Physico-chemical and techno-functional properties of proteins isolated from the green microalgae *Tetraselmis* sp.

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Abstract

In this thesis, the mild isolation of an algae soluble protein isolate (ASPI) and the characterisation of its techno-functional properties are described. The ASPI was isolated from the green microalgae *Tetraselmis* sp. by beadmilling and subsequent anion exchange adsorption. The isolate obtained contained 59 ± 7% (w/w) protein and 20 ± 6% (w/w) carbohydrates, the latter composed for approximately one fourth of uronic acids (4.8 ± 0.4% [w/w]). In the pH range 5.5 – 6.5, in which currently used legumin seed protein isolates (e.g. soy) show low solubility, ASPI retained high solubility independent of ionic strength. In the soluble pH range, the foam stability of ASPI is superior to the foam stabilities of whey protein isolate (WPI) and egg white albumin (EWA). At pH 7, ASPI stabilized foams are 1.7 times more stable than WPI stabilized foams. Further fractionation of APSI results in foams even 3 times more stable than WPI stabilized foams. In addition, emulsions stabilised with ASPI are stable against droplet aggregation around pH 5 at low ionic strength, while emulsions stabilised by WPI are not stable at this pH. The stability of ASPI emulsions at this pH is attributed to the co-adsorption of the charged polysaccharide fraction present in ASPI. The role of the charged polysaccharides on stabilisation of the emulsions was confirmed by fractionating ASPI into protein-rich and charged polysaccharide-rich fractions. The combination of charged polysaccharides and proteins in ASPI results in good techno-functional properties that are between that of pure proteins and that of the naturally occurring protein-polysaccharide hybrid gum arabic (GA). It is concluded that ASPI represents an attractive substitute for currently used high-value food protein isolates. Due to the combination of the positive interfacial properties of its protein fraction with the broad pH stability of its charged polysaccharide fraction, ASPI possesses the positive attributes of two types of techno-functional ingredients.

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1 General Introduction

Microalgae have been considered a promising alternative protein source in foods already since the early 1950's. Initially, the interest in microalgae protein was based on its high nutritional quality (e.g. Fabregas & Herrero, 1985). Nevertheless, the application of microalgae as protein supplements in foods did not gain significant importance, since the presence of non-protein components (e.g. chlorophyll) led to undesirable changes in colour, taste and structure (Becker, 2007; Davies, 1971). Recently, the interest in microalgae protein has been renewed in connection with the interest in producing biofuels from microalgae. Feasibility studies have indicated that the extraction of high-value protein fractions for techno-functional application could contribute considerably to the value of the microalgae biomass and, thus, to the cost-efficient production of microalgae biofuels (Williams & Laurens, 2010). In spite of the continuing interest in microalgae proteins, no adequate isolation procedures are available to enable studies of protein compositions and resulting techno-functional properties of microalgae protein isolates. Therefore, in this thesis, first a mild isolation process was developed to obtain a soluble protein isolate free from any intense colour from the green microalgae *Tetraselmis* sp. Subsequently, the physico-chemical and techno-functional properties of the protein isolate obtained were studied. To assess the techno-functional quality of the studied microalgae protein isolate, its techno-functional properties were described in reference to currently used food proteins like, e.g., whey protein isolate.

1.1. Microalgae

The taxonomic term "algae" refers to a highly heterogeneous group of simple eukaryotic organisms defined neither as plants nor as bacteria. Typically, algae are classified into different groups based on the types of chlorophyll and accessory pigments present in their photosynthetic membranes. Chlorophyta (green algae), for example, are characterized by the presence of the green pigment chlorophyll, while rhodophyta (red algae) owe their red colour to the phycobiliprotein phycoerythrin (van den Hoek et al., 1995). In addition to this classification system, single cell or undifferentiated multicellular algae are referred to as microalgae, while more complex multicellular algae are called macroalgae. Due to their simple form of organization microalgae can reach considerably higher growth rates than macroalgae (Williams & Laurens, 2010). In addition, compounds like, e.g., lipids, starch or different pigments can be easily accumulated in microalgae by varying growth conditions (Singh & Gu, 2010). For these reasons, microalgae have been identified as promising biomass producers.

1.2. Microalgae protein

While green macroalgae typically contain 14 - 25% (w/w) protein (Harnedy & Fitzgerald, 2011), green microalgae possess with 40 – 50% (w/w) higher protein contents (Table 1.1). Most commonly these protein contents are estimated as so called crude protein by multiplying the total nitrogen content of the microalgae biomass by a nitrogen-to-protein conversion factor (N-Prot factor). The conventionally used N-Prot factor of 6.25 is based on the assumption that the samples contain proteins with 16% (w/w) nitrogen and hardly any non-protein nitrogen (NPN). Microalgae, however, do contain considerable amounts of NPN in the form of inorganic nitrogen, nucleic acids, and other organic nitrogen. Thus, the use of the conventional factor results in an overestimation of the true protein content (Becker, 1994; Lourenço et al., 2004). To determine the protein contents of microalgae correctly, specific N-Prot factors need to be calculated from the amino acid composition of the microalgae of interest. Based on the average of N-Prot factors determined for different microalgae species in different growths phases, Lourenço et al. (2004) have recommended the use of an average N-Prot factor of 4.78 (± 0.36) for microalgae species with unknown specific N-Prot factors.

Table 1.1 - General composition of different green microalgae in comparison to different food protein sources (w/w% dry matter) (based on Becker, 1994, Belitz et al., 2009; Schwenzfeier et al., 2011)

Commodity	Protein	Carbohydrates	Lipids
Tetraselmis sp.	36[a]	24	22
Chlamydomonas rheinhardii	48[b]	17	21
Chlorella vulgaris	55[b]	15	18
Dunaliella salina	57[b]	32	6
Scendesmus obliquus	53[b]	14	13
Egg white	88[c]	7	< 1
Milk	26[c]	38	28
Soybean	37[c]	30	20

[a] Nx5.60
[b] Nx6.25
[c] not specified

Table 1.2 – Protein fractions identified in different microalgae species by SDS-PAGE analysis and immunoblotting

Algae species	Identified proteins	MW [kDa]	Location	Reference
Chlamydomonas reinhardtii	Light harvesting Chlorophyll a/b protein	24.5 / 23.5	Thylakoid membrane	Kuchitsu, 1988
	Possibly photosystem II herbicide binding protein	32		
Chlorella *Chlamydomonas reinhardtii*	Hydroxy-proline-O-glycosides	n.d.	Cell wall	Miller, 1972
Haematococcus pluvialis	81 proteins: 8 functional categories, mainly cell-wall modifying enzymes and transport/binding proteins	15 - < 225	Cell wall	Wang, 2004
Chlorella vulgaris	Rubisco 38-kDa polypeptide (periplasmic carbonic anhydrase)	52 / 14 38	Not specified	Villarejo, 1998
Dunaliella salina	520 proteins in total, mainly involved in motor, signalling , cell division, metabolic processes	n.d.	Flagella	Jia, 2010
Spirulina platensis (cyanobacteria)	Four main phycobiliproteins	18 - 19	Not specified	Simo, 2005
Spirulina platensis (cyanobacteria)	None, suggested: part of or associated with photsynthesis	14.4 - 116	Not specified	Chronakis, 2000
	Phycobiliproteins	(most pronounced: 20.1, 43)		
Porphyridium cruentum (red microalgae)	B-phycoerythrin	α, β and γ-subunits of about 17, 18 and 27 kDa	Not specified	Bermejo Román, 2002

1.2.1. Composition of microalgae protein

Protein isolates derived from animal sources or different terrestrial crops are typically characterized by the presence of a major storage protein fraction (e.g. milk with 80% casein soy with 55% glycinin + β-conglycinin). Microalgae, however, do not accumulate distinct storage proteins as N-source for the next generation, since as eukaryotic single cell organisms they reproduce by cell division. Therefore, most proteins present in microalgae are expected to be enzymes involved in photosynthesis or other metabolic activities (Contreras et al., 2008; Wang et al., 2004). The exact composition of microalgae protein, however, is still largely unknown and no quantitative data is available yet. Some of the proteins already identified in different microalgae are summarized in table 1.2.

