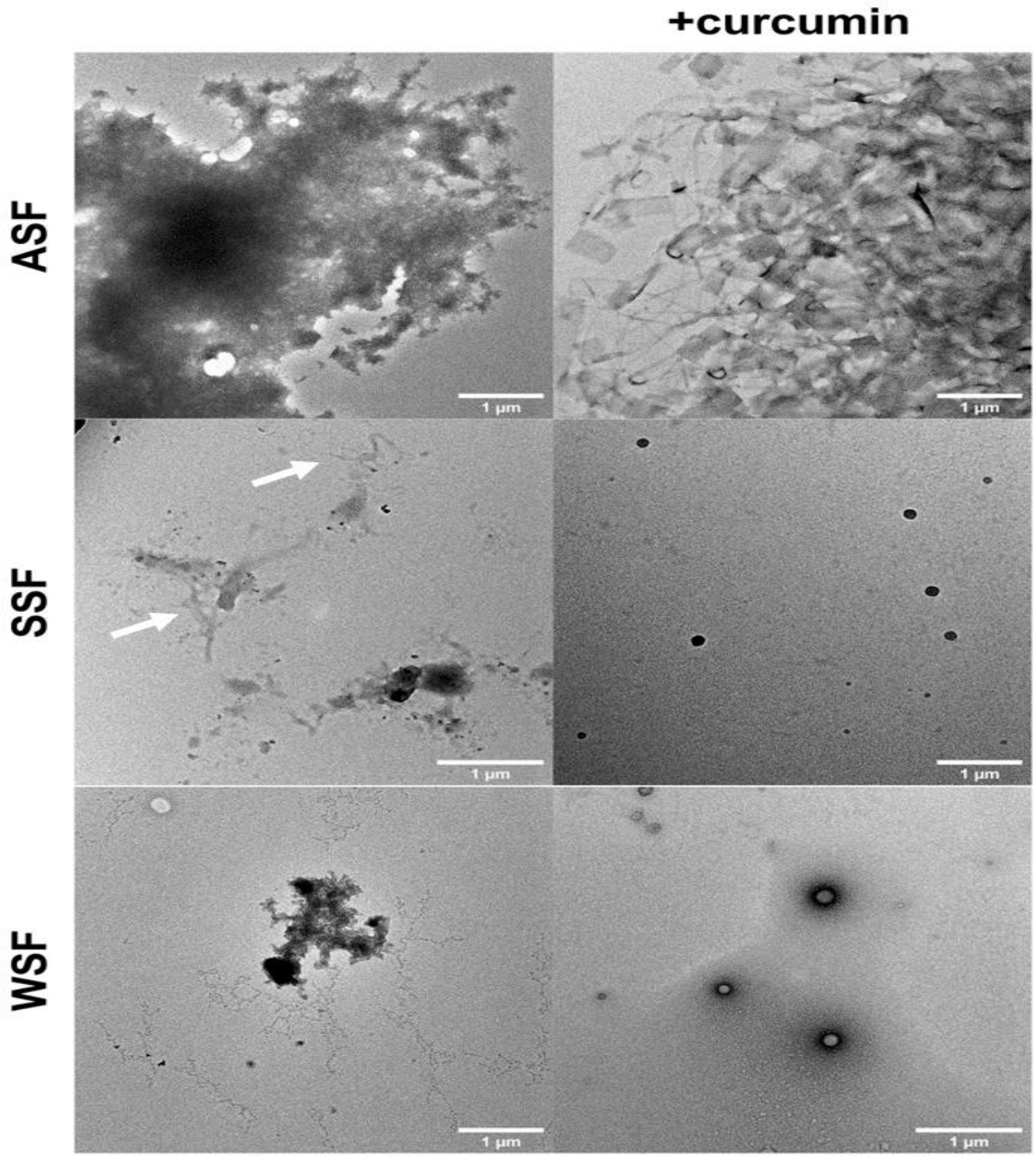

Fig. 2.5. Transmission electron microscopy of ASF, SSF, and WSF protein fractions alone and in the presence of 100 µM curcumin. Scale bar represents 1 µm. White arrows indicate the presence of protein fibrillar structures in SSF.

2.3.6. In vitro gastric digestibility of pea protein fractions-curcumin complexes

The influence of pea protein fractions-curcumin interaction on the *In vitro* gastric protein digestibility was assessed by pepsin hydrolysis. These interactions are unique for each protein fraction. In (Fig. 2.6), the effects of curcumin interaction on protein digestibility are apparent with

increasing curcumin concentration. The polyphenol significantly reduced the protein digestibility of WSF, SSF, and ASF to 76.64%, 58.67%, and 100%, respectively, as compared to their respective controls. WSF (albumin) displayed the most significant decrease in protein digestibility (Fig. 2.6a), which may be ascribed to the tightly folded, non-spiral and globular structure of albumin, which hindered the active regions of digestive enzymes, or the protein cleavage sites which are in accordance with the reduced protein digestibility of bovine serum albumin after complexation with tannic acid.[35] Notably, curcumin decreased the hydrolysis of ASF (glutelin) drastically, and no protein was hydrolyzed at the highest concentration of curcumin. There was a gradual decrease at lower curcumin concentrations (≤50 µM), however, digestion was completely diminished above 80 µM of curcumin (Fig. 2.6b). This suggests that the curcumin-induced highly ordered sheet-like structures tightly connected by a network of fibrils significantly hindered proteolytic cleavage by blocking pepsin cleavage sites, or by increasing the hydrophobicity of the protein surface, preventing digestive enzymes from accessing protein target sites. Thus, the negative effects of curcumin on protein digestibility is more pronounced with ASF compared to WSF and SSF. Furthermore, earlier studies found a substantial curcumin ASF binding as a result of high protein surface hydrophobicity, which inhibits the accessibility of pepsin.[17]

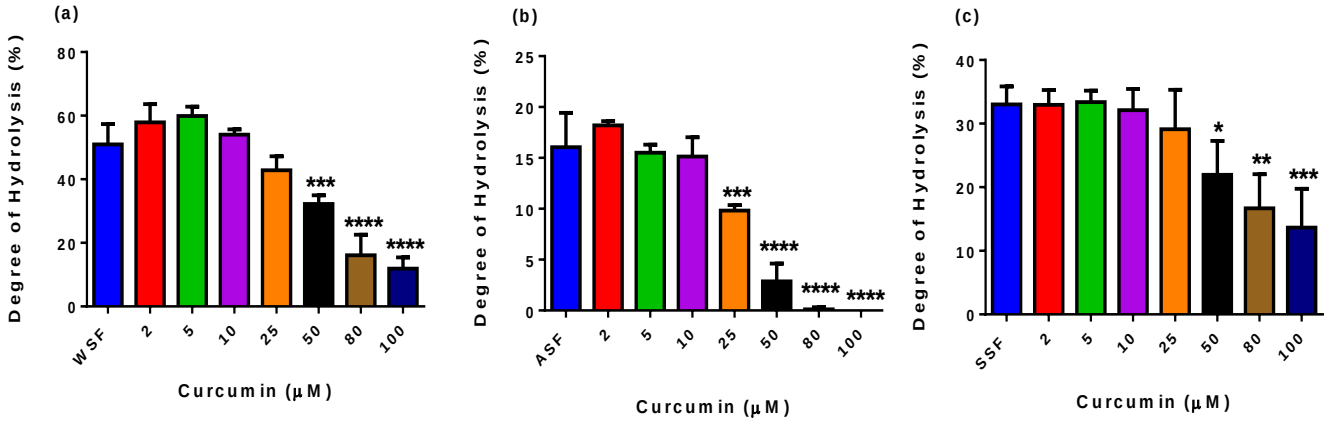

Fig. 2.6. Degree of Hydrolysis for pea (a) albumin (WSF), (b) glutelin (ASF), and (c) globulin (SSF) respectively at different curcumin concentrations in µM after pepsin hydrolysis.

2.4. Conclusion

The interaction between curcumin and pea protein fractions, WSF, ASF and SSF, and its effect on the *In vitro* gastric protein digestibility was studied. Results showed the formation of insoluble

nanocomplexes influenced by binding with curcumin which resulted in ordered structures as seen in DLS, TEM and surface hydrophobicity. It is most likely that covalent bonding did not occur, indicating that non-covalent interactions facilitated the complexes generated by the protein and curcumin. Considering the framework of the food matrix, protein-polyphenol complexation is as crucial as their formation; interactions with curcumin were demonstrated to drastically hinder protein digestion due to the restricted accessibility of digestive enzymes. Protein digestibility can be reduced, and digestion activity can be rendered inactive by polyphenols when combined with digestive enzymes to form a complex. Digestibility is influenced by the concentration of curcumin, the structure of proteins, and the nature of binding. Meanwhile, there might be promising effects as regards the gastrointestinal absorption of curcumin. The results of this study provide insight into how interactions across various food structures impact the nutritional content of protein in food products and their behavior in the gastric phase of digestion. This information can be utilized to understand physiological traits related to the use of proteins as polyphenol delivery systems. There is a need to balance the amount of curcumin to achieve the goals of effective release of curcumin after encapsulation and maintain its biological and therapeutic properties without detrimental effects on protein digestibility.

CRediT Author Statement

Judith Oballa: Conceptualization, Investigation, Methodology, Formal analysis, Validation and Writing – original draft preparation, Writing – review and editing. Raliat Abioye: Visualization, and Writing - review and editing. Joy Obeme-Nmom: Writing – review and editing. Chibuike Udenigwe: Funding acquisition, Conceptualization, Validation, Resources, Supervision and Writing- Review and editing.

2.5. References

(1) Ge, J.; Sun, C.; Corke, H.; Gul, K.; Gan, R.; Fang, Y. The Health Benefits, Functional Properties, Modifications, and Applications of Pea (Pisum Sativum L.) Protein: Current Status, Challenges, and Perspectives. *Compr Rev Food Sci Food Saf* **2020**, *19* (4), 1835–1876. https://doi.org/10.1111/1541-4337.12573.

(2) Huntrakul, K.; Yoksan, R.; Sane, A.; Harnkarnsujarit, N. Effects of Pea Protein on Properties of Cassava Starch Edible Films Produced by Blown-Film Extrusion for Oil Packaging. *Food Packag Shelf Life* **2020**, *24*. https://doi.org/10.1016/j.fpsl.2020.100480.

(3) Adebiyi, A. P.; Aluko, R. E. Functional Properties of Protein Fractions Obtained from Commercial Yellow Field Pea (Pisum Sativum L.) Seed Protein Isolate. *Food Chem* **2011**, *128* (4), 902–908. https://doi.org/10.1016/j.foodchem.2011.03.116.

(4) Warnakulasuriya, S. N.; Nickerson, M. T. Review on Plant Protein–Polysaccharide Complex Coacervation, and the Functionality and Applicability of Formed Complexes. *J Sci Food Agric* **2018**, *98* (15), 5559–5571. https://doi.org/10.1002/jsfa.9228.

(5) McCarthy, N. A.; Kennedy, D.; Hogan, S. A.; Kelly, P. M.; Thapa, K.; Murphy, K. M.; Fenelon, M. A. Emulsification Properties of Pea Protein Isolate Using Homogenization, Microfluidization and Ultrasonication. *Food Research International* **2016**, *89*, 415–421. https://doi.org/10.1016/J.FOODRES.2016.07.024.

(6) Lan, Y.; Chen, B.; Rao, J. Pea Protein Isolate–High Methoxyl Pectin Soluble Complexes for Improving Pea Protein Functionality: Effect of PH, Biopolymer Ratio and Concentrations. *Food Hydrocoll* **2018**, *80*, 245–253. https://doi.org/10.1016/j.foodhyd.2018.02.021.

(7) Lu, Z. X.; He, J. F.; Zhang, Y. C.; Bing, D. J. Composition, Physicochemical Properties of Pea Protein and Its Application in Functional Foods. *Crit Rev Food Sci Nutr* **2020**, *60* (15), 2593–2605. https://doi.org/10.1080/10408398.2019.1651248.

(8) Burger, T. G.; Zhang, Y. Recent Progress in the Utilization of Pea Protein as an Emulsifier for Food Applications. *Trends Food Sci Technol* **2019**, *86*, 25–33. https://doi.org/10.1016/j.tifs.2019.02.007.

(9) Lam, A. C. Y.; Can Karaca, A.; Tyler, R. T.; Nickerson, M. T. Pea Protein Isolates: Structure, Extraction, and Functionality. *Food Reviews International* **2018**, *34* (2), 126–147. https://doi.org/10.1080/87559129.2016.1242135.

(10) Shanthakumar, P.; Klepacka, J.; Bains, A.; Chawla, P.; Dhull, S. B.; Najda, A. The Current Situation of Pea Protein and Its Application in the Food Industry. *Molecules* **2022**, *27* (16), 5354. https://doi.org/10.3390/molecules27165354.

(11) Tulbek, M. C.; Lam, R. S. H.; Wang, Y. C.; Asavajaru, P.; Lam, A. Pea: A Sustainable Vegetable Protein Crop. *Sustainable Protein Sources* **2017**, 145–164. https://doi.org/10.1016/B978-0-12-802778-3.00009-3.

(12) Klupšaitė, D.; Juodeikienė, G. Legume: Composition, Protein Extraction and Functional Properties. A Review. *Chemical Technology* **2015**, *66* (1), 5–12. https://doi.org/10.5755/j01.ct.66.1.12355.

(13) Boachie, R. T.; Okagu, O. D.; Abioye, R.; Hüttmann, N.; Oliviero, T.; Capuano, E.; Fogliano, V.; Udenigwe, C. C. Lentil Protein and Tannic Acid Interaction Limits In Vitro Peptic Hydrolysis and Alters Peptidomic Profiles of the Proteins. *J Agric Food Chem* **2022**, *70* (21), 6519–6529. https://doi.org/10.1021/acs.jafc.2c00197.

(14) Yonar, M. E.; Yonar, M. S.; İspir, Ü.; Ural, M. Ş. Effects of Curcumin on Haematological Values, Immunity, Antioxidant Status and Resistance of Rainbow Trout (Oncorhynchus Mykiss) Against Aeromonas Salmonicida Subsp. Achromogenes. *Fish Shellfish Immunol* **2019**, *89*, 83–90. https://doi.org/10.1016/j.fsi.2019.03.038.