1.2.1.1. Rubisco

Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) is suggested to be one of the most important and most abundant enzymes in photosynthetic organisms like microalgae, since it catalyses the initial reaction during the conversion of inorganic CO_2 to organic matter (Ellis, 1979). The enzyme complex typically adopts a hexadecameric quaternary structure by the association of 8 large (55 kDa) and 8 small (15 kDa) polypeptide chains, resulting in a total molecular mass around 560 kDa (form I) (Taylor et al., 2001). In some photosynthetic bacteria, however, the enzyme is a dimer composed of two large polypeptide chains (form II) (Luo et al., 2001). In both cases, the substrate binding site is located at the interface of two large polypeptide chains. Although the major activity of Rubisco is that of a carboxylase, its affinity for CO_2 is rather low. In addition to its inefficiency in binding CO_2, the carboxylation activity of Rubisco is compromised by its oxygenase side activity. To correct for its low activity Rubisco occurs abundantly in photosynthetic tissues (Ellis, 1979). Due to this abundance Rubisco in its purified, native form is often considered one of most promising candidates for the techno-functional applications of photosynthetic proteins from leaves and algae. On analytical scale Rubisco is readily isolated from the cytoplasm-stroma mix obtained from leafy tissues by ammonium sulphate precipitation and subsequent gel filtration (e.g. Ranty & Cavalie, 1982). In green microalgae, however, Rubisco is often stored for a large part (depending on growth conditions) in a cell organelle denoted the pyrenoid (Griffiths, 1970). The pyrenoid is typically surrounded by a starch sheath, which might hinder the accessibility of Rubisco even after disintegration of the strong cellulosic cell wall.

Table 1.3 - Isolation strategies applied to extract protein from green microalgae

Method	Used algae species	Applied condtions during			Reference	Protein yield reported	Final form of isolated protein
		Pretreatment	Cell disintegration	Protein purification			
Alkali treatment	*Scendesmus acutus*	n.a.	0.5 M NaOH, 10 min, 80 -100 °C, repeatedly	n.a.	Rausch, 1981	Indirect, ~ 24% (w/w)	Soluble
Organic extraction + enzymatic hydrolysis	*Chlorella, Scenedesmus*	100% EtOH, 50°C, 3h	n.a.	Protease incubation	Tchorbanov, 1987	No	Pellet, solubilized by subsequent protease incubation
Sonication	*Chlorella*	n.a.	Sonication, 1% SDS, 55 - 70 W	n.a.	Meijer, 1998	Indirect, strongly dependent on detection method	Soluble
Homogenization + TCA precipitation	15 marine algae species	n.a.	0.1 M NaOH, 5 min, 4 °C	25 % TCA	Barbarino, 2005	No	Pellet, re-suspended in NaOH/SDS PAGE sample buffer for protein analysis
Pressurized liquid extraction + ammonium sulphate precipitation	*Spirulina platensis*	n.a.	1500 psi, 45 min	70% $(NH_4)_2SO_4$	Herrero, 2005	No	Soluble
Bead milling	*Chlorella*	n.a.	External cooling	n.a.	Doucha, 2008	No	Not reported (expectedly soluble)
Enzymatic degradation of algal cell wall	Red seaweeds				Fleurence, 1995	Indirect, max 2.1% (w/w)	Soluble

n.a. = not applied

1.2.1.2. Other proteins

As shown in table 1.2, most proteins identified in microalgae have either structural or metabolic functions. During proteome analysis of the proteins present in the cell wall of the green microalgae *Haematococcus pluvialis* 38 proteins were identified and divided in 9 categories. The two major protein categories (9 proteins each) were cell-wall modifying enzymes and transport/binding proteins (Wang et al., 2004). Next to their catalytic role in metabolic processes, proteins present in microalgae serve a structural role. In the so-called light harvesting complexes of the chloroplast, for example, proteins provide a matrix for the assembly of chlorophyll molecules (Williams & Laurens, 2010; Gantt, 1980). Next to pigments, proteins present in microalgae can also be complexed with sugar moieties. For the two green microalgae *Chlorella* and *Chlamydomonas reinhardtii* it has been shown that their cell wall consists partly of cross-linked hydroxyl-proline-rich glycoproteins (Miller et al., 1972).

1.2.2. Extraction of microalgae protein

The extraction of intracellular proteins from unicellular microalgae such as *Tetraselmis* sp. is usually hindered by their strong cellulosic cell walls (Becker, 2007; Doucha & Lívanský, 2008). To disintegrate these cell walls, various harsh chemical conditions, e.g. alkali cell dissolution or organic solvent extraction, have been applied (Table 1.3). In addition, the use of cell wall degrading enzymes has been suggested to facilitate cell disintegration. This biotechnological approach, however, would most probably have to be validated and optimized for each algae species because of differences in cell wall compositions (Fleurence, 1999). In addition, to prevent the degradation of proteins, only enzyme preparations devoid of proteolytic side activity should be applied. As third extraction strategy, physical cell disintegration, e.g. by sonication or homogenisation, has been tested. Typically, these method were combined with a subsequent precipitation step to further purify the protein (e.g. 10% trichloroacetic acid (w/v) [TCA] or 70% (w/v) $(NH_4)_2SO_4$ precipitation).

Since most of the methods summarized in table 1.3 were aimed at increasing protein accessibility for digestive enzymes or developed for analytical purposes, protein denaturation was not considered an issue in those studies. As a consequence, the isolates obtained often lacked solubility as a direct result of the harsh isolation conditions applied. One method applied to re-solubilize the precipitated proteins was their degradation to

peptides using either NaOH at high temperatures or a proteolytic enzyme. To cover all possible techno-functional applications of microalgae proteins, however, they need to be isolated as intact proteins in a soluble form. Although the extraction methods described by Meijer & Wijffels (1998) and Herrero et al. (2005) yield soluble protein fractions, the use of sodium dodecyl sulphate (SDS) and ammonium sulphate, respectively, is undesirable. SDS strongly binds to the proteins present and might alter their techno-functional properties, especially their interfacial properties. Ammonium sulphate precipitations are usually not suitable for large-scale applications. In conclusion, out of the methods summarized in table 1.3 physical cell disintegration by bead milling seems to be the most suitable method for the extraction of a soluble and techno-functional protein fraction from microalgae and is, therefore, applied in this research.