(15) Ribnicky, D. M.; Roopchand, D. E.; Oren, A.; Grace, M.; Poulev, A.; Lila, M. A.; Havenaar, R.; Raskin, I. Effects of a High Fat Meal Matrix and Protein Complexation on the Bioaccessibility of Blueberry Anthocyanins Using the TNO Gastrointestinal Model (TIM-1). *Food Chem* **2014**, *142*, 349–357. https://doi.org/10.1016/J.FOODCHEM.2013.07.073.

(16) Siviero, A.; Gallo, E.; Maggini, V.; Gori, L.; Mugelli, A.; Firenzuoli, F.; Vannacci, A. Curcumin, a Golden Spice with a Low Bioavailability. *J Herb Med* **2015**, *5* (2), 57–70. https://doi.org/10.1016/J.HERMED.2015.03.001.

(17) Okagu, O. D.; Udenigwe, C. C. Molecular Interactions of Pea Globulin, Albumin and Glutelin With Curcumin: Formation and Gastric Release Mechanisms of Curcumin-Loaded Bio-Nanocomplexes. *Food Biophys* **2022**, *17* (1), 10–25. https://doi.org/10.1007/s11483-021-09697-5.

(18) Ghayour, N.; Hosseini, S. M. H.; Eskandari, M. H.; Esteghlal, S.; Nekoei, A. R.; Hashemi Gahruie, H.; Tatar, M.; Naghibalhossaini, F. Nanoencapsulation of Quercetin and Curcumin in Casein-Based Delivery Systems. *Food Hydrocoll* **2019**, *87*, 394–403. https://doi.org/10.1016/J.FOODHYD.2018.08.031.

(19) Esmaili, M.; Ghaffari, S. M.; Moosavi-Movahedi, Z.; Atri, M. S.; Sharifizadeh, A.; Farhadi, M.; Yousefi, R.; Chobert, J. M.; Haertlé, T.; Moosavi-Movahedi, A. A. Beta Casein-Micelle as a Nano Vehicle for Solubility Enhancement of Curcumin; Food Industry Application. *LWT - Food Science and Technology* **2011**, *44* (10), 2166–2172. https://doi.org/10.1016/J.LWT.2011.05.023.

(20) Xie, H.; Xiang, C.; Li, Y.; Wang, L.; Zhang, Y.; Song, Z.; Ma, X.; Lu, X.; Lei, Q.; Fang, W. Fabrication of Ovalbumin/κ-Carrageenan Complex Nanoparticles as a Novel Carrier for Curcumin Delivery. *Food Hydrocoll* **2019**, *89*, 111–121. https://doi.org/10.1016/J.FOODHYD.2018.10.027.

(21) Moghadam, M.; Salami, M.; Mohammadian, M.; Delphi, L.; Sepehri, H.; Emam-Djomeh, Z.; Moosavi-Movahedi, A. A. Walnut Protein–Curcumin Complexes: Fabrication, Structural Characterization, Antioxidant Properties, and in Vitro Anticancer Activity. *Journal of Food Measurement and Characterization* **2020**, *14* (2), 876–885. https://doi.org/10.1007/s11694-019-00336-9.

(22) Zhang, H.; Wang, T.; He, F.; Chen, G. Fabrication of Pea Protein-Curcumin Nanocomplexes via Microfluidization for Improved Solubility, Nano-Dispersibility and Heat Stability of Curcumin: Insight on Interaction Mechanisms. *Int J Biol Macromol* **2021**, *168*, 686–694. https://doi.org/10.1016/j.ijbiomac.2020.11.125.

(23) Okagu, O. D.; Jin, J.; Udenigwe, C. C. Impact of Succinylation on Pea Protein-Curcumin Interaction, Polyelectrolyte Complexation with Chitosan, and Gastrointestinal Release of Curcumin in Loaded-Biopolymer Nano-Complexes. *J Mol Liq* **2021**, *325*. https://doi.org/10.1016/j.molliq.2020.115248.

(24) Aditya, N. P.; Yang, H.; Kim, S.; Ko, S. Fabrication of Amorphous Curcumin Nanosuspensions Using β-Lactoglobulin to Enhance Solubility, Stability, and Bioavailability. *Colloids Surf B Biointerfaces* **2015**, *127*, 114–121. https://doi.org/10.1016/J.COLSURFB.2015.01.027.

(25) Liu, Y.; Cai, Y.; Ying, D.; Fu, Y.; Xiong, Y.; Le, X. Ovalbumin as a Carrier to Significantly Enhance the Aqueous Solubility and Photostability of Curcumin: Interaction and Binding

Mechanism Study. *Int J Biol Macromol* **2018**, *116*, 893–900. https://doi.org/10.1016/J.IJBIOMAC.2018.05.089.

(26) Tang, C.-H. Nanocomplexation of Proteins with Curcumin: From Interaction to Nanoencapsulation (A Review). *Food Hydrocoll* **2020**, *109*. https://doi.org/10.1016/j.foodhyd.2020.106106.

(27) Abioye, R. O.; Obeme-Nmom, J. I.; Udenigwe, C. C. Pea Protein–Curcumin Interactions and Their Effects on In Vitro Protein Digestibility. *ACS Food Science & Technology* **2024**, *4* (3), 711–718. https://doi.org/10.1021/acsfoodscitech.3c00614.

(28) Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; Tinevez, J.-Y.; White, D. J.; Hartenstein, V.; Eliceiri, K.; Tomancak, P.; Cardona, A. Fiji: An Open-Source Platform for Biological-Image Analysis. *Nat Methods* **2012**, *9* (7), 676–682. https://doi.org/10.1038/nmeth.2019.

(29) Nielsen, P. M.; Petersen, D.; Dambmann, C. Improved Method for Determining Food Protein Degree of Hydrolysis. *J Food Sci* **2001**, *66* (5), 642–646. https://doi.org/10.1111/J.1365-2621.2001.TB04614.X.

(30) García Arteaga, V.; Demand, V.; Kern, K.; Strube, A.; Szardenings, M.; Muranyi, I.; Eisner, P.; Schweiggert-Weisz, U. Enzymatic Hydrolysis and Fermentation of Pea Protein Isolate and Its Effects on Antigenic Proteins, Functional Properties, and Sensory Profile. *Foods* **2022**, *11* (1), 118. https://doi.org/10.3390/foods11010118.

(31) Hao, L.; Sun, J.; Pei, M.; Zhang, G.; Li, C.; Li, C.; Ma, X.; He, S.; Liu, L. Impact of Non-Covalent Bound Polyphenols on Conformational, Functional Properties and in Vitro Digestibility of Pea Protein. *Food Chem* **2022**, *383*. https://doi.org/10.1016/j.foodchem.2022.132623.

(32) Ramírez-García, G.; Trapiella-Alfonso, L.; D'Orlyé, F.; D'Orlyé, A. Electrophoretic Methods for Characterizing Nanoparticles and Evaluating Their Bio-Interactions for Their Further Use as Diagnostic, Imaging, or Therapeutic Tools. *Capillary Electromigration Separation Methods* **2018**, 397–421. https://doi.org/10.1016/B978-0-12-809375-7.00019-8.

(33) Gumustas, M.; Sengel-Turk, C. T.; Gumustas, A.; Ozkan, S. A.; Uslu, B. Effect of Polymer-Based Nanoparticles on the Assay of Antimicrobial Drug Delivery Systems. *Multifunctional Systems for Combined Delivery, Biosensing and Diagnostics* **2017**, 67–108. https://doi.org/10.1016/B978-0-323-52725-5.00005-8.

(34) Alizadeh-Pasdar, N.; Li-Chan, E. C. Y. Comparison of Protein Surface Hydrophobicity Measured at Various PH Values Using Three Different Fluorescent Probes. *J Agric Food Chem* **2000**, *48* (2), 328–334. https://doi.org/10.1021/jf990393p.

(35) Kaspchak, E.; Bonassoli, A. B. G.; Iwankiw, P. K.; Kayukawa, C. T. M.; Igarashi-Mafra, L.; Mafra, M. R. Interactions of Antinutrients Mixtures with Bovine Serum Albumin and Its Influence on in Vitro Protein Digestibility. *J Mol Liq* **2020**, *315*. https://doi.org/10.1016/j.molliq.2020.113699.

CHAPTER 3 - IMPACT OF IONIC STRENGTH ON THE BIOMOLECULAR INTERACTIONS OF PEA PROTEIN ALBUMIN-CURCUMIN COMPLEXES AND THEIR INFLUENCE ON *IN VITRO* PROTEIN DIGESTIBILITY

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Abstract

This study investigated the influence of ionic strength on the biomolecular interaction of pea albumin (*WSF*-water-soluble fraction)-curcumin complex and their underlying effects on protein digestibility. Pea albumin-curcumin solutions (0.125mg/mL) were prepared and mixed with varying concentrations of NaCl (0, 0.15, 15 and 150 mM). The dynamic light scattering analysis reveals particle aggregation of complexes relative to protein alone due to increasing salt concentration. Ultraviolet-visible spectroscopy and a decrease in surface hydrophobicity and transmittance show complex formation and support “salting out” phenomena. Fluorescence microscopy revealed that increased salt concentration co-localized complexes and protein aggregates enlarged in size and quantity. There was a concentration-dependent protein tryptophan fluorescence quenching upon binding with curcumin. The complexes with and without salt showed a decrease in Stern−Volmer apparent binding constant (K_A) from 0.4 ± 0.06 to 0.2 ± 0.04 and a decrease in the number of binding sites *(n*) from 0.5 ± 0.05 to 0.8 ± 0.8, indicating that the concentration of NaCl had an impact on the interaction. Notably, the degree of hydrolysis remained unchanged, suggesting that protein digestibility is independent of the gastrointestinal tract's ionic strength and that molecular interactions may affect physicochemical properties but not protein digestibility. These findings provide prospective insights into how physiological conditions, notably ionic strength, influence food matrix interactions and the effect this could exert on the digestibility of proteins.

Keywords: Molecular interactions, ionic strength, salting-out, binding, agglomeration, protein digestibility.

3.1. Introduction

Improving plant protein digestibility and bioavailability has become more interestingly relevant due to its impact on human nutrition and health. Protein digestibility determines the extent to which dietary protein can be effectively broken down and absorbed by the digestive system, allowing it to be readily used for metabolism.[1] For instance, the benefits of legumes can be maximized depending on how their proteins are easily digested.[2] Higher digestibility of proteins which leads to improved biological value is a major factor in maximizing protein utilization, boosting multiple aspects of health, and contributing to a nutritious and balanced diet.[1] Nonetheless, some dynamic mechanisms, such as enzymatic and physicochemical interactions, exist within the gastrointestinal tract that regulate the degradation of proteins. Among others, a critical environmental factor that plays a significant role in protein digestibility in the gastrointestinal tract is ionic strength. The main ions in the fluids involved in gastrointestinal absorption and secretion are sodium (Na+) and chloride (Cl-), which are typically linked during this process.[3] It has been estimated that the ionic strength of the gastric fluid varies between 0.051 and 0.151 mol/L, whereas that of the intestine is between 0.070 and 0.166 mol/L.[4,5] Protein conformation, solubility, and tendency for agglomeration can all be modified by salts, consequently impacting the physical characteristics and stability of proteins.[6] Monovalent salts, such as sodium chloride (NaCl), can alter protein stability by modulating the ionic strength of the solution. This tends to be mildly stable or unstable based on the characteristics of the electrostatic charge distribution within the bounds of the protein.[7] Research demonstrates that at a given ionic strength, the interaction is highly dependent on the type of salt used, this phenomenon is governed by the Hofmeister effect. The Hofmeister series categorizes ions based on their precipitating capacity on proteins.[8]

Ionic strength can influence protein behavior and result in either salting-in (higher solubility) or salting-out (lower solubility).[9] An increase in protein solubility by incorporating small amounts of salt (e.g., NaCl), is known as "salting-in" and this is attributed to an acceleration of the ionic strength of the solution. Here, protein molecules are stabilized by salt molecules via the decrease of the electrostatic relationship between protein molecules, thereby enhancing repulsion forces and improving protein-water interactions. At a low ionic strength, solubility of charged molecules are increased by the salt due to screening out of charge-charge interactions. Thus, a decreased ionic strength prevents molecules from precipitation and aggregation.[10] On the contrary, the addition of larger amount of salts to the solution comes a decrease in the solubility, hence, “salting-out”

effects. At this point, the molecules of proteins and salt are involved in a competition to bind to water. i.e. high ionic strength induces rapid electrostatic interaction of protein molecules leading to reduction in proteins solubility and increased precipitation as well as agglomeration.[11] Apparently, protein molecules are inclined to form associations with each other since protein-protein interactions are more energetically advantageous than protein-solvent interactions. In addition, salting-out also emerges due to hydrophobic interactions.[12] Various proteins have distinct salting-out thresholds that are utilized to fractionate them in raw extracts. Theoretically, depending on the concentration, the addition of NaCl can trigger both salt-in and salt-out reactions in a protein solution.[13]

Generally, there exists a wide variation in the digestibility of proteins across different foods. These variations can be driven by factors such as the source of protein, the food matrix, the molecular relationships between proteins and other food components (i.e., food formulations), as well as the physiological conditions for digestion.[14] Understanding the mechanisms of food component interactions during gastrointestinal digestion is necessary for the effective development of functional foods.[15] Pea albumin, the water-soluble fraction (WSF) of pea protein, constitutes approximately 18–25% of the total protein in field pea seeds and is renowned for its excellent nutritive, biological, and techno-functional values.[16] It is abundant in essential amino acids, particularly sulfur-containing amino acids such as cysteine and methionine.[17,18] The use of pea protein albumin in food applications offers several advantages due to its smaller molecular weight, low protein charge, and distinct hydrophobic distributions. These qualities make it suitable for emulsifying, gelling, and foaming capacities.[19–22] Although studies on plant-based albumins are limited, existing pilot studies have highlighted their possible utilization in foods as technologically beneficial proteins.[23–25] Additionally, numerous studies on the pea albumin fraction have reported good emulsifying, foam-stabilizing and gelation properties.[21,22]

Plant polyphenols are well-known for their bioactive properties and beneficial health impacts, including antioxidant, antimicrobial, and anti-cancerous effects. Food protein and polyphenolic constituents generally coexist and interact easily with one another either via non-covalent or covalent mechanisms forming complexes. The physicochemical characteristics of the two reacting compounds are significantly influenced by the generated complexes, impacting properties like conformation, function, nutritional value, digestibility, absorption, and bioavailability.[26] Recently,

the interaction and complexation of the hydrophobic polyphenol curcumin with proteins from various sources have been extensively studied.[27–30] Environmental factors commonly regulate transitory interactions to yield more stable protein-polyphenol complexes.[31] However, little information is available in the literature associated with the influence of ionic strength on these physicochemical interactions.

The stability, solubility, aggregation of proteins, as well as their interactions with various ligands, are greatly influenced by the ionic strength of the solution.[32,33] It was reported that the inclusion of ions significantly improved the freeze-thaw stability of glycosylated soy protein emulsions.[34] Concerning the interfacial characteristics, there was an improvement in the surface activity of pea protein hydrolysate with increasing ionic strength.[35] Similarly, results for walnut protein-xanthan gum complexes showed high values of interfacial pressure at 0.35 M of NaCl and maximum dilatational modulus at 0.15 M of NaCl.[36] As ionic strength increased based on the addition of NaCl, the binding affinity of quercetin to bovine serum albumin reduced. This is possibly due to a stronger hydration shell of the protein with dissociated electrostatic interactions.[37] However, with higher ionic strength, the interaction between chlorogenic acid and bovine serum albumin in a buffering system improved. This interaction was also predominantly facilitated by hydrophobic forces.[38] Furthermore, high ionic strength amplified the binding of tannic acid to bovine serum albumin, further reducing the in vitro digestibility of protein.[39]

Ionic strength can enhance the emulsifying abilities of complexes of protein and polyphenol interactions. In a study involving complexes from moringa seed residue protein and tannic acid of which the physicochemical parameters of the emulsions were determined with conditions of pH (3-9) and ionic strength (0-0.4 M NaCl), results indicated that stable emulsions were formed by the complexes at reduced ionic strength.[40] Likewise, the optimal use of sufficient ionic strength promoted protein-curcumin interactions and improved the characteristics of the particles (smaller size, monodispersity), thereby stabilizing Pickering emulsions.[41]

In recent years, various studies have reported the effects of ionic strength on the physicochemical properties of proteins and bioactive compounds including their complexes. Interestingly, there is no systematic study of this influence on in vitro protein digestibility. Therefore, this study investigated the impact of ionic strength on pea albumin-curcumin complexes and the influence on protein digestibility was investigated. By adjusting the ionic strength, protein-curcumin

complexes were prepared and characterized by their particle and surface properties, morphology, nature of binding and simulated gastric and intestinal digestibility. The context for the choice of salt concentrations 0 mM, 0.15 mM, 15 mM and 150 mM was to mimic the physiological condition of the gastrointestinal tract.[42,44] The salt concentration in the gastrointestinal tract varies depending on the region, hence, the use of NaCl salt concentration ranges was primarily to identify possibilities of dose-dependent effects. This information would provide valuable insights into foodmatrix interactions under physiological conditions (particularly the microstructural modifications triggered by sodium salt) and the potential impacts on gastrointestinal digestion.

3.2. Materials and methods

3.2.1. Chemicals and reagents

The pea protein fraction (albumin)-WSF derived from field pea seeds was generously provided by Pulse Canada, Manitoba. Curcumin (≥94%), ethanol, sodium hydroxide (NaOH), hydrochloric acid (HCl), 8-anilino-1-naphthalenesulfonic acid (ANS), L-serine, dithiothreitol (DTT), disodium tetraborate decahydrate, sodium dodecyl sulfate (SDS), *O*-phthaldialdehyde (OPA), tris (hydroxymethyl) aminomethane (Tris-base), pepsin (≥250 units/mg solid) from porcine gastric mucosa, pancreatin (8X USP specification) from porcine pancreas and Fast Green FCF (≥85%) were purchased from Millipore Sigma (Ontario, Canada). Sodium chloride (NaCl, ≥99%) was purchased from Fisher Scientific (Ontario, Canada). The chemicals and reagents used were of analytical grade (≥95%) with no additional purification performed.

3.2.2. Sample preparation

Water-soluble fraction (WSF) was isolated from yellow field pea seeds as previously described.[28] Curcumin solution was also prepared as previously reported.[29] Briefly, a stock solution of 2.7 mM curcumin in ethanol was prepared, then serially diluted to 1 mM and 0.5 mM in 60% and 30% ethanol respectively and shielded from light to avoid degradation. Thereafter, a diluted curcumin concentration was prepared in water to achieve 400 μM. Following that, a 0.5 mg/mL protein stock solution was prepared in water and mixed with an equal volume of curcumin to obtain a final concentration of 0.125 mg/mL of protein and 100 μM of curcumin in 0, 0.15, 15 and 150 mM of NaCl. For the control, WSF in the absence of curcumin under the same NaCl concentrations was prepared. All experiments were performed in triplicate at standard ambient temperature (25 °C).

3.2.3. Surface property characteristics

Dynamic light scattering (DLS), using a Zetasizer Nano-Zs (Malvern Instruments Ltd., Worcestershire, UK), was used to assess the surface property characteristics of the protein-curcumin mixtures. Protein solutions in the presence and absence of curcumin at varying ionic strengths alongside their respective control solutions were prepared in disposable polystyrene cuvettes, equilibrated at room temperature of 25 °C for 10 s (set at a backscattered angle of 173°, a refractive index of 1.330, a viscosity of 0.8872 cP, and a dielectric constant of 78.5) in Milli Q water (pH 7.0). All samples were analyzed for their average particle size, size distribution, polydispersity index (PDI), and surface charge (zeta potential).

3.2.4. Determination of surface hydrophobicity

The effect of ionic strength on the surface hydrophobicity of pea albumin-curcumin complexes and pea albumin (alone in water) at various NaCl concentrations was assessed using ANS. Samples (200 µL) and ANS (5 µL) were combined in a black 96-well clear-bottom microplate, following a 15-minute incubation in the dark. Fluorescence intensity was measured at λ_{ex} 390 nm and λ_{em} 470 nm using a Spark multimode microplate reader (Tecan, Stockholm, Sweden).

3.2.5. Ultraviolet-visible spectroscopy (turbidity assay)

The mixtures of pea albumin fraction and curcumin at different ionic strengths were prepared and determined for their complexation. 100 µL of the solutions and water (as blank) were added to a 96-well transparent microplate. The plate was placed in a microplate reader (Tecan, Stockholm, Switzerland) and shaken for 30 seconds. The absorbance spectral analysis of the mixtures was measured at wavelengths between 280 and 600 nm. At the maximum absorbance wavelength of curcumin (430 nm), the interactions between pea protein fraction and curcumin were evaluated and represented as percent transmittance using Eq. (*1*):

$$absorbance = -\log_{10}(\%transmittance) \quad \textbf{(1)}$$

3.2.6. Fluorescence microscopy

Fluorescence microscopy was employed to visualize the resulting effect on ionic strength on the complexes formed by pea albumin-curcumin interaction. All samples were stained by mixing with Fast Green FCF (0.1% w/v) at equal volumes. Thereafter, a 10 µL aliquot of the stained samples was transferred onto microscope slides, secured with a coverslip, then sealed with clear nail polish.

Slides were imaged with an Axio Imager 2 fluorescence microscope fitted with an Axiocam 506 camera (Carl Zeiss, Oberkochen, Germany). The Fluorescein Isothiocyanate (FITC) channel (λ_{ex} 495 nm, λ_{em} 519 nm) was used to detect curcumin while the Cyanine-5 (Cy5) channel (λ_{ex} 650 nm, λ_{em} 673 nm) was used for protein. The images were further processed using the Zen 2.3 pro software (Carl Zeiss, Oberkochen, Germany) and Image J software (NIH, Bethesda, Maryland, USA).[43]

3.2.7. Fluorescence quenching analysis

The tryptophan fluorescence quenching method using the Varian Cary Eclipse fluorescence spectrophotometer instrument from Agilent, Santa Clara, USA, was employed to investigate the influence of ionic strength on curcumin and protein interaction. The fluorescence spectra analysis for tryptophan was measured at λ_{ex} = 280 nm and λ_{em} = 300 – 450 nm, with a 5 nm slit width. Using the Stern-Volmer (Eqs. (*2*)−(*4*)), the binding parameters were calculated at the highest tryptophan fluorescence intensities.[28,29,44]

$$F_0/_F = 1 + K_Q t_0[Q] = 1 + K_D[Q] \quad \textbf{(2)}$$

$$F_0/_{F_0 - F} = 1/_{fK[Q]} + 1/_f \quad \textbf{(3)}$$

$$\log\left[F_0 - F/_F\right] = \log K_A + n \log[Q] \quad \textbf{(4)}$$

Where, F_0 and F are the fluorescence intensities of the protein fraction without and with the quencher (curcumin), respectively; $[Q]$ is the concentration of the quencher (curcumin); f represents the accessible fluorophore fraction; t_0 represents the protein fluorophore lifetime in the absence of curcumin (quencher), which is typically in 10^{-9} secs;[44] K is the effective quenching constant of the accessible fluorophores; K_D is the Stern-Volmer quenching constant; K_Q represents the biomolecular quenching rate constant; n represents the significant number of binding sites for curcumin and K_A is the apparent binding constant. The plots of linear regression of (Eqs. (*2*) − (*4*)), was used to ascertain the binding parameters and further examine the effects of ionic strength.

3.2.8. In Vitro gastrointestinal digestion

To mimic the gastric conditions, 5 mL containing equal volumes of protein fraction-curcumin complexes at varying concentrations of NaCl was prepared. Using 1 M HCl, the samples were

initially adjusted to pH 3 then 30 µL of pepsin solution (25,000 U/mL) in water was added. Following that, digestion was initiated by incubation at an optimal temperature of 37 ºC for 2 h while shaking constantly at 90 rpm. After the gastric phase, the mixtures were further adjusted to pH 7 with 1 M NaOH then the addition of 4.1 µL of pancreatin solution (800 U/mL) depending on pepsin activity; to incorporate the intestinal phase for 2 hours at 37 ºC, with continuous shaking at 90 rpm. The digest was agitated and frozen at −20 ºC to stop enzymatic hydrolysis.

3.2.9. Degree of hydrolysis

The extent of enzymatic hydrolysis was ascertained using the *O*-phthaldialdehyde (OPA) analysis based on previous studies.[45] In brief, mixtures of blank (water) or the samples, 30 µL of serine standard combined with 225 µL of OPA reagent were each placed in a clear bottom black microplate of 96 wells. After that, fluorescence intensity was measured using a Spark multimode microplate reader instrument (Tecan, Stockholm, Sweden) at excitation and emission wavelengths of 360 nm and 460 nm, respectively.

A serine (0.5–5 mM) standard curve was used to determine the serine equivalents (NH_2/g protein). The number of hydrolyzed bonds, h, was calculated using Eq. (5):

$$h = \frac{serine - NH_2 - \beta}{\alpha}\ megq/g\ protein \quad (5)$$

Serine-NH_2 represents the serine equivalent calculated from the serine standard curve; and α and β denote constants (α = 1.00, β = 0.4, the theoretical general values for unexamined raw materials).[45,46] The degree of hydrolysis (DH %) was further determined with h and h_{tot} (total number of peptide bonds per protein equivalent) in Eq. (6):

$$DH\ (\%)\ =\ {}^{h}/_{h_{tot}}\ \times 100 \quad (6)$$

3.2.10. Statistical Analysis

Experiments were conducted in triplicate and data were reported as means ± standard deviation of triplicates. A one-way analysis of variance (ANOVA) with GraphPad Prism version 10.0.2 for Windows (GraphPad Software, La Jolla, CA, USA), was employed for further statistical analysis. The Šídák test was used for multiple comparisons, and significant differences between means were defined at $\alpha = 0.05$.