1.3. Techno-functional properties of food proteins

Proteins are often used in food applications, since they can have good water binding, structuring (aggregation, gelation) and surface (foaming, emulsifying) properties. One prerequisite for these properties is typically high protein solubility. All these techno-functional properties depend on the molecular properties of the protein, but are also strongly influenced by parameters like pH, ionic strength and the presence of other molecules, e.g. polysaccharides.

For microalgae derived proteins, hardly any information is available on the techno-functional properties, since they have not been isolated for this purpose yet. However, since Rubisco is one of the proteins considered to be abundantly present in microalgae protein isolates, some information may be obtained from studies on Rubisco-rich leaf protein isolates. For protein isolates from different leaf species (tobacco, alfalfa, soy, sugar beet), it was already shown that they possess similar techno-functional properties (Sheen, 1991). This might be caused by the abundance of Rubisco in these isolates. Protein isolates from tobacco and alfalfa leaves showed high solubility except in the pH range 4 – 6, around the isoelectric point of Rubisco (Knuckles & Kohler, 1982; Sheen & Sheen, 1985). In another study, the techno-functional properties (water-binding, emulsification, gelation) of tobacco leaf protein isolate were compared to those of egg white and soy protein isolates (Sheen & Sheen, 1985). The leaf protein fractions were found to have better properties, as illustrated for instance by the higher foam stability.

The above indicates that Rubisco containing extracts from microalgae may have positive techno-functional properties. Nevertheless, there is very little information available on the techno-functional properties of Rubisco containing protein isolates from leaves, or from microalgae. Especially the effects of different system conditions (such as pH, ionic strength) have not been studied. In addition, also sample composition (e.g. the presence of polysaccharides) can have an effect on these properties. The influence of both factors on the techno-functional properties of proteins is discussed in the next sections, using existing information from more commonly used food proteins.

1.3.1. Protein solubility

One of the most important prerequisites for the successful application of protein isolates in foods is high solubility. The solubility of a single protein is typically minimal at its isoelectric point (pI), since at this point the overall net charge is zero and electrostatic repulsion is low. At all other pH values, the electrostatic repulsion between the individual protein molecules is high and they are kept in solution as long as they retain their native conformation (Fennema et al., 2008). Typically, this pH dependent solubility behaviour of a single protein results in a bell-shaped solubility curve with a minimum around the pI (Figure 1.1A, B) (González-Pérez et al., 2005; Lakemond et al., 2000). Some proteins (e.g. β-lactoglobulin, ovalbumin, sunflower albumins), however, retain high solubility even around their pI. Upon denaturation (e.g. by heat), however, the solubility for those proteins typically decreases around the pI due to the increased exposure of hydrophobic groups at the protein surface. (Zhu & Damodaran, 1994).The solubility of a single protein can also be altered by the presence of other molecules (e.g. other proteins, polysaccharides). Sunflower albumins (SFA), for example, are highly soluble over a broad pH range in their purified form (Figure 1.1B). If present as part of complete sunflower isolate, however, the electrostatic interactions between SFA and helianthinin (the two proteins are oppositely charged at pH values between 5.5 and 7.0) result in decreased solubility at pH 4.0 - 5.5.

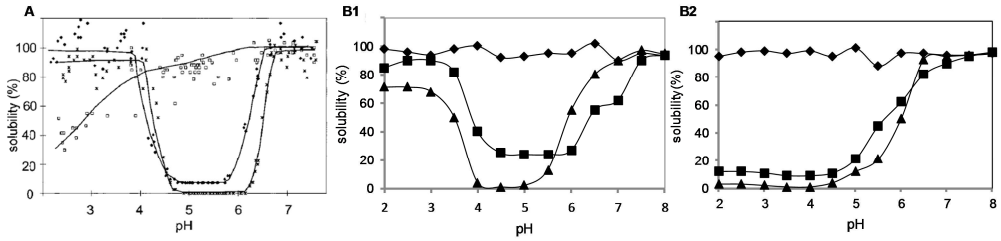

Figure 1.1 – pH dependent solubility profiles of glycinin [I = 0.5 M (□), I = 0.2 M (♦), I = 0.03 M (*)] (A) and helianthinin (▲), sunflower albumins (♦) and sunflower isolate (■) [I = 0.03 M (B1), I = 0.25 M (B2], (reproduced and adapted from González-Pérez et al., 2005; Lakemond et al., 2000)

Next to pH, also ionic strength (I) influences the solubility of proteins. Generally, it is suggested that a moderate increase in ionic strength tends to increase solubility. For glycinin, for example, it was shown that an ionic strength of 0.5 M is sufficient to ensure high solubility (~ 90%) around the pI (~pH 5). For helianthinin, in contrast, an increase in ionic strength to 0.25 M has shown to decrease solubility at pH < 5 (Figure 1.1).

1.3.2. Formation and stability of protein stabilized foams and emulsions

Foams as well as emulsions are colloidal systems, in which one phase (air in foam, oil in oil-in-water emulsions) is dispersed in another phase. The addition of a protein facilitates the droplet/bubble break-up during the production of foams and emulsions by adsorption to the droplet/bubble surface. As a result, it lowers surface tension and prevents immediate coalescence (Tcholakova et al., 2003). The emulsifying and foaming ability of a protein can be characterized by the minimum protein concentration needed to produce stable emulsions and foams, respectively. Above this concentration protein-stabilized oil-in-water-emulsions typically show an average droplet size of ≤ 1μm, dependent on homogenization conditions. In this so-called, 'emulsifier-rich' regime (Tcholakova et al., 2003), the final droplet size is independent of the isolate concentration, since sufficient protein is present to cover the surface of the smallest droplets formed within the homogenizer valve. For emulsions stabilized with the potato protein patatin, for example, this protein concentration was 2 mg/mL (Van Koningsveld et al., 2006) (Figure 1.2). Below this concentration, the average droplet size increases steeply with decreasing patatin concentration. In this so-called 'emulsifier-poor' regime, the isolate concentration is insufficient to stabilize the smallest droplets formed. This resulted in droplets ≥ 1 μm and, thus, emulsions prone to instability.

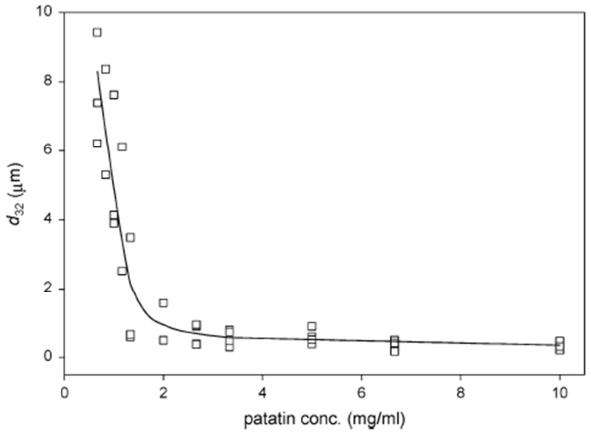

Figure 1.2 – Average droplet diameter (d_{32}) of patatin stabilized emulsions (10% oil (w/v), pH 7, I = 50 mM) as function of protein concentration (mg/mL) (reproduced from Van Koningsveld et al., 2006)

1.3.2.1. Effect of pH, ionic strength and charged polysaccharides on protein stabilized foams and emulsions

The aggregation of protein-covered emulsion droplets typically occurs close to the pI of the protein and at increased ionic strength due to low electrostatic repulsion between the individual droplets (Figure 1.3).