3.3. Results and discussion

3.3.1. Characterization of particle and surface properties

Using dynamic light scattering, each sample was characterized for its average particle size, particle size distribution (polydispersity index) and zeta potential. (Fig. 3.1a-d) depicted the different effects of ionic strength on the protein alone and protein-curcumin complexes. With increasing ionic strength, pea fraction alone displayed an increase in the average particle size. However, this increase was more apparent with protein-curcumin mixtures. Interestingly, at 0 mM NaCl, the protein-curcumin mixtures formed homogenous complexes, indicated by the relatively low (> 0.5) polydispersity. This can be attributed to the formation of more compact structures upon introducing curcumin as previously reported, where nanoparticles were observed under similar conditions.[28] The gradual addition of salt facilitated the process of agglomeration among the protein and its curcumin-loaded complex. This can be linked to the creation of large aggregates following the addition of curcumin. At 150 mM NaCl concentration, the increase from the ordered structures of protein-curcumin complexes became evident, unlike their counterpart, supporting a phenomenon known as "salting out". Notably, the presence of salt is a driving force for protein aggregation as demonstrated in (Fig. 3.1a). This induces protein-protein interactions as well as protein-curcumin interactions. At varying salt concentrations, sodium ions (Na+) which are positively charged can interact with the negatively charged protein molecules via electrostatic screening, reducing electrostatic repulsions, decreasing protein solubility, and increasing the tendency for particle aggregates to form.[47] Ionic strength is one physiological condition that can trigger proteins to agglomerate; however, the extent of this aggregation may depend on the salt type or concentration. The salt concentration is another essential factor necessary for protein stability.[48] A high concentration of salt was shown to induce the protein agglomeration of potato proteins while facilitating the protein-protein interactions across surface films.[49] The formation of aggregates of varying sizes is confirmed by the particle size distribution, wherein smaller nanoaggregates converge to produce large aggregates. Polydispersity seems to increase alongside the salt concentration. In (Fig. 3.1b and d), at varying salt concentrations (0, 0.15, 15 and 150 mM) the polydispersity index increased accordingly, ranging from 0.35 at 0 mM NaCl to 0.97 at 150 mM NaCl for both WSF alone and WSF-Cur. complexes (Fig. 3.1b). Interestingly, a bimodal distribution is seen exclusively in the presence of curcumin at the lower concentrations (≤0.15 mM) of salt, an effect that diminishes at the highest concentrations of salt (Fig. 3.1d). Coinciding

with the average particle size and polydispersity index is a reduction in the zeta potential. Higher relative zeta potential results in stronger electrostatic repulsion and to a large extent, an extreme divergence between molecules in suspension; further bringing out reduced flocculation and aggregation that may be driven via Van der Waals interactions.[50] In contrast, upon increasing the NaCl concentration from 0 mM to 150 mM, the zeta potential significantly decreased from -25.2 to -5.8 mV and -23.4 to -4.48 mV for the WSF alone and WSF-Cur., respectively (Fig. 3.1c). Surface charge decreased with increasing aggregation. The aggregative propensity exhibited by the particles is highly dependent on the salt concentration. Zeta potential values of soy protein particles in suspensions with higher quantities of NaCl salt were similarly significantly influenced by ionic strength (-24.15 to -4.97 mV).[51] WSF alone exhibited similar trends with its curcumin-loaded complex. The addition of salt plays a role in screening charge-charge interactions by reducing electrostatic repulsion across proteins enabling them to converge more easily.[52] The absolute decrease in the zeta potential values indicates that a high ionic strength solution influences the conformational or colloidal stability by the acceleration of rapid coagulation or flocculation of particles. Protein agglomeration constitutes one prevalent pathway for protein instability.[53] Zeta potential is often employed to assess the stability of protein in suspensions.[54] This present data validates that ionic strength facilitated charge-charge interactions; repulsive forces are weakened whereas attractive forces are enhanced (electrostatic screening) which may thus influence the functional characteristics.[55]

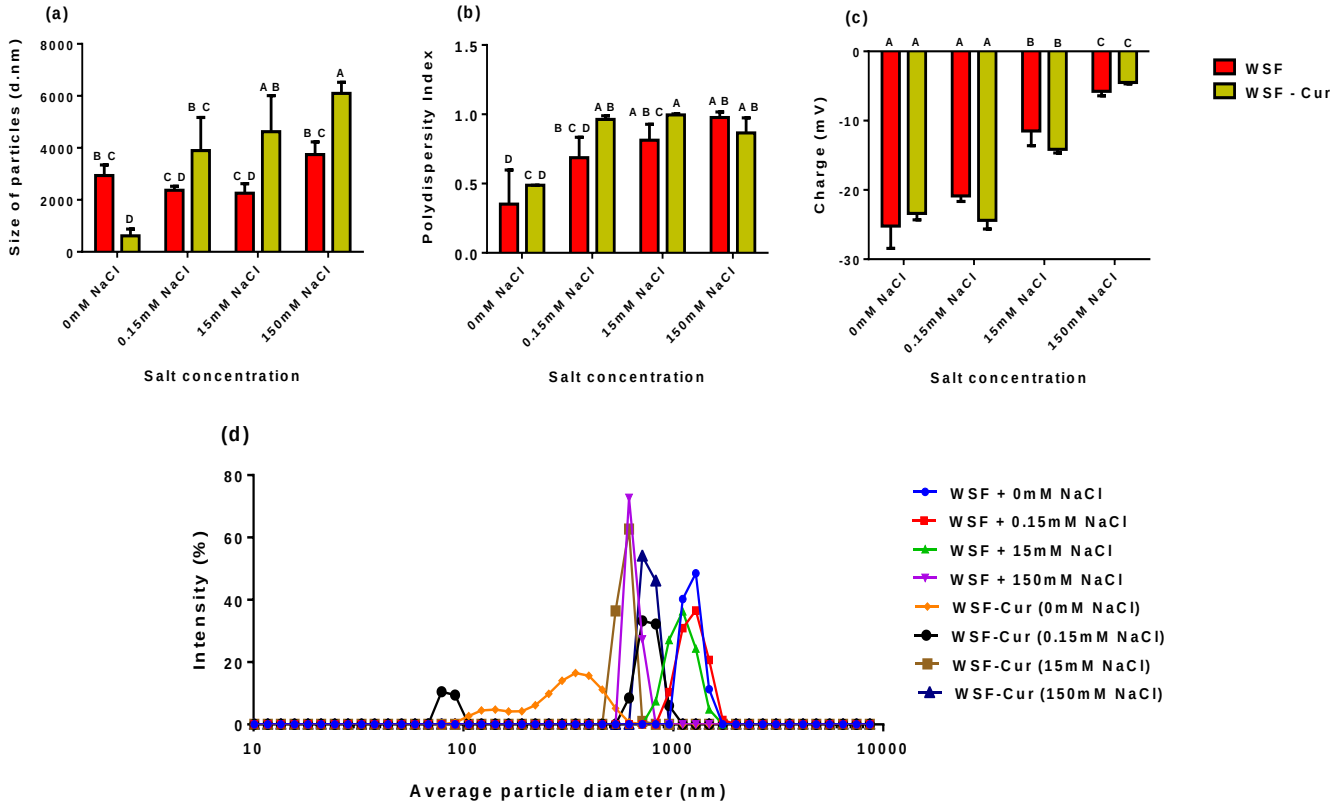

Fig. 3.1. (a) Average particle size, (b) polydispersity index, (c) zeta potential (charge), and (d) particle size distribution by intensity of pea protein -albumin (WSF)-curcumin mixtures and pea protein -albumin (WSF) only at varying salt concentrations in mM. Bars with different letters indicate significantly different mean values ($p < 0.05$).

3.3.2. Surface hydrophobicity

Protein surface hydrophobicity was performed to assess the effect of salt concentration on protein propensity to engage in intermolecular interactions and impacts on the functional characteristics that correlate with their interfaces.[56] This is primarily contingent on exposed hydrophobic surface domains, which are highly susceptible to environmental conditions.[55] As illustrated in (Fig. 3.2), the effect of ionic strength on the surface hydrophobicity of WSF alone and WSF-Cur. complexes using an ANS fluorescent probe were evaluated. WSF alone experienced a fluctuating increase in surface hydrophobicity upon the addition of salt. At 0 mM NaCl, there was an instant increase in surface hydrophobicity relative to WSF-Cur. On the introduction of 0.15 mM NaCl concentration, there was an initial reduction compared to the control followed by a gradual increase which was more pronounced at 150 mM NaCl concentration. This can be attributed to the fact that with the initial addition of salt (at a low concentration of 0.15 mM), the surface hydrophobicity of the

proteins was reduced due to the introduction of salt ions in the solution which somewhat shielded protein molecules from one another, hindered electrostatic interactions between them and strengthened protein-water interactions resulting in slight increase in protein solubility. As a result, the hydrophobicity of the protein aggregate exterior was reduced.[12] When the salt concentration was increased further up to 150 mM NaCl, there was a significant increase in surface hydrophobicity. At higher salt concentrations, proteins might undergo partial unfolding, exposing more hydrophobic residues on their surface and raising their overall hydrophobicity.[57] Low hydrophobicity leads to increased solubility and reduced protein agglomeration and vice versa. There was an initial unfolding of the hydrophobic core of the protein, hence ANS was able to bind resulting in increased fluorescence intensity. The introduction of salt to the environment facilitated more exposure of the hydrophobic residues, thus at the highest ionic strength, higher hydrophobicity of proteins was encouraged. A similar result was observed for green pea protein fractions and chickpea protein isolates at increasing salt concentrations.[58,59] Conversely, a gradual decrease in surface hydrophobicity was observed for WSF-Cur. complexes with increasing salt concentration. This supports the data obtained from DLS (Fig. 3.1). The formation of nanocomplexes between protein and curcumin reduces protein surface hydrophobicity compared to the protein fraction alone, making hydrophobic interaction a vital parameter. The reduced fluorescence intensity is a result of the strong masking of hydrophobic regions of the protein by bound curcumin, limiting the binding of ANS to the proteins.[28] However, salt concentration further facilitates curcumin-protein interactions by exposing the hydrophobic regions of the protein, increasing curcumin binding and thus, significantly decreasing surface hydrophobicity in the presence of 150 mM NaCl (Fig. 3.2). The surface hydrophobicity of alkaline soluble fraction (ASF) from pea protein isolate also displayed a similar trend from 0 to 150 mM NaCl concentrations.[44] This result confirms that surface hydrophobicity is dependent on salt concentration.

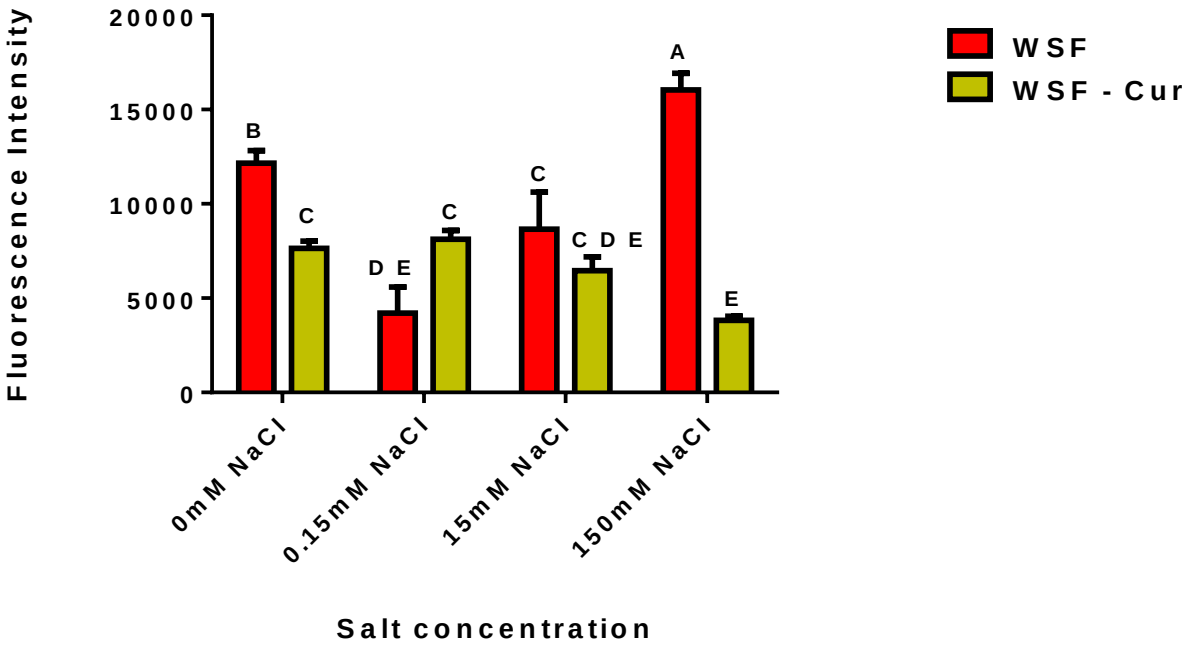

Fig. 3.2. Surface hydrophobicity using 1-Anilonaphthalene-8-Sulphonic acid (ANS) of pea protein -albumin (WSF)-curcumin mixtures and pea protein -albumin (WSF) only measured by fluorescence intensity at λem 470 nm at varying salt concentrations in mM. Bars with different letters indicate significantly different mean values ($p < 0.05$).

3.3.3. Effect of ionic strength on pea albumin-curcumin complexation

To examine the effect of salt on protein-curcumin interactions, ultraviolet-visible spectroscopy was employed (Fig. 3.3a and b). This extensive and relatively simple technique is utilized to investigate conformational changes in proteins and their complex formation.[60] Interestingly, an increase in salt concentration resulted in a slight increase in absorbance, even at a constant curcumin concentration (100 μM). The protein demonstrated the ability to interact with curcumin and form protein-polyphenol complexes, but the salt concentration had a unique influence on these interactions. The concentration of salt also seemed to affect the turbidity of the complexes to an extent, judging from the slight gradual increase in absorbance from 0 to 150 mM NaCl (Fig. 3.3a). As previously indicated by the data obtained from DLS (Fig. 3.1a-d), the shift in turbidity emanates from the variations in the mass and size of aggregates.[61] The concept that complexation occurred is supported by the general tendency for the increase in turbidity with the inclusion of salt; nonetheless, salting out further induced the solubility of curcumin. This enables curcumin to bind likely via hydrophobic interactions with the pea proteins, promoting its stability over time.

Hydrophobic interactions between curcumin and the protein core enhance the solubility and stability of curcumin. Furthermore, the complexation of curcumin with food proteins has been developed as an effective and prospective strategy to improve the solubility, chemical stability, and bioavailability of curcumin in aqueous environments. For instance, it was observed that complexing with soy protein isolate drastically increased the aqueous solubility of curcumin by 812-fold.[62] Notably, no remarkable difference was observed for the transmittance expressed at the absorbance maxima of curcumin (430 nm) with increasing ionic strength. This also supports the data of the initial nanocomplexation (Fig. 3.1b) formed by protein and curcumin which increased in size at high salt concentrations (> 0.15 mM) and obstructed the transmission of light. Equally, no new shift was observed which indicates that this complexation was governed by non-covalent interactions.

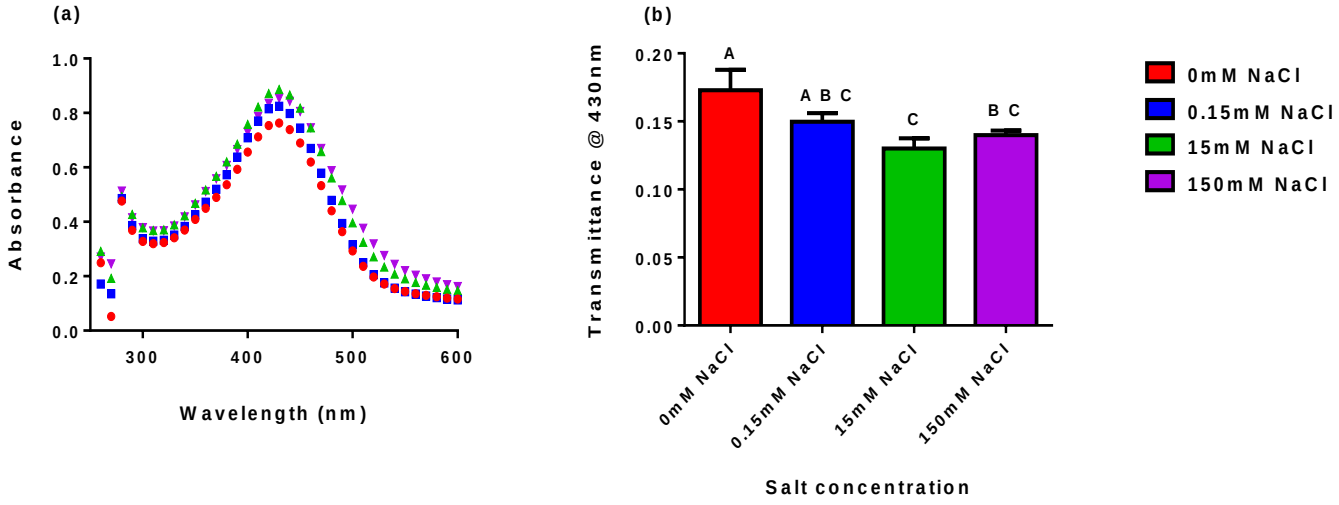

Fig. 3.3. (a) Ultraviolet-visible spectroscopy (absorbance spectra) and (b) percent transmittance (at curcumin λmax 430 nm) of WSF-curcumin mixtures at varying salt concentrations in millimolar. Bars with different letters indicate significantly different mean values ($p < 0.05$).

3.3.4. Fluorescence microscopy

The influence of ionic strength on the complexation of pea albumin and curcumin at 0, 015, 15, and 150 mM NaCl concentrations was visualized via fluorescence microscopy (Fig. 3.4). Pea protein fraction and curcumin were represented by FITC (green) and Cy5 (pink) channels, respectively. There was an initial formation of protein-curcumin nanoparticles as seen in 0 mM

NaCl, however, as the concentration of salt increased, these nanoaggregates produced looked like large clumps. This change confirms the salt-enhancing effect at varying ionic strength and explains the maximal increase in the size and quantity of complexes. Additionally, at lower salt concentrations, there is colocalization between protein and curcumin, which verifies the complexation resulting from their interactions. Interestingly, regions where curcumin is present (indicated by the white arrows) in regions where the protein is absent as the concentration of salt increases are observed especially at 150 mM NaCl (Fig. 3.4). It might be explained by the fact that the presence of salt caused some unbound curcumin to self-aggregate. It is probable that at the high concentration of curcumin (100 μM), it surpassed its solubility threshold, thus resulting in aggregation.[29]

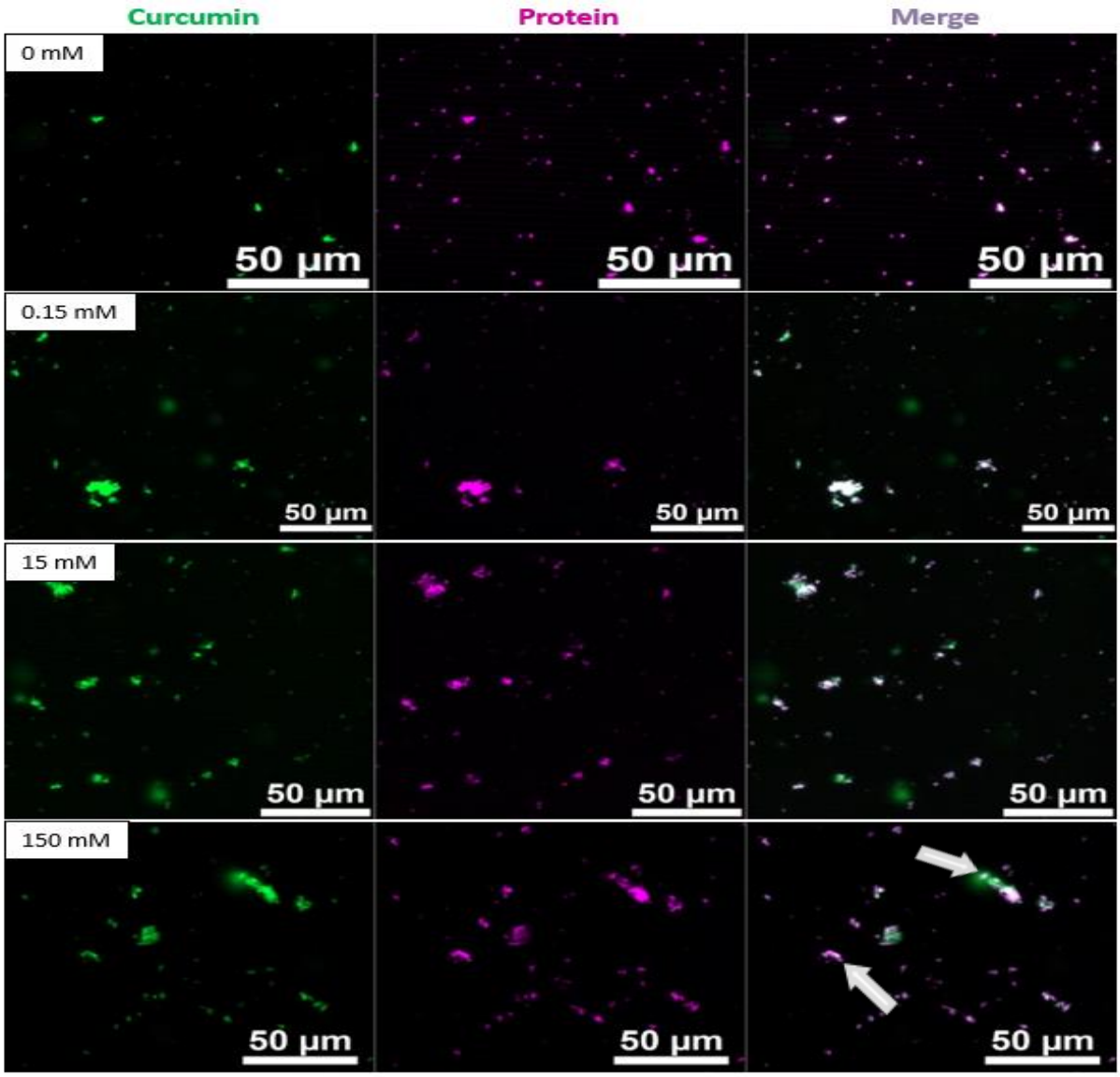

Fig. 3.4. Fluorescence microscopy of curcumin, pea-albumin (WSF) and pea-albumin-curcumin complexes (WSF-Cur.) at 0, 0.15, 15, 150mM NaCl salt concentrations. Scale bars represent 50 µm.

3.3.5. Influence of ionic strength on the interaction and binding parameters of pea albumin-curcumin complexes

Tryptophan residues are intrinsic fluorophores, often incorporated in the hydrophobic core of proteins. The fluorescence spectroscopy of proteins extensively reveals the binding characteristics of small compounds to proteins.[63] The spectral properties of the fluorescence emission can change when the aromatic amino acid residues are subjected to exposure to various solvent environments.[60] From the fluorescence emission spectrum characteristics, the Stern-Volmer

binding parameters can be deduced (Table 3.1). The Stern-Volmer equations (Eqs. 2-4) can be used to quantify the binding affinity of phenolic compounds on proteins and to determine quenching constants.

The nature and strength of binding between pea albumin and curcumin were assessed by the tryptophan fluorescence quenching technique at 0 mM and 150 mM NaCl concentrations. Fig. 3.5 reveals the intrinsic fluorescence spectra at constant pea albumin concentration and varying concentrations of curcumin (0–100 μM) in the absence and presence of ionic strength. With increasing curcumin concentration, there was a significant concentration-dependent decrease in tryptophan fluorescence which led to a red shift of the maximum fluorescence emission wavelength. This confirms the binding interaction of curcumin to the protein hydrophobic core, which is probably the site for protein-curcumin interaction. Notably, the variations in fluorescence intensity either blue shift or red shift reveal substantial information regarding the influence of the microenvironment of Tryptophan as well as the structure of proteins and how they interact with other molecules.[64] In line with earlier research on protein-curcumin complexation, a red shift, sometimes referred to as a bathochromic shift, further demonstrates the protein unfolding and the exposure of tryptophan residues.[28,44] However, this concentration-dependent decrease is different at 0 mM compared to 150 mM NaCl concentrations. The emission wavelength of WSF-Cur. at 0 mM exhibited a similar trend with previous studies.[28,44] In contrast, WSF-Cur. at 150 mM experienced a slightly higher fluorescence intensity compared to 0 mM, at all curcumin concentrations. It could be that a higher concentration of salt, induced slight exposure of tryptophan residue initially. Similarly, it was reported that an increased concentration of NaCl resulted in the rearrangement of protein structure as evident in the higher fluorescence intensity at 100 mM.[59]

The fluorescence quenching data in the presence and absence of NaCl was analyzed using the Stern-Volmer equation (Eqs. 2–4) to ascertain the mechanism of interactions between curcumin and pea protein fraction. Using (Eq. 2), the Stern-Volmer quenching constant, K_D, and the biomolecular quenching rate constant, K_Q, were determined. As shown in (Table 3.1), the K_D and K_Q values of the complexes at 0 mM and 150 mM NaCl displayed a static quenching behavior as evident in the linear plot (Fig. 3.6a and d). However, this linearity was predominant at low (≤ 10 μM) curcumin concentrations, whereas a dynamic quenching behavior was observed at curcumin

concentrations above 25 μM. Previous studies reported a comparable pattern for pea protein fractions.[28,44] The static quenching mechanism results in the ground state complexation between protein and curcumin formed at the lower concentration of curcumin whereas the dynamic quenching refers to excited state collisions between tryptophan residues in proteins and curcumin, which occurs at high concentrations of curcumin where there is a propensity for the saturation of the protein binding site and fluctuations in chemical balance.[44] This implies that the rate of association of protein-curcumin complexation is more prominent at lower curcumin concentrations than at higher concentrations. Thus, dissociation is more apparent at high concentrations, which could have an impact on complex stability. Moreso, the K_Q values for the complexes exceed the limiting diffusion rate constant ($K_{diff} = 2.0 \times 10^{10}\ M^{-1}\ S^{-1}$), indicating that static binding, as opposed to dynamic binding, was likely the primary cause of the fluorescence quenching. Stated differently, the ground state complex formation leads to the predicted binding parameters shown in (Table 3.1). The K_D value increased by two-fold at 150 mM compared to 0 mM salt. Higher salt concentration encouraged the dynamic quenching mode, whereas low ionic strength supported static binding, similar to previously reported research.[44] Protein hydrophobicity is a major factor in the binding as observed by the values of K_D and K_Q, however, at 150 mM, ionic strength altered the nature of protein and curcumin interaction. This signifies that the K_D and K_Q values of the complexes at 0 mM rely on hydrophobic interactions. The occurrence of stable complexes at lower concentrations can be explained via hydrophobic interactions rather than electrostatic interactions.[44]

Given that quenching is directly associated with the extent of binding, K, K_A, K_D and K_Q are interrelated. Equation 3 was used to determine the effective quenching constant K, also known as the associative binding constant for the protein-curcumin complex and f, the accessible fluorophore fractions. The effective quenching constant for the accessible fluorophore is significantly higher in 0 mM than in 150 mM signifying a better interaction and binding in the absence of ionic strength. The increase in the K value of the complex at 0 mM NaCl corroborates with the increased surface hydrophobicity of the complex at 0 mM (Table 3.1, Fig. 3.2). More quenching results in more binding. This particular parameter is frequently used in the fluorescence quenching approach to quantify the degree of protein-ligand interaction.[28] Also, with increased NaCl concentration to 150 mM, a two-fold decrease in apparent binding constant, K_A, was observed (Table 3.1). This decline in binding may have resulted from a salt-induced alteration in the protein tertiary

structure.[65] Previous studies showed that increasing salt concentration decreased the binding of whey protein isolate/hyaluronic complexes with curcumin.[66] The accessible fluorophore fraction *f*, was estimated as 0.7 ± 0.09 and 1.3 ± 0.35 for 0 mM and 150 mM NaCl respectively. This corroborates the slight increase in the maximum emission fluorescence intensity in the presence and absence of ionic strength as seen in (Fig. 3.6b and e). The number of binding sites of the complexes *n*, estimated from the plot of (Eq. 4), was approximately 1 for both conditions (0 mM and 150 mM NaCl), which implies that a single curcumin molecule was statically linked to a protein molecule in the complex via fluorescence quenching.