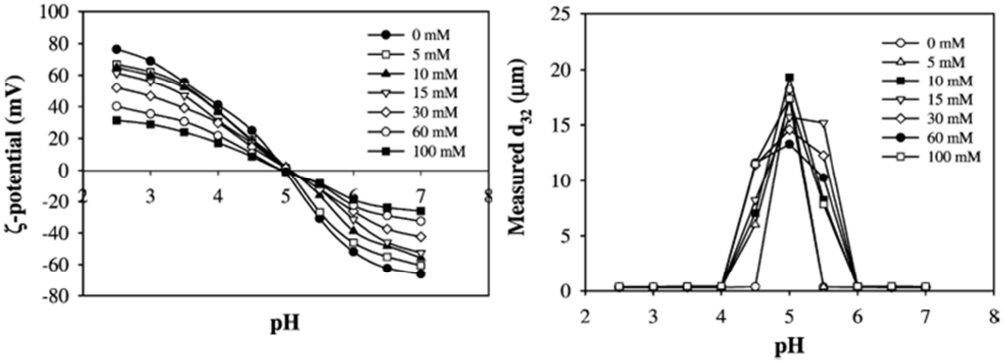

Figure 1.3 – Droplet ζ-potenials (A) and mean droplet diameter (B) of oil-in-water emulsions stabilized with 0.04% (w/w) whey protein isolate as function of pH at different KCl concentrations (reproduced from Kulmyrzaev & Schubert, 2004)

To enhance emulsion stability around the pI or at increased ionic strength, polysaccharides can be added to the protein solution. Typically, the presence of a polysaccharide enhances emulsion stability by increasing the viscosity of the continuous phase (Dickinson, 2003). In addition, anionic polysaccharides, typically not surface active, can co-adsorb to the droplets surface by the formation of soluble complexes with a surface-active protein above the pK_a of the polysaccharide and below the isoelectric point of the protein (Ganzevles et al., 2006). For oil-in-water-emulsion stabilized by β-lactoglobulin-pectin adsorbed layers, for example, droplet ζ-potential measurements indicated that the co-adsorption of the pectin molecules results in high electrostatic repulsion in the pH range 4.0 – 5.5 and, thus, prevents flocculation (Guzey & McClements, 2007). Similar to emulsions purely stabilized by β-lactoglobulin, the addition of 50 mM NaCl decreased electrostatic repulsion between the β-lactoglobulin-pectin covered droplets and promotes flocculation. This example indicates the potential of protein-polysaccharide complexes to enhance emulsion stability, provided that pH and ionic strength are carefully controlled.

In contrast to emulsions, the effects of pH and ionic strength on the foam properties of a protein have been almost exclusively investigated indirectly by studying interfacial

properties. Generally, it is assumed that those interfacial properties reflect the actual foam properties. For the proteins bovine serum albumin (BSA) and ovalbumin it was shown that their adsorption to the air-water interface increased around their respective pI and at high ionic strength (Cho et al.; 1997, Wierenga et al.; 2005, Engelhardt et al., 2012) (Figure 1.4). The increased protein adsorption under these conditions was found to result in faster adsorption kinetics, which in turn resulted in higher foam formation and stability (Wierenga & Gruppen, 2010).

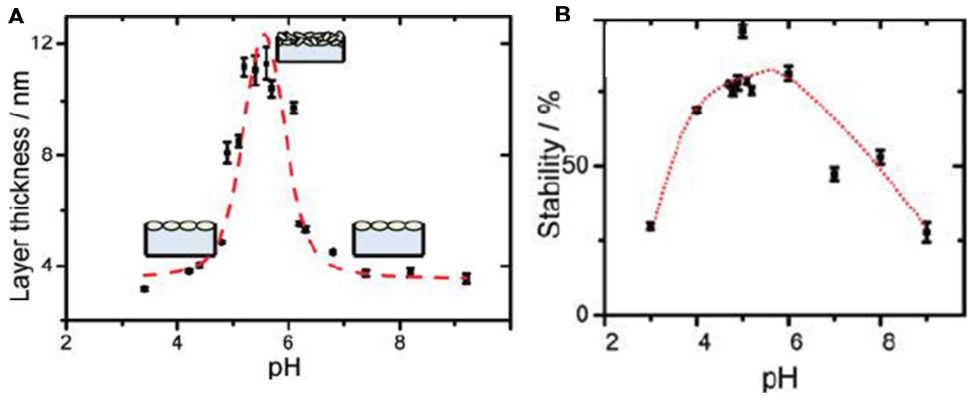

Figure 1.4 – Thickness of adsorbed BSA layers at the air-water interface (A) and corresponding foam stability (B) as function of pH (reproduced from Engelhardt et al., 2012)

In contrast to the results of those studies, which suggest a general link between reduced electrostatic repulsion and increased foam stability, Kuropatwa et al. (2009) found diverging effects of low electrostatic repulsion on foams stabilized for WPI and egg white protein. While egg white protein foams were indeed more stable at the pI of the isolate, the stability of WPI stabilized foams was minimal at the pI of WPI. The latter was attributed to the formation of β-lactoglobulin octamers.

Similar to emulsion stability, foam stability can be enhanced by the addition of a polysaccharide, primarily by decreasing the rate of disproportion and drainage by increasing the bulk viscosity (Yang & Foegeding, 2010). In addition, the adsorption of electrostatically stabilized protein-polysaccharides adsorption was shown to result in equal or superior interfacial properties and subsequent foam stabilities compared to pure protein (Benichou et al., 2007; Miquelim et al., 2010; Schmitt & Turgeon, 2011).

1.4. Aim and outline of the thesis

Although the extraction of high-value protein fractions for techno-functional applications in foods is expected to contribute considerably to the commercial value of the microalgae biomass, the structural and techno-functional properties of microalgae proteins are only poorly documented. Therefore, the research described in this thesis aims to provide knowledge about the physico-chemical and techno-functional properties of microalgae protein.

The mild isolation process used to obtain an algae soluble protein isolate (ASPI) from *Tetraselmis* sp. free from any intense colour or taste is described in **Chapter 2**. Chemical composition, amino acid composition and pH-dependent solubility of all intermediate fractions and the final ASPI are reported. In addition, specific nitrogen-to-protein conversion factors are determined for all fractions in order to follow the removal of non-protein nitrogen. **Chapter 3** describes the effects of isolate concentration, pH, ionic strength and calcium ion concentration on the formation and stability of emulsions prepared using ASPI as emulsifier. Whey protein isolate (WPI) and gum arabic (GA) are used as reference emulsifiers, since WPI represents one of the most classes of proteinaceous emulsifiers and GA is, like ASPI, a naturally occurring polysaccharide-protein conjugate able to stabilize emulsions. In **Chapter 4** the effects of isolate concentration, pH and ionic strength on the interfacial properties as well as the foam properties of ASPI are discussed. In this study WPI and egg white albumin (EWA), two foaming agents commonly applied in food industry, are used as reference. In **Chapter 5** the influence of the polysaccharides present in ASPI on the techno-functional properties of the isolate is investigated. Therefore, ASPI was fractionated using centrifugal membrane filtration. The fractions obtained were characterized with regard to their chemical composition and their foaming and emulsion properties were studied as function of pH. Finally, **Chapter 6** addresses remaining questions about the representativeness of the isolation process developed for *Tetraselmis* sp. In addition, the compositional analysis of microalgae protein isolates and the study of their techno-functional properties are discussed.

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2 Isolation and characterization of soluble protein from the green microalgae *Tetraselmis* sp.

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Abstract

Extraction of high-value protein fractions for techno-functional applications in foods can considerably increase the commercial value of microalgae biomass. Proteins from *Tetraselmis sp.* were extracted and purified after cell disintegration by bead milling, centrifugation, ion exchange chromatography using the absorbent Streamline DEAE, and final decolourization by precipitation at pH 3.5. The algae soluble isolate was free from the intense colour typical for algae products and contained 64% (w/w) proteins and 24% (w/w) carbohydrates. The final isolate showed solubility independent of ionic strength and 100% solubility at and above pH 5.5. Since most plant proteins used in foods show poor solubility in the pH range 5.5 - 6.5, the algae soluble protein isolate could be useful for techno-functional applications in this pH range.

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2.1. Introduction

Microalgae are a potential feedstock for the next generation of biofuels, but recent feasibility studies (Wijffels & Barbosa, 2010; Williams & Laurens, 2010) have shown that an integrated biorefinery approach is needed to make the microalgae biofuel production profitable. As one of the major constituents of the algae biomass, proteins (up to 50% [w/w]) are expected to play an important role in algae biorefinery (Williams & Laurens, 2010). Due to their abundance and their amino acid profile microalgae proteins have long been considered as an alternative protein source in foods (Spolaore et al., 2006). Nevertheless, the application of microalgae protein did not gain significant importance, since the presence of non-protein components (e.g. chlorophyll) led to undesired changes in colour, taste, and structure (Becker, 2007). To increase the potential applications in food and to add to its commercial value, microalgae proteins have to be isolated from the microalgae cell free from any intense colour and taste and without changes in the molecular structure.

Mild isolation techniques are necessary to recover proteins with high solubility and good techno-functional properties (Løkra et al., 2008), but the rigid cell wall of green microalgae such as *Tetraselmis* hinders the extraction of intact intracellular proteins (Doucha & Lívanský, 2008). To access them various harsh physical or chemical conditions including, e.g., alkali cell dissolution, organic solvent extraction or high-pressure homogenization have been applied. Protein denaturation was not considered a problem in those studies, since most of the methods described were developed for analytical purposes or were aiming at the production of enzymatic protein hydrolysates (Contreras et al., 2008; Morris et al., 2008; Tchorbanov & Bozhkova, 1988). Bead milling, in contrast, is an effective way to release intracellular protein in their native form from microbial cells (Chisti & Moo-Young, 1986), provided heat development inside the grinding chamber can be prevented. To separate the liberated intracellular protein from other soluble cell components ion exchange chromatography can be used. For many different microbial feedstocks including cyanobacteria (Ramos et al., 2010) it was carried out as Expanded Bed Absorption (EBA) to allow direct protein capture from the crude cellular lysate still containing insoluble cell debris. If applied during microalgae protein isolation EBA could help to reduce the production costs by eliminating at least one centrifugation step and possibly also other purification steps.

This study describes the isolation of soluble proteins from the commercially available microalgae, *Tetraselmis sp.*, under mild, non-denaturing conditions to enable the study of

their techno-functional properties. During isolation special attention is paid to the removal of coloured substances to allow a broad range of applications of the final protein isolate. Solubility of the algae protein isolate was tested at different pH values, and the amino acid composition was determined at different stages of isolation. Since the conventional nitrogen-to protein conversion factor (N-Prot factor) of 6.25 is unsuitable for microalgae due to their high concentrations of non-protein nitrogen (NPN), the conversion factors k_p (actual protein content from total measured nitrogen) and k_a (based purely on the amino acid composition of the respective protein fraction) (Mossé, 1990) were determined at every processing step in order to follow the removal of NPN.

2.2. Material and Methods

All chemicals were purchased from either Merck (Darmstadt, Germany) or Sigma Aldrich (St. Louis, MO, USA) if not stated otherwise. The algae material used was commercially available nonviable Instant Algae® *Tetraselmis* (Reed Mariculture, Campbell, CA, USA). It was kept frozen prior to use.

2.2.1. Preparation of the algae soluble protein isolate

Algae paste containing 24 g dry matter / 100 g paste (as determined by AACC method 44-15.02) was diluted 1:1 (w/v) with 100 mM Tris/HCl buffer, pH 8.0, 1 mM EDTA, 50 mM DTT, and 5 mM $MgCl_2$. For cell disintegration the diluted algae suspension was recirculated through the grinding chamber of the agitation bead mill DYNO®-Mill Type MULTI LAB (Willy A. Bachofen AG Maschinenfabrik, Muttenz, Switzerland) for 30 min with a pump speed of 1.5 L/min. The 0.3 L grinding chamber was filled with the maximum amount (approximately 65 % [v/v]) of ceramic beads made of Zirconia, Yttria stabilized, 0.4 - 0.6 mm. Water cooled to 5 °C was continuously circulating in the cooling jacket of the grinding chamber to avoid elevated temperatures inside the chamber. Outside the grinding chamber the algae suspension was kept at 5 °C. Using these process conditions the temperature of the algae suspension at the mill outlet never exceeded 20 °C. After bead milling the algae suspension was centrifuged (4 °C, 30 min, 40.000 x g). The supernatant obtained (algae juice) was dialyzed (MWCO 12.000 – 14.000) extensively against 35 mM potassium phosphate buffer, pH 7.6 at 4°C. Five-hundred mL of the dialyzed solution were applied on a glass filter (pore size 2) containing 200 mL of (settled) EBA ion exchange adsorbent Streamline DEAE (GE Healthcare, Uppsala, Sweden) previously washed with an excess of demineralized water and then equilibrated with 500 mL 35 mM potassium

phosphate buffer, pH 7.6. The effluent was reapplied twice to ensure maximum protein binding. After sample application the absorbent was washed with 35 mM potassium phosphate buffer, pH 7.6 to remove unbound components. Bound protein was eluted by applying 400 mL of the same buffer containing 2 M NaCl. The absorption material was cleaned and regenerated using 1 M NaOH. The eluate was first dialyzed (MWCO 12.000 – 14.000) against water and subsequently against 35 mM potassium phosphate buffer, pH 7.6 at 4°C to yield the crude algae soluble protein isolate (CASPI). Decolourization of the isolate was done by adjusting the pH of 400 mL CASPI to 3.5 with 1 M HCl at room temperature. The acidified sample was kept at 4 °C for at least one hour and centrifuged at 4700 x g at 4 °C for 10 min. The pellet was washed with 200 mL water and centrifuged again under the same conditions. The pellet was dissolved in 200 mL water by adjusting the pH to 7.6 with 0.1 M or 1 M NaOH at room temperature and the obtained algae soluble protein isolate (ASPI) was freeze-dried.