Consistent with these findings, the impact of curcumin interaction on protein conformations varies depending on the type of protein and even the environmental conditions. Binding affinity is affected by non-covalent interactions such as hydrophobicity, electrostatic interactions, hydrogen bonding as well as Van der Waals forces between the protein and curcumin which may be modified with the existence of other molecules. It is commonly measured and analyzed by the equilibrium dissociation constant (K_D). The biomolecular interaction strengths are ranked and evaluated using this parameter. The lower the K_D value, the more improved the ability of the ligand to bind to its target molecule (i.e. binding affinity). Likewise, the higher the value of K_D, the weaker the mutual attraction and binding of the ligand and target molecule as illustrated in Table 3.1.[67] It is possible that some curcumin-protein complexes were formed, however, given the significant increase in K_D relative to the absence of salt, it is probable that these complexes were weakly bound, likely favoring dissociation more at a salt concentration of 150 mM. This leads to less static quenching at higher concentrations of curcumin resulting in less stable complexes. Ionic strength regulates the extent of electrostatic interactions: higher ionic strength exposes tryptophan residues on the protein exterior, which is evident with an increase in the fluorescent intensity at 150 mM. Surprisingly, this increase is not reflected in the binding affinity at 150 mM. Notably, pea albumin is known to have a low molecular weight, which suggests that it has fewer functional groups to interact with curcumin and, consequently, may have caused a significant decrease in the binding strength of protein–curcumin interactions.[28] Similarly, the WSF-curcumin complex showed lower binding affinity compared to its counterparts, which was reported to have likely been a result of its decreased hydrophobicity, the location of the tryptophan residue proximal to the surface of the protein, or the mechanism of the interactions.[28] Variations in WSF-Cur. the binding process at high salt concentrations may be due to detrimental polar conditions, thus the binding strength is more

favored at 0 mM than at 150 mM NaCl. Future studies in identifying optimal physiological conditions that are critical for effective complex stability and thus required for functional food compositions should be explored

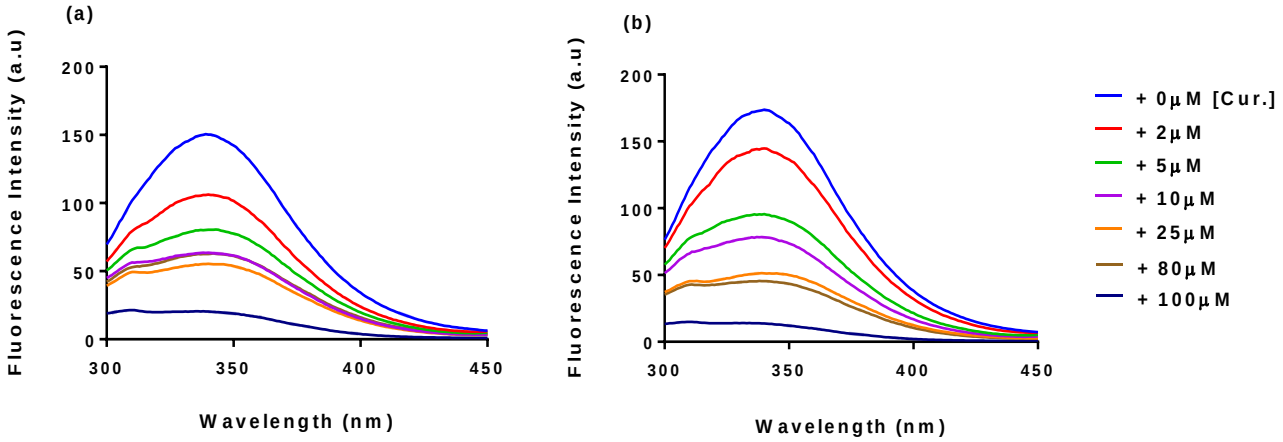

Fig. 3.5. Fluorescence emission spectra of (a) 0 mM NaCl (b) 150 mM NaCl of pea protein albumin (WSF) at varying curcumin concentrations in µM.

Table 3.1. The binding parameters of pea albumin-curcumin complexes by tryptophan fluorescence quenching.[a]

WSF+Curcumin	K_A	K $(\times 10^4 M^{-1})$	K_D $(\times 10^4 M^{-1})$	K_Q $(\times 10^4 M^{-1} S^{-1})$	n	f
0 mM NaCl	0.4 ± 0.06^a	47 ± 1.14^a	3.8 ± 0.70^b	3.8 ± 0.70^b	0.5 ± 0.05^b	0.7 ± 0.09^b
150 mM NaCl	0.2 ± 0.04^b	5.0 ± 0.38^b	7.8 ± 0.38^a	7.8 ± 0.38^a	0.8 ± 0.08^a	1.3 ± 0.35^a

[a]Significant differences ($p < 0.05$) are indicated by superscripts with distinct letters in a column, which represent the mean values. (WSF) water-soluble fraction (pea albumin), K effective quenching constant for the accessible fluorophore, K_D Stern-Volmer quenching constant, K_Q biomolecular quenching rate constant, K_A apparent binding constant, n number of binding sites for curcumin in WSF and f accessible fluorophore fraction.

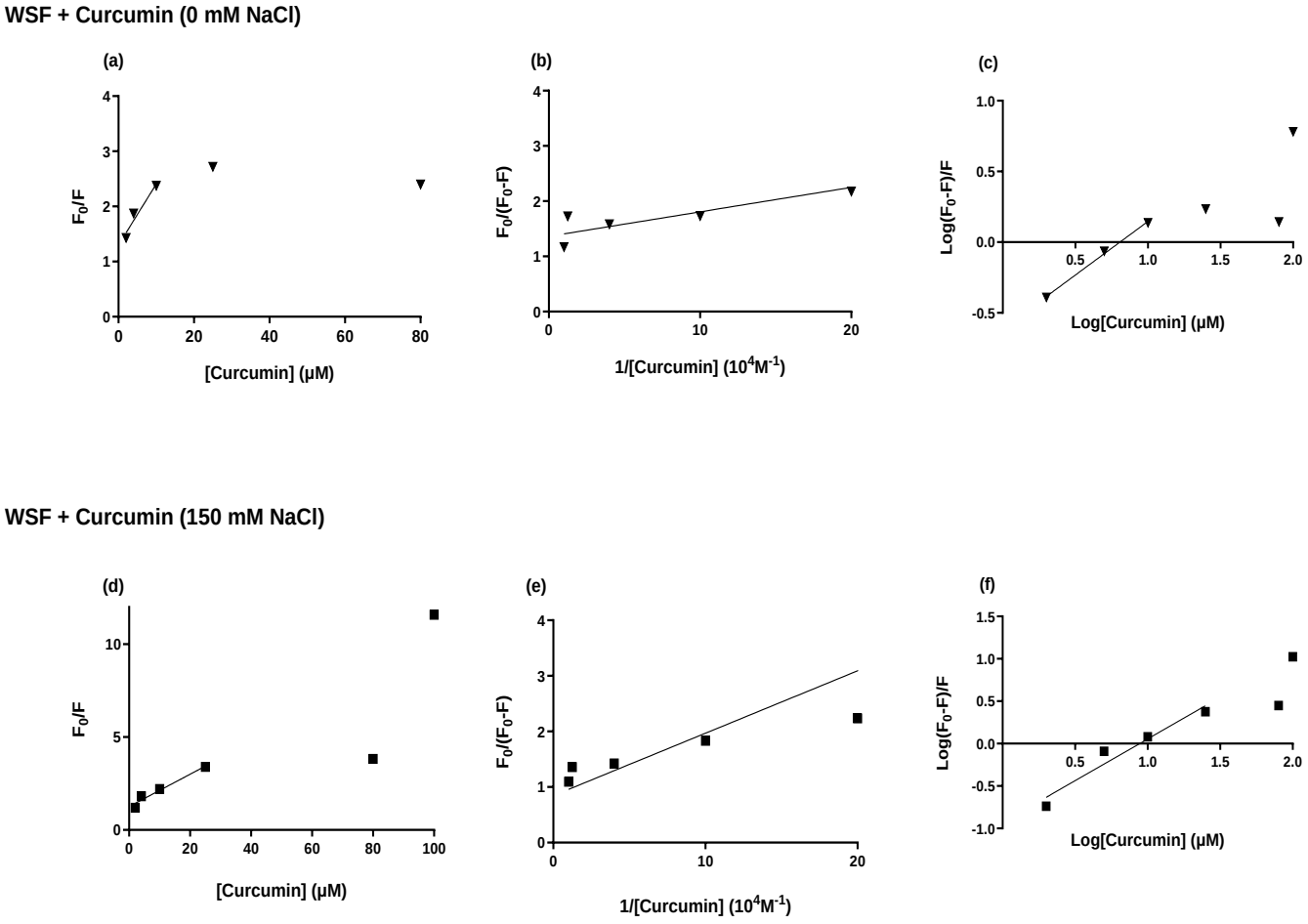

Fig. 3.6. Plots to determine the (a and d) Stern-Volmer quenching constant, K_D through F_0/F against [Curcumin] in μM; (b and e) accessible fluorophore fraction, *f* and the effective quenching constant for the accessible fluorophore fractions *K*, through $F_0/(F_0-F)$ against 1/[Curcumin] and (c and f) the significant number of binding sites for curcumin n in WSF through Log$(F_0-F)/F$ against Log[Curcumin] of WSF-curcumin complexes at 0 mM and 150 mM NaCl concentrations.

3.3.6. Simulated gastrointestinal digestion

Digestibility is a key consideration when employing proteins in the production of novel functional foods.[68] Given its relevance, the effect of complexation with curcumin on the digestion characteristics of food protein was investigated under simulated gastrointestinal conditions. Fig. 3.7 presents the influence of ionic strength on WSF alone and its curcumin-loaded complexes *In vitro*. The extent of protein hydrolysis after gastrointestinal digestion was not statistically different even at varying ionic strength. The amount of NaCl was increased up to 150 mM which is equivalently the same concentration in the gastrointestinal tract. Degree of hydrolysis increased

significantly for protein alone compared to protein-curcumin complexes. Surprisingly, there was no obvious change in protein hydrolysis rate between 0.15, 15 and 150 mM NaCl concentrations. The increase in NaCl up to 150 mM did not have any effect on protein digestibility. The changes in the physicochemical characteristics of the complexes at different concentrations of salt did not affect the extent of protein digestion *in vitro*. Even though slightly lower *In vitro* protein digestion was observed in the absence of NaCl (0 mM), on the addition of salt up the 150 mM, the increase remained unchanged for both protein alone and protein-curcumin complexes. This could have been as a result of unstable stable complexes that were formed as indicated by fluorescence quenching. Also, the drastic increase in particle size as seen in DLS (Fig. 1a-d) which resulted in protein agglomeration could have contributed to low digestibility and the unchanging behavior of the mechanism even after ionic strength was altered. The degree of hydrolysis of lentil proteins was reported to decrease suggesting that aggregation played a major role in this effect.[69] In addition, the variation in surface hydrophobicity is directly linked to protein digestibility. It was reported that an increase in surface hydrophobicity accelerated the precipitation of proteins, which subsequently hindered the enzymes from adequate hydrolysis, and as a result diminished protein digestion; as seen in the control (0 mM in Fig. 3.7).[70]

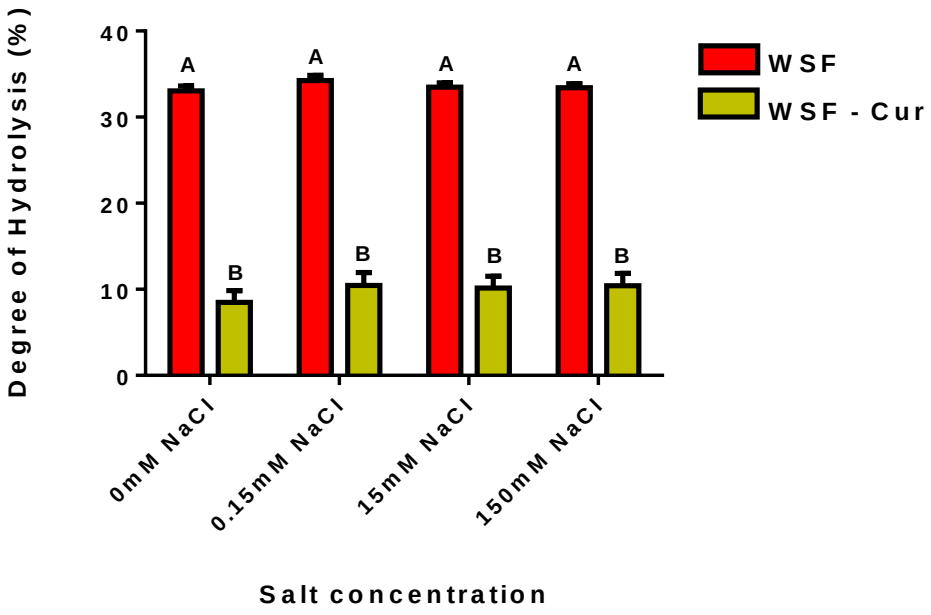

Fig. 3.7. Degree of hydrolysis of pea protein -albumin (WSF)-curcumin mixtures at varying salt concentrations in mM. Significant differences ($p < 0.05$) are denoted by letters above bars.

3.4. Conclusion

From the present results, the influence of ionic strength on pea albumin-curcumin complexes and its underlying effect on protein digestibility was assessed. Hydrophobicity is predicted to facilitate the complexation between the protein fraction and curcumin. High salt concentrations impacted the surface and particle characteristics of the protein-curcumin complexes by the production of large protein aggregates with increasing NaCl compared to their counterpart due to the effect of salting out as seen in the dynamic light scattering analysis. The surface hydrophobicity revealed the unfolding of proteins with salt concentration which increased the protein hydrophobicity. However, this was not seen for the protein-curcumin complex, suggesting strong protein-curcumin interaction and that hydrophobic pockets of protein were buried in the interior of the protein. Increasing ionic strength also caused a slight increase in the absorbance spectroscopy of curcumin in the complex even at a constant concentration (100µM), suggesting that the solubility of curcumin was improved as a result of salt-enhanced curcumin-protein complexation. Fluorescence microscopy correlates the results from dynamic light scattering with the formation of large, aggregated complexes upon the increase in salt concentration. Nonetheless, it was also observed

that some curcumin molecules were found in areas where proteins were absent. This implies that curcumin has surpassed its solubility limit at a high concentration resulting in aggregation. Further understanding of the nature of interactions was provided using the Stern-Volmer equations. The binding of curcumin to the proteins led to an intrinsic tryptophan fluorescence quenching at a constant protein concentration and increasing curcumin concentrations. Static quenching mechanism was observed at low (≤ 10 μM) curcumin concentrations whereas high (≥ 25 μM) curcumin concentrations exhibited more dynamic quenching. The nature of binding formed a 1:1 complexation of curcumin to proteins (number of binding sites). However, the binding strength was stronger at low salt concentration than at high salt concentration suggesting the formation of weak complexes that dissociated at high salt concentrations. Additionally, the binding ability between plant proteins and curcumin at various NaCl concentrations needs further investigation. Even though physiological conditions impacted the characteristics of the complexes, negligible changes were seen in protein digestibility. These changes are mainly attributed to the existence of rigid aggregates of proteins or the formation of some unstable complexes that influence digestibility. This study provides insights into the ionic conditions in which various bioactive compounds such as curcumin interact with proteins, the possibility of salt-induced modifications in protein-curcumin delivery systems in gastrointestinal environments and potential impacts on protein digestibility.

CRediT Author Statement

Judith Oballa: Conceptualization, Investigation, Methodology, Formal analysis, Validation and Writing – original draft preparation, Writing – review and editing. Raliat Abioye: Visualization, and Writing - review and editing. Joy Obeme-Nmom: Writing – review and editing. Chibuike Udenigwe: Funding acquisition, Conceptualization, Validation, Resources, Supervision and Writing- Review and editing.

3.5. References

(1) Pu, Y.; Long, Y.; Xu, D.; Niu, Y.; Wu, Q.; Chen, S.; Wang, R.; Ge, R. Influence of Thermal Denaturation on Whey Protein Isolates in Combination with Chitosan for Fabricating Pickering Emulsions: A Comparison Study. *Front Nutr* **2024**, *11*. https://doi.org/10.3389/fnut.2024.1418120.

(2) Misquitta, S. A.; Kshirsagar, D. N.; Dange, P. R.; Choudhari, V. G.; Kabra, M. M. Digestibility of Proteins in Legumes. *Production and Utilization of Legumes - Progress and Prospects* **2023**. https://doi.org/10.5772/INTECHOPEN.110372.

(3) Hornbuckle, W. E.; Simpson, K. W.; Tennant, B. C. Gastrointestinal Function. *Clinical Biochemistry of Domestic Animals* **2008**, 413. https://doi.org/10.1016/B978-0-12-370491-7.00014-3.

(4) Johnson, J. L.; Holinej, J.; Williams, M. D. Influence of Ionic Strength on Matrix Integrity and Drug Release from Hydroxypropyl Cellulose Compacts. *Int J Pharm* **1993**, *90* (2), 151–159. https://doi.org/10.1016/0378-5173(93)90151-5.

(5) Lindahl, A.; Ungell, A. L.; Knutson, L.; Lennernäs, H. Characterization of Fluids from the Stomach and Proximal Jejunum in Men and Women. *Pharm Res* **1997**, *14* (4), 497–502. https://doi.org/10.1023/A:1012107801889/METRICS.

(6) Curtis, R. A.; Ulrich, J.; Montaser, A.; Prausnitz, J. M.; Blanch, H. W. Protein–Protein Interactions in Concentrated Electrolyte Solutions. *Biotechnol Bioeng* **2002**, *79* (4), 367–380. https://doi.org/10.1002/BIT.10342.

(7) Kohn, W. D.; Kay, C. M.; Hodges, R. S. Salt Effects on Protein Stability: Two-Strandedα-Helical Coiled-Coils Containing Inter- or Intrahelical Ion Pairs. *J Mol Biol* **1997**, *267* (4), 1039–1052. https://doi.org/10.1006/jmbi.1997.0930.

(8) Marek, P. J.; Patsalo, V.; Green, D. F.; Raleigh, D. P. Ionic Strength Effects on Amyloid Formation by Amylin Are a Complicated Interplay among Debye Screening, Ion Selectivity, and Hofmeister Effects. *Biochemistry* **2012**, *51* (43), 8478–8490. https://doi.org/10.1021/BI300574R/SUPPL_FILE/BI300574R_SI_001.PDF.

(9) Shire, S. J. Formulation of Proteins and Monoclonal Antibodies (MAbs). *Monoclonal Antibodies* **2015**, 93–120. https://doi.org/10.1016/B978-0-08-100296-4.00004-X.

(10) Hansen, L. The Optimization and Scale-Up of Pea Protein Extractions and Impact on Structural and Functional Properties. **2020**.

(11) Kaur, L.; Lamsar, H.; López, I. F.; Filippi, M.; Min, D. O. S.; Ah-Sing, K.; Singh, J. Physico-Chemical Characteristics and in Vitro Gastro-Small Intestinal Digestion of New Zealand Ryegrass Proteins. *Foods* **2021**, *10* (2). https://doi.org/10.3390/FOODS10020331.

(12) Duong-Ly, K. C.; Gabelli, S. B. Salting out of Proteins Using Ammonium Sulfate Precipitation. *Methods Enzymol* **2014**, *541*, 85–94. https://doi.org/10.1016/B978-0-12-420119-4.00007-0.

(13) Nahar, M. K.; Zakaria, Z.; Hashim, U.; Bari, M. F. Effect of PH and Salt Concentration on Protein Solubility of Slaughtered and Non-Slaughtered Broiler Chicken Meat. *Sains Malays* **2017**, *46* (5), 719–724. https://doi.org/10.17576/jsm-2017-4605-06.

(14) Orlien, V.; Aalaei, K.; Poojary, M. M.; Nielsen, D. S.; Ahrné, L.; Carrascal, J. R. Effect of Processing on in Vitro Digestibility (IVPD) of Food Proteins. *Crit Rev Food Sci Nutr* **2023**, *63* (16), 2790–2839. https://doi.org/10.1080/10408398.2021.1980763.

(15) Cirkovic Velickovic, T. D.; Stanic-Vucinic, D. J. The Role of Dietary Phenolic Compounds in Protein Digestion and Processing Technologies to Improve Their Antinutritive Properties. *Compr Rev Food Sci Food Saf* **2018**, *17* (1), 82–103. https://doi.org/10.1111/1541-4337.12320.

(16) McCarthy, N. A.; Kennedy, D.; Hogan, S. A.; Kelly, P. M.; Thapa, K.; Murphy, K. M.; Fenelon, M. A. Emulsification Properties of Pea Protein Isolate Using Homogenization, Microfluidization

and Ultrasonication. *Food Research International* **2016**, *89*, 415–421. https://doi.org/10.1016/J.FOODRES.2016.07.024.

(17) Dziuba, J.; Szerszunowicz, I.; Nałecz, D.; Dziuba, M. Proteomic Analysis of Albumin and Globulin Fractions of Pea (Pisum Sativum L.) Seeds. *Acta Sci Pol Technol Aliment* **2014**, *13* (2), 181–190. https://doi.org/10.17306/J.AFS.2014.2.7.

(18) Djemaoune, Y.; Cases, E.; Saurel, R. The Effect of High-Pressure Microfluidization Treatment on the Foaming Properties of Pea Albumin Aggregates. *J Food Sci* **2019**, *84* (8), 2242–2249. https://doi.org/10.1111/1750-3841.14734.

(19) Yang, J.; Kornet, R.; Diedericks, C. F.; Yang, Q.; Berton-Carabin, C. C.; Nikiforidis, C. V.; Venema, P.; van der Linden, E.; Sagis, L. M. C. Rethinking Plant Protein Extraction: Albumin - From Side Stream to an Excellent Foaming Ingredient. *Food Structure* **2022**, *31*, 100254. https://doi.org/10.1016/J.FOOSTR.2022.100254.

(20) Djoullah, A.; Husson, F.; Saurel, R. Gelation Behaviors of Denaturated Pea Albumin and Globulin Fractions during Transglutaminase Treatment. *Food Hydrocoll* **2018**, *77*, 636–645. https://doi.org/10.1016/J.FOODHYD.2017.11.005.

(21) Lu, B.-Y.; Quillien, L.; Popineau, Y. Foaming and Emulsifying Properties of Pea Albumin Fractions and Partial Characterisation of Surface-Active Components. *J Sci Food Agric* **2000**, *80* (13), 1964–1972. https://doi.org/https://doi.org/10.1002/1097-0010(200010)80:13%3C1964::AID-JSFA737%3E3.0.CO;2-J.

(22) Kornet, R.; Yang, J.; Venema, P.; van der Linden, E.; Sagis, L. M. C. Optimizing Pea Protein Fractionation to Yield Protein Fractions with a High Foaming and Emulsifying Capacity. *Food Hydrocoll* **2022**, *126*, 107456. https://doi.org/10.1016/j.foodhyd.2021.107456.

(23) Cheung, L.; Wanasundara, J.; Nickerson, M. T. Effect of PH and NaCl on the Emulsifying Properties of a Napin Protein Isolate. *Food Biophys* **2015**, *10* (1), 30–38. https://doi.org/10.1007/S11483-014-9350-7.

(24) Wong, A.; Pitts, K.; Jayasena, V.; Johnson, S. Isolation and Foaming Functionality of Acid-Soluble Protein from Lupin (Lupinus Angustifolius) Kernels. *J Sci Food Agric* **2013**, *93* (15), 3755–3762. https://doi.org/10.1002/JSFA.6249.

(25) Yang, J.; Sagis, L. M. C. Interfacial Behavior of Plant Proteins — Novel Sources and Extraction Methods. *Curr Opin Colloid Interface Sci* **2021**, *56*, 101499. https://doi.org/10.1016/J.COCIS.2021.101499.

(26) Zhang, Q.; Cheng, Z.; Wang, Y.; Fu, L. Dietary Protein-Phenolic Interactions: Characterization, Biochemical-Physiological Consequences, and Potential Food Applications. *Crit Rev Food Sci Nutr* **2021**, *61* (21), 3589–3615. https://doi.org/10.1080/10408398.2020.1803199.

(27) Tang, C. H. Nanocomplexation of Proteins with Curcumin: From Interaction to Nanoencapsulation (A Review). *Food Hydrocoll* **2020**, *109*, 106106. https://doi.org/10.1016/J.FOODHYD.2020.106106.

(28) Okagu, O. D.; Udenigwe, C. C. Molecular Interactions of Pea Globulin, Albumin and Glutelin With Curcumin: Formation and Gastric Release Mechanisms of Curcumin-Loaded Bio-Nanocomplexes. *Food Biophys* **2022**, *17* (1), 10–25. https://doi.org/10.1007/s11483-021-09697-5.

(29) Okagu, O. D.; Jin, J.; Udenigwe, C. C. Impact of Succinylation on Pea Protein-Curcumin Interaction, Polyelectrolyte Complexation with Chitosan, and Gastrointestinal Release of Curcumin in Loaded-Biopolymer Nano-Complexes. *J Mol Liq* **2021**, *325*. https://doi.org/10.1016/j.molliq.2020.115248.

(30) Abioye, R. O.; Obeme-Nmom, J. I.; Udenigwe, C. C. Pea Protein–Curcumin Interactions and Their Effects on In Vitro Protein Digestibility. *ACS Food Science & Technology* **2024**, *4* (3), 711–718. https://doi.org/10.1021/acsfoodscitech.3c00614.

(31) Brudzynski, K.; Maldonado-Alvarez, L. Polyphenol-Protein Complexes and Their Consequences for the Redox Activity, Structure and Function of Honey. A Current View and New Hypothesis – a Review. *Pol J Food Nutr Sci* **2015**, *65* (2), 71–80. https://doi.org/10.1515/PJFNS-2015-0030.

(32) Von Staszewski, M.; Jara, F. L.; Ruiz, A. L. T. G.; Jagus, R. J.; Carvalho, J. E.; Pilosof, A. M. R. Nanocomplex Formation between β-Lactoglobulin or Caseinomacropeptide and Green Tea Polyphenols: Impact on Protein Gelation and Polyphenols Antiproliferative Activity. *J Funct Foods* **2012**, *4* (4), 800–809. https://doi.org/10.1016/j.jff.2012.05.008.

(33) Jaldappagari, S.; Balakrishnan, S.; Hegde, A. H.; Teradal, N. L.; Narayan, P. S. Interactions of Polyphenols with Plasma Proteins: Insights from Analytical Techniques. *Curr Drug Metab* **2013**, *14* (4), 456–473. https://doi.org/10.2174/1389200211314040009.

(34) Wang, Y.; Wang, X.; Wang, G.; Wang, X. Effect of Ionic Strength on Freeze–Thaw Stability of Glycosylated Soy Protein Emulsion. *J Am Oil Chem Soc* **2021**, *98* (9), 891–901. https://doi.org/10.1002/AOCS.12393.

(35) Sarigiannidou, K.; Odelli, D.; Jessen, F.; Mohammadifar, M. A.; Ajalloueian, F.; Vall-llosera, M.; de Carvalho, A. F.; Casanova, F. Interfacial Properties of Pea Protein Hydrolysate: The Effect of Ionic Strength. *Colloids and Interfaces* **2022**, *6* (4), 76. https://doi.org/10.3390/COLLOIDS6040076/S1.

(36) Tan, Y.; Deng, X.; Liu, T.; Yang, B.; Zhao, M.; Zhao, Q. Influence of NaCl on the Oil/Water Interfacial and Emulsifying Properties of Walnut Protein-Xanthan Gum. *Food Hydrocoll* **2017**, *72*, 73–80. https://doi.org/10.1016/J.FOODHYD.2017.05.031.

(37) Rawel, H. M.; Meidtner, K.; Kroll, J. Binding of Selected Phenolic Compounds to Proteins. *J Agric Food Chem* **2005**, *53* (10), 4228–4235. https://doi.org/10.1021/JF0480290/SUPPL_FILE/JF0480290SI20050322_101120.PDF.

(38) Prigent, S. V. E.; Gruppen, H.; Visser, A. J. W. G.; Van Koningsveld, G. A.; De Jong, G. A. H.; Voragen, A. G. J. Effects of Non-Covalent Interactions with 5-O-Caffeoylquinic Acid (Chlorogenic Acid) on the Heat Denaturation and Solubility of Globular Proteins. *J Agric Food Chem* **2003**, *51* (17), 5088–5095. https://doi.org/10.1021/JF021229W/ASSET/IMAGES/LARGE/JF021229WF00006.JPEG.

(39) Kaspchak, E.; Mafra, L. I.; Mafra, M. R. Effect of Heating and Ionic Strength on the Interaction of Bovine Serum Albumin and the Antinutrients Tannic and Phytic Acids, and Its Influence on in Vitro Protein Digestibility. *Food Chem* **2018**, *252*, 1–8. https://doi.org/10.1016/J.FOODCHEM.2018.01.089.