2.2.2. Analyses

All samples were freeze-dried prior to analysis except for the aliquots needed for moisture content determination. All analysis results are expressed on a dry weight basis, assuming a residual moisture content of 10% after freeze-drying. Moisture and ash content were determined gravimetrically according to AACC methods 44-15.02 and 08-16.01, respectively (AACC, 1995). For moisture content determinations, the samples were dried in the oven at 80 °C over night, followed by 3 hours at 100 °C. The lipid content was determined gravimetrically after exhaustive extraction of freeze-dried samples by Soxhlet extraction using chloroform/methanol (2:1 v/v) as solvent. All analyses mentioned were carried out in triplicate.

2.2.2.1. Amino acid composition and protein content

Amino acid composition analysis was carried out in duplicate using samples from independent isolation runs by Ansynth Service BV (Berkel en Roodenrijs, The Netherlands) according to the method of (Moore & Stein, 1963). Standard deviations were found to be on average < 3% of the mean. In the worst case the standard deviation was 9%. Protein contents of samples from every processing step were calculated as the sum of amino acid residue weights. For soluble fractions, concentrations were also determined with the Pierce® BCA protein assay according to the producer's instructions (Pierce, Rockford, IL, USA). BSA was used as standard. All protein content determinations were at least carried out in duplicate.

2.2.2.2. Total nitrogen content and Nitrogen – to - Protein conversion factors

Two different N-Prot factors were determined as described previously (Mossé, 1990). The first factor, k_p, was calculated as the ratio of the sum of amino acid residue weights (actual protein present in the cell) to total nitrogen (N_T). N_T contents were determined by the Dumas method using a Flash EA 1112 NC analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol. The second factor, k_a, was calculated as ratio of the sum of amino acid residue weights to nitrogen from recovered amino acids. Amide nitrogen from asparagine and glutamine was not determined separately[1]. Therefore, the nitrogen recovered from amino acids was calculated assuming that ASX and GLX were present as either 100% ASN/GLN or 100% ASP/GLU.

2.2.2.3. Sugar composition and total uronic acid content

Sugar composition was determined according to De Ruiter et al. (1992). Samples were dried at 40 °C under vacuum over P_2O_5 and hydrolyzed with 2 N HCl in dry methanol for 16 h at 80 °C followed by evaporation and subsequent 2 M TFA hydrolysis at 121 °C for 1 h. Monomeric sugars were analyzed using high-performance anion-exchange chromatography − pulsed amperometric detection (Dionex, Sunnyvale, CA, USA) analysis as described previously (Sengkhamparn et al., 2009). Total uronic acid contents were determined after pre-hydrolysis in aqueous 72% (w/w) H_2SO_4 (1h, 30°C) and subsequent hydrolysis in 1 M H_2SO_4 (3h, 100°C) using an automated *m*-hydroxydiphenyl assay (Ahmed & Labavitch, 1978). A galacturonic acid standard curve (12.5–100 µg/mL) was used for quantification. Analyses were carried out in duplicate

2.2.2.4. Spectrophotometric analysis

The presence of coloured compounds in the algae extracts was measured in solutions centrifuged for 5 min at 16.100 x g at 20 °C and containing protein concentration of 5 mg/mL in Milli-Q water. UV spectra from 350 – 750 nm were recorded in 1 cm quartz cuvettes using a UV-1601 Shimadzu spectrophotometer and UV Probe 2.00 software (Shimadzu, Kyoto, Japan).

[1] During acid hydrolysis asparagine (ASN) and glutamine (GL|N) are deamidated, resulting in aspartic acid (ASP) and glutamic acid (GLU). To illustrate that subsequently determined ASP and GLU values represent originally present ASP + ASN and GLU + GLN values, they are labelled ASX and GLX, respectively.

2.2.2.5. SDS-PAGE and immunoblotting

The protein compositions of the algae extracts were visualized using SDS-PAGE under reducing conditions (10 mM 2-mercaptoethanol) on a Mini-protean II system (Bio-Rad Laboratories, Hercules, CA, USA) according to the instructions of the manufacturer. Gels were stained with either Coomassie brilliant blue (CBB) or Periodic acid - Schiff's reagent (PAS) or proteins were transferred electrophoretically to a PVDF membrane (Bio-Rad Laboratories) for immunoblotting. Immunoblot assays were carried out with standard reagents according to the protocol of Bio-Rad Laboratories. Chicken polyconal antibodies against the large subunit of Rubisco (Abcam, Cambridge, UK) on the membrane were detected with a rabbit polyclonal antibody to chicken IgY horseradish-peroxidase-conjugate (Abcam) using 3,3',5,5'-tetramethylbenzidine as substrate. The Rubisco standard used was partially purified Rubisco from spinach (Sigma). Precision Plus Protein Dual Colors Standard (Biorad) was used as molecular weight marker on all gels.

2.2.2.6. Protein solubility

Prior to solubility determinations the ionic strength of the protein solutions was adjusted. For the I = 0.03 M buffer the protein solution was either dialyzing against 35 mM potassium phosphate buffer, pH 7.6 or freeze-dried protein was dissolved in the same buffer. For the I = 0.5 M buffer the ionic strength of the initial 35 mM potassium phosphate buffer, pH 7.6 was adjusted by adding NaCl to reach a final concentration of 0.4 M NaCl, for the I = 0.2 M buffer to reach a final concentration of 0.1 M NaCl. At all ionic strengths no protein precipitation was observed at the initial pH 7.6. The pH of the algae protein solution (~ 5 mg/mL) in pH 7.6 buffer at I = 0.5, 0.2 or 0.03 M was adjusted at 25 °C by adding 0.1 M HCl or 0.1 M NaOH to obtain final pH values ranging from 2.5 to 8.5 with 0.5 unit intervals. As shown previously (Lakemond et al., 2000), the pH adjustments did not considerably influence the ionic strength. Added amounts and actual pH values were controlled using a pH-stat unit. After pH adjustment samples were kept at 4 °C for at least one hour to ensure complete precipitation and to prevent possible protease activity. Afterwards they were centrifuged (10 min; 4700 x g; 4 °C) and the protein concentrations of the supernatants were determined using the BCA protein assay. Calculated concentrations were corrected for dilutions made during pH adjustment. Protein solubility was defined as protein concentration of the respective supernatant relative to the initial protein concentration of the pH 7.6 supernatant; hence the latter was set as 100%. All solubility curves were prepared in duplicate and their standard deviations were found to be on average ≤ 6 % of the mean. In the worst case the standard deviation was 17 %.

2.3. Results and Discussion

2.3.1. Extraction of soluble protein from the algae cell

Microscopic analysis of the *Tetraselmis* cell suspension showed complete disruption of cells after milling (*data not shown*). Prior to bead milling of the algae biomass contained 36% (w/w) proteinaceous material (i.e. proteins, peptides and free amino acids), 24% (w/w) carbohydrates and 19% (w/w) lipids (Table 2.1). After bead milling and centrifugation 21% of the total proteinaceous material was present in a soluble form in the algae juice (Table 2.2), and 63% of this material was retained after dialysis with a membrane with a 14 kDa cut-off.

Table 2.1 – Gross chemical composition of the algae starting material and the final algae soluble protein isolate (in w/w%), parentheses: according standard deviations

Component	Algae paste	Protein isolate
Proteins	36 (<1)	64 (2)
Sugars	24 (1)	24 (<1)
Lipids[a]	19 (<1)	n.d.[b]
Ash	15 (<1)	n.d.

[a] Determined as $CHCl_3$/MeOH soluble material

[b] n.d. = not determined

2.3.2. Preparation and characterization of the protein isolate

2.3.2.1. Chemical composition of the protein isolate

The dialyzed juice was further purified using IEC and acidic precipitation. As shown in table 2.2, the final ASPI contained ~ 50% of the soluble proteins present in the dialyzed juice which amounted to an increase in protein content up to 64% (w/w). The ASPI also contained 24% (w/w) carbohydrates. The total carbohydrate content (w/w) of the final isolate was the same as for the algae starting material, but the carbohydrate-to-protein ratio decreased. This shows a clear enrichment in protein. The polysaccharides still present in ASPI are mainly composed out of 41 mol% galactose and 22 mol% uronic acids. This sugar composition suggests that the polysaccharides were possibly charged carbohydrate with uronic acids as building blocks or attached to the protein as part of a

Table 2.2 – Protein yield, total protein and sugar contents and molar sugar composition of the mono-, oligo-, and polysaccharides of samples at each step of the isolation procedure, parentheses: according standard deviations

Processing step	Proteinaceous material [w/w%]		Proteinaceous yield [%]	Neutral sugars [w/w%]		Uronic acids [w/w%]	
Paste	36.23	(0.40)	100	19.42	(1.31)	4.66	(0.33)
Pellet	39.47	(0.44)	79	17.35	(5.67)	10.39	(1.77)
Algae juice	24.53	(1.61)	21	n.d.		n.d.	
Dialyzed algae juice	58.64	(0.34)	13	41.16	(1.86)	5.64	(0.46)
CASPI	60.91	(2.82)	8	29.05	(3.63)	4.19	(0.03)
ASPI	64.40	(1.72)	7	18.49	(0.38)	5.28	(n.a.)

Processing step	Sugar composition											
	Rha		Ara		Gal		Glc		Man		Xyl		UA	
Paste	1.85	(1.44)	3.40	(0.62)	37.65	(0.03)	29.52	(0.98)	7.44	(0.01)	0.72	(0.01)	19.41	(1.06)
Pellet	0.19	(0.14)	2.23	(1.71)	30.36	(2.15)	23.25	(2.56)	5.65	(0.96)	0.21	(0.29)	38.12	(7.81)
Algae juice	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Dialyzed algae juice	9.48	(1.53)	13.04	(1.06)	51.70	(0.25)	2.57	(0.78)	9.50	(0.64)	1.60	(0.44)	12.11	(0.48)
CASPI	9.96	(0.20)	9.63	(0.12)	58.28	(0.22)	2.97	(1.19)	4.06	(0.03)	2.33	(0.11)	12.77	(1.39)
ASPI	14.77	(1.20)	8.22	(0.03)	40.63	(0.06)	8.45	(0.90)	3.30	(0.16)	2.23	(0.01)	22.39	(0.36)

[a] n.d. = not determined
[b] n.a. = not available

glycoprotein. The presence of charged carbohydrates was expected, since the principle of the selected IEC is based on the recovery of anionic molecules. The presence of glycoproteins was shown by PAS staining after SDS PAGE analysis (Figure 2.1, PAS).

2.3.2.2. Protein composition of the protein isolate

The proteins present in the ASPI as determined with SDS PAGE analysis were diverse and resembled those of the intermediate isolate (CASPI) and the algae juice (Figure 2.1). The SDS PAGE results show, that the overall protein composition was hardly influenced by IEC. Bands with the highest intensity represented proteins with a molecular size of about 50, 40, 25 and 15 kDa under reducing conditions. The distinct band with a molecular mass of about 50 kDa was identified by immunoblot analysis as the large subunit of Rubisco (Figure 2.1, WB).

The high diversity in protein composition can be explained by the fact that microalgae do not accumulate distinct storage proteins as N-source. Based on proteomics data from different microalgae species (Contreras et al., 2008; Wang et al., 2004) most proteins present in the isolate obtained would be expected to be enzymes involved in photosynthesis or other metabolic activities. These enzymes (e.g. Rubisco) can consist of multiple polypeptide chains. The SDS PAGE results imply that the majority of these polypeptide chains are smaller than 50 kDa.

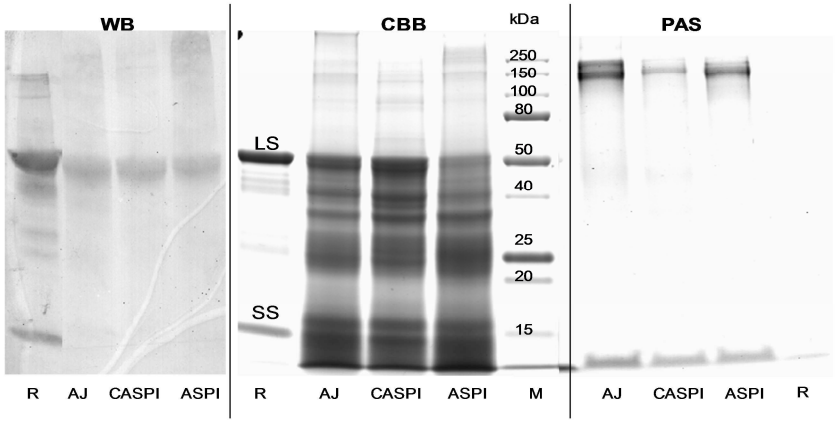

Figure 2.1 - SDS-PAGE gels stained by Coomassie Brillant Blue (CBB) / Periodic acid – Schiff's reagent (PAS) and corresponding Westernblot (WB). Protein concentration for all samples is 5 mg/mL ; AJ = Algae juice, CASPI = Crude algae soluble protein isolate, ASPI = Soluble algae protein isolate, R = Rubisco with large and small subunit (LS, SS), M = Molecular weight maker

Table 2.3 – Amino acid profile of samples obtained during the different isolation steps (g per 100 g protein), parentheses: according standard deviations

	ILE		LEU		VAL		LYS		PHE		TYR		MET		CYS		THR	
Algae paste	4.06	(0.02)	9.45	(0.52)	5.73	(0.02)	6.52	(0.04)	5.62	(0.03)	3.63	(0.07)	2.78	(0.04)	1.39	(0.02)	5.17	(0.05)
Pellet	4.32	(0.03)	9.99	(0.02)	5.54	(0.02)	5.60	(0.01)	6.84	(0.06)	4.25	(0.02)	3.07	(0.01)	1.07	(0.01)	5.25	(0.01)
Dialyzed algae juice	4.14	(0.02)	7.69	(0.01)	6.03	(0.01)	5.97	(0.01)	4.63	(0.02)	4.03	(0.02)	2.62	(0.03)	1.94	(0.01)	6.65	(0.01)
CASPI	4.34	(0.17)	8.02	(0.93)	6.04	(0.06)	5.74	(0.06)	4.91	(0.05)	4.38	(0.36)	2.88	(0.02)	1.91	(0.05)	6.34	(0.10)
ASPI	4.69	(0.12)	8.31	(0.01)	6.07	(0.27)	6.00	(0.27)	5.32	(0.02)	4.42	(0.40)	3.05	(0.13)	1.92	(0.07)	6.14	(0.06)
	ALA		**ARG**		**ASX**[a]		**GLX**[a]		**GLY**		**HIS**		**PRO**		**SER**		**TRP**	
Algae paste	9.39	(0.03)	5.01	(0.03)	9.27	(0.04)	11.34	(0.13)	6.40	(0.01)	2.01	(0.01)	6.22	(0.03)	4.39	(0.04)	1.61	(0.01)
Pellet	7.00	(0.01)	5.65	(0.03)	9.83	(0.02)	10.62	(0.01)	6.47	(0.01)	2.19	(0.01)	5.14	(0.01)	5.01	(0.01)	2.16	(0.03)
Dialyzed algae juice	7.28	(0.01)	5.50	(0.01)	11.27	(0.01)	13.49	(0.01)	5.69	(0.01)	1.85	(0.01)	4.86	(0.01)	5.01	(0.01)	1.36	(0.01)
CASPI	7.03	(0.06)	5.33	(0.07)	11.18	(0.14)	13.46	(0.32)	5.58	(0.08)	1.82	(0.03)	4.58	(0.13)	4.87	(0.02)	1.60	(0.01)
ASPI	6.83	(0.01)	5.97	(0.30)	10.66	(0.18)	12.56	(0.16)	5.50	(0.06)	1.87	(0.02)	4.35	(0.09)	4.70	(0.01)	1.63	(0.02)

[a] No separate analysis of ASP/ASN and GLU/GLN

2.3.2.3. Nitrogen–to–Protein conversion factors

To enable the evaluation of the efficiency of future isolation runs without prior amino acid analysis two different N-Prot factors were determined based on the results of the amino acid analysis (Table 2.3) for every processing step. The first factor, k_a, describes the actual N-Prot factor calculated from the amino acid composition and is, therefore, often only valid for pure protein. Since amide nitrogen from asparagine and glutamine was not determined separately, nitrogen recovered from amino acids was calculated assuming that ASX and GLX were present as either 100% ASN/GLN or 100% ASP/GLU. As a result two k_a values are obtained for each sample. Consequently, two values for the ratios between proteinaceous (N_{AA}) and total nitrogen (N_T) are calculated. The actual values of these parameters are within the interval of these theoretical minimum and maximum values (Table 2.4)**.** The intervals for actual k_a values are constant ($5.4 < y < 6.4$ [$y = k_a$]) over the whole isolation process. This consistency reflects the findings of the SDS PAGE analysis.

Table 2.4 – Proteinaceous nitrogen and nitrogen-to-protein conversion factors k_a and k_p at each step of the isolation procedure

Processing step	N_{AA}/N_T [%][a,b]	N-Prot factor k_p	N-Prot factor k_a[c]
Algae paste	$70 < x < 80$	4.38	$5.45 < y < 6.28$
Pellet	$75 < x < 87$	4.76	$5.49 < y < 6.33$
Algae juice	$48 < x < 56$	3.08	$5.44 < y < 6.31$
Dialyzed algae juice	$80 < x < 95$	5.08	$5.36 < y < 6.36$
CASPI	$83 < x < 98$	5.32	$5.41 < y < 6.42$
ASPI	$88 < x < 103$	5.60	$5.41 < y < 6.35$

[a] Proteinaceous nitrogen (N_{AA}) as proportion of total nitrogen (N_T)

[b] Lower limit represents theoretical value calculated with ASX/GLX = 100% ASP/GLU, upper limit calculated with ASX/GLX = 100% ASN/GLN

[c] Lower limit represents theoretical value calculated with ASX/GLX = 100% ASN/GLN, upper limit calculated with ASX/GLX = 100% ASP/GLU

The k_p value of 4.38 for the algae paste is comparable to previously reported k_p values for different microalgae species (Lourenço et al., 2004). It is much lower than k_a due to the presence of high amounts of NPN, such as inorganic nitrogen, nucleic acids and other organic nitrogen. After cell disintegration and centrifugation most of these NPN components were present in the juice. The majority of the initial proteinaceous material (79%), however, was present in the pellet. This disproportionate distribution resulted in a

decrease of proteinaceous nitrogen (N_{AA}) from 75% < x < 87% to 48% < x < 56 % and a k_p factor of 3.08 for the juice. After dialysis the relative proteinaceous material increases to 80% < x < 95% resulting in a k_p factor of 5.08 due to the presence of a high amount of low molecular weight NPN components. For ASPI k_p was found to be 5.60. NPN could be removed almost completely; values for k_p and k_a converged and k_p was now located within the calculated interval for k_a. This shows an almost complete removal of NPN. Assuming that all ASX/GLX = ASN/GLN results in even ~ 100% proteinaceous N (N_{AA}/N_T). The presence of glycoproteins (Figure 2.1, PAS) suggests, however, that a small part of N_T is not amino acid associated, but present in form of amino sugars, either linked to the protein fraction or present as individual aminoglycoside.

2.3.2.4. Colour of the protein isolate

A major obstacle for the application of microalgae protein in food systems is the dark green colour of the algal biomass. During protein isolation from *Tetraselmis sp.*, colour removal was followed visually and spectrophotometrically. The intense green colour of the original biomass was already removed during the first processing step by discarding chlorophyll containing chloroplastic fragments in the pellet fraction (Insert in Figure 2.2). Consequently, the UV-spectrum of the dialyzed juice (Figure 2.2 - 2) did not show the two intense absorption maxima characteristic for the most abundant chlorophyll in microalgae, chlorophyll a (~430 and ~680 nm) (Scheer, 2006). Instead, the most dominant peaks were a double peak between 500 nm - 560 nm and a single peak around 418 nm. Carotenoids are abundant pigments in microalgae and typically absorb light from 400 – 500 nm (Britton, 1995). Although normally only soluble in organic solvents, carotenoids can be present in algae juice when associated with water soluble molecules like proteins (Zagalsky, 1995).

At comparable protein concentrations (~5 mg/mL) the protein isolate sample showed a residual absorption intensity of 15% for the three absorption maxima of the dialyzed algae juice. Acid precipitation caused 75% of the decrease in intensity. Direct protein precipitation from the juice did not remove colour sufficiently and thus centrifugation and IEC needed to be applied subsequently