(40) Huang, Z.; Liao, L.; McClements, D. J.; Li, J.; Li, R.; Zou, Y.; Li, M.; Zhou, W. Utilizing Protein-Polyphenol Molecular Interactions to Prepare Moringa Seed Residue Protein/Tannic Acid Pickering Stabilizers. *LWT* **2022**, *154*, 112814. https://doi.org/10.1016/J.LWT.2021.112814.

(41) Nayak, A.; Genot, C.; Meynier, A.; Dorlando, A.; Capron, I. Impact of Process and Physico-Chemical Conditions on the Formation of Curcumin-Whey Protein Composite Particles Capable to Stabilize Food-Compatible Oil in Water Emulsions. *LWT* **2022**, *153*, 112421. https://doi.org/10.1016/J.LWT.2021.112421.

(42) *Why is Ionic Strength Relevant to DLS Measurements?* https://www.brookhaveninstruments.com/why-is-ionic-strength-relevant-to-dls-measurements/ (accessed 2024-07-19).

(43) Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; Tinevez, J. Y.; White, D. J.; Hartenstein, V.; Eliceiri, K.; Tomancak, P.; Cardona, A. Fiji: An Open-Source Platform for Biological-Image Analysis. *Nat Methods* **2012**, *9* (7), 676–682. https://doi.org/10.1038/NMETH.2019.

(44) Okagu, O. D.; Abioye, R. O.; Udenigwe, C. C. Molecular Interaction of Pea Glutelin and Lipophilic Bioactive Compounds: Structure-Binding Relationship and Nano-/Microcomplexation. *J Agric Food Chem* **2023**, *71* (12), 4957–4969. https://doi.org/10.1021/ACS.JAFC.3C00047/ASSET/IMAGES/MEDIUM/JF3C00047_0011.GIF.

(45) Nielsen, P. M.; Petersen, D.; Dambmann, C. Improved Method for Determining Food Protein Degree of Hydrolysis. *J Food Sci* **2001**, *66* (5), 642–646. https://doi.org/10.1111/J.1365-2621.2001.TB04614.X.

(46) García Arteaga, V.; Demand, V.; Kern, K.; Strube, A.; Szardenings, M.; Muranyi, I.; Eisner, P.; Schweiggert-Weisz, U. Enzymatic Hydrolysis and Fermentation of Pea Protein Isolate and Its Effects on Antigenic Proteins, Functional Properties, and Sensory Profile. *Foods* **2022**, *11* (1), 118. https://doi.org/10.3390/foods11010118.

(47) Xu, H.-N.; Liu, Y.; Zhang, L. Salting-out and Salting-in: Competitive Effects of Salt on the Aggregation Behavior of Soy Protein Particles and Their Emulsifying Properties. *Soft Matter* **2015**, *11* (29), 5926–5932. https://doi.org/10.1039/C5SM00954E.

(48) Rajan, R.; Ahmed, S.; Sharma, N.; Kumar, N.; Debas, A.; Matsumura, K. Review of the Current State of Protein Aggregation Inhibition from a Materials Chemistry Perspective: Special Focus on Polymeric Materials. *Mater Adv* **2021**, *2* (4), 1139–1176. https://doi.org/10.1039/D0MA00760A.

(49) Dachmann, E.; Nobis, V.; Kulozik, U.; Dombrowski, J. Surface and Foaming Properties of Potato Proteins: Impact of Protein Concentration, PH Value and Ionic Strength. *Food Hydrocoll* **2020**, *107*, 105981. https://doi.org/10.1016/J.FOODHYD.2020.105981.

(50) Thaiphanit, S.; Schleining, G.; Anprung, P. Effects of Coconut (Cocos Nucifera L.) Protein Hydrolysates Obtained from Enzymatic Hydrolysis on the Stability and Rheological Properties of Oil-in-Water Emulsions. *Food Hydrocoll* **2016**, *60*, 252–264. https://doi.org/10.1016/J.FOODHYD.2016.03.035.

(51) Zhang, Y. H.; Tang, C. H.; Wen, Q. B.; Yang, X. Q.; Li, L.; Deng, W. L. Thermal Aggregation and Gelation of Kidney Bean (Phaseolus Vulgaris L.) Protein Isolate at PH 2.0: Influence of Ionic Strength. *Food Hydrocoll* **2010**, *24* (4), 266–274. https://doi.org/10.1016/J.FOODHYD.2009.10.002.

(52) Lu, X.; Lu, Z.; Yin, L.; Cheng, Y.; Li, L. Effect of Preheating Temperature and Calcium Ions on the Properties of Cold-Set Soybean Protein Gel. *Food Research International* **2010**, *43* (6), 1673–1683. https://doi.org/10.1016/J.FOODRES.2010.05.011.

(53) Berrill, A.; Biddlecombe, J.; Bracewell, D. Product Quality During Manufacture and Supply. In *Peptide and Protein Delivery*; Elsevier, 2011; pp 313–339. https://doi.org/10.1016/B978-0-12-384935-9.10013-6.

(54) Jachimska, B.; Wasilewska, M.; Adamczyk, Z. Characterization of Globular Protein Solutions by Dynamic Light Scattering, Electrophoretic Mobility, and Viscosity Measurements. *Langmuir* **2008**, *24* (13), 6867–6872. https://doi.org/10.1021/LA800548P/ASSET/IMAGES/MEDIUM/LA-2008-00548P_0003.GIF.

(55) Deng, Y.; Huang, L.; Zhang, C.; Xie, P.; Cheng, J.; Wang, X.; Li, S. Physicochemical and Functional Properties of Chinese Quince Seed Protein Isolate. *Food Chem* **2019**, *283*, 539–548. https://doi.org/10.1016/J.FOODCHEM.2019.01.083.

(56) Chen, J.; Mu, T.; Zhang, M.; Goffin, D.; Sun, H.; Ma, M.; Liu, X.; Zhang, D. Structure, Physicochemical, and Functional Properties of Protein Isolates and Major Fractions from Cumin (Cuminum Cyminum) Seeds. *Int J Food Prop* **2018**, *21* (1), 685–701. https://doi.org/10.1080/10942912.2018.1454467.

(57) Siow, H. L.; Lim, T. S.; Gan, C. Y. Development of a Workflow for Screening and Identification of α-Amylase Inhibitory Peptides from Food Source Using an Integrated Bioinformatics-Phage Display Approach: Case Study – Cumin Seed. *Food Chem* **2017**, *214*, 67–76. https://doi.org/10.1016/J.FOODCHEM.2016.07.069.

(58) Zhang, T.; Jiang, B.; Mu, W.; Wang, Z. Emulsifying Properties of Chickpea Protein Isolates: Influence of PH and NaCl. *Food Hydrocoll* **2009**, *23* (1), 146–152. https://doi.org/10.1016/J.FOODHYD.2007.12.005.

(59) Chang, L.; Lan, Y.; Chen, B.; Rao, J. Interfacial, and Emulsifying Properties Nexus of Green Pea Protein Fractions: Impact of PH and Salt. *Food Hydrocoll* **2023**, *140*, 108652. https://doi.org/10.1016/J.FOODHYD.2023.108652.

(60) Poklar Ulrih, N. Analytical Techniques for the Study of Polyphenol-Protein Interactions. *Crit Rev Food Sci Nutr* **2017**, *57* (10), 2144–2161. https://doi.org/10.1080/10408398.2015.1052040.

(61) Ru, Q.; Wang, Y.; Lee, J.; Ding, Y.; Huang, Q. Turbidity and Rheological Properties of Bovine Serum Albumin/Pectin Coacervates: Effect of Salt Concentration and Initial Protein/Polysaccharide Ratio. *Carbohydr Polym* **2012**, *88* (3), 838–846. https://doi.org/10.1016/J.CARBPOL.2012.01.019.

(62) Tapal, A.; Tiku, P. K. Complexation of Curcumin with Soy Protein Isolate and Its Implications on Solubility and Stability of Curcumin. *Food Chem* **2012**, *130* (4), 960–965. https://doi.org/10.1016/J.FOODCHEM.2011.08.025.

(63) Pan, K.; Luo, Y.; Gan, Y.; Baek, S. J.; Zhong, Q. PH-Driven Encapsulation of Curcumin in Self-Assembled Casein Nanoparticles for Enhanced Dispersibility and Bioactivity. *Soft Matter* **2014**, *10* (35), 6820–6830. https://doi.org/10.1039/c4sm00239c.

(64) Rahimi Yazdi, S.; Corredig, M. Heating of Milk Alters the Binding of Curcumin to Casein Micelles. A Fluorescence Spectroscopy Study. *Food Chem* **2012**, *132* (3), 1143–1149. https://doi.org/10.1016/J.FOODCHEM.2011.11.019.

(65) Zhang, Q.; Jeganathan, B.; Dong, H.; Chen, L.; Vasanthan, T. Effect of Sodium Chloride on the Thermodynamic, Rheological, and Microstructural Properties of Field Pea Protein Isolate/Chitosan Complex Coacervates. *Food Chem* **2021**, *344*, 128569. https://doi.org/10.1016/J.FOODCHEM.2020.128569.

(66) Zhong, W.; Zhang, T.; Dong, C.; Li, J.; Dai, J.; Wang, C. Effect of Sodium Chloride on Formation and Structure of Whey Protein Isolate/Hyaluronic Acid Complex and Its Ability to Loading Curcumin. *Colloids Surf A Physicochem Eng Asp* **2022**, *632*, 127828. https://doi.org/10.1016/J.COLSURFA.2021.127828.

(67) *Binding Affinity | Malvern Panalytical*. https://www.malvernpanalytical.com/en/products/measurement-type/binding-affinity (accessed 2024-08-12).

(68) Tomé, D. Digestibility Issues of Vegetable versus Animal Proteins: Protein and Amino Acid Requirements-Functional Aspects. *Food Nutr Bull* **2013**, *34* (2), 272–274. https://doi.org/10.1177/15648265130340022 5/ASSET/15648265130340022 5.FP.PNG_V03.

(69) Boachie, R. T.; Capuano, E.; Oliviero, T.; Udenigwe, C. C.; Fogliano, V. Undigested Glycated Lentil Proteins Modulate the Gut Microbiota Profile but Not the Metabolites in Vitro. *J Funct Foods* **2023**, *107*, 105667. https://doi.org/10.1016/J.JFF.2023.105667.

(70) Liu, K.; Ning, M. Antioxidant Activity Stability and Digestibility of Protein from Se-Enriched Germinated Brown Rice. *LWT* **2021**, *142*, 111032. https://doi.org/10.1016/J.LWT.2021.111032.

CHAPTER 4 – CONCLUSION

In addition to contributing nutrients and biologically active compounds that are beneficial in terms of nutrition and health, proteins and polyphenols further impact food quality and stability via physical and chemical relationships. In general terms, enhancing the digestibility of plant proteins will enable the optimal utilization of their nutritional ingredients, eliminate food waste, and promote sustainability. Pea proteins are in increasing popularity in food systems owing to their extensive industrial uses, including their techno-functional qualities such as gelling, foaming, emulsifying, and water and oil-holding abilities. The complexity of pea protein fractions with curcumin modified the microstructures and physicochemical characteristics of individual protein fractions, each having unique variations during digestion. The complexation was mediated by non-covalent interactions such as hydrophobic interactions. There was an overall decrease in protein digestibility, which confirms the mechanism of interaction that occurred. Among the three protein fractions, pea glutelin had no protein hydrolysis due to the ordered sheet-like structures formed upon complexation with curcumin which inhibited digestive enzymes from accessing cleavage sites of the protein. Notably, pea glutelin has been shown to serve as a potential delivery system for curcumin based on its high binding strength. Understanding the physiological conditions of the gastrointestinal tract is essential to recognizing their critical role in protein absorption, bioavailability, and digestion. In this context, the effect of a significant factor in the gastrointestinal tract (ionic strength) on the water-soluble fraction (pea albumin) and curcumin complexes and the extent of this influence on protein digestibility was investigated. Ionic strength drastically modulated the physiochemical characteristics even though the effect on protein digestibility was negligible. Tryptophan fluorescence quenching revealed the nature and strength of binding of the curcumin to protein in the presence and absence of ionic strength. It turns out that binding was more favorable in the absence of ionic strength. There were more weakly unstable complexes at high ionic strength. Regarding the composition of the food matrix, protein-polyphenol complexation is similarly significant as its formation. However, there is limited information on food matrix effects on protein digestibility. Owing to the complexity of food matrices. i.e. different food components, technological processing of food products, there exist contradicting results or findings of the impact on protein in the gastrointestinal tract. Additionally, variations in protein digestibility could stem from the existence of components that modify its digestion such as antinutrients. Antinutrients appear to exist in the make-up of proteins. They are secondary

metabolites that are produced by plants to protect themselves from external stress like pathogenic attacks, and ultraviolet light. In this case, it is ideal that proteins all contain some amount of antinutrients which are present all through the complexation of proteins with other food components. They might not exert their effects during food processing; however, they are shown to have adverse effects on protein bioavailability and digestibility. These components tend to inhibit the access of digestive enzymes to proteins. They include protease inhibitors, lectins, saponins, and phytates. This might also be part of the reasons for reduced protein digestibility.

For future research, it is vital to carefully design and optimize protein pretreatment processing procedures such as non-thermal treatment to increase overall protein digestibility while retaining the nutritional and functional properties of the proteins. Despite the recorded reduction in protein digestibility, the released peptides might possess biologically active qualities, further studies ought to inquire into bioactivity utilizing different assays, such as the antioxidant assay. To deepen insights into protein-curcumin binding mechanisms and accurately assess binding strength, future studies should adopt complementary techniques such as isothermal titration calorimetry (ITC) and circular dichroism (CD). The results indicate that protein binding to curcumin led to a decrease in protein digestibility across different curcumin concentrations. Notably, lower concentrations of curcumin caused a greater enhancement in protein digestion compared to higher concentrations. Moving forward, future research should focus on optimizing curcumin loading into proteins for the development of delivery systems that maximize encapsulation efficiency, improve curcumin bioavailability, and minimize adverse effects on protein digestibility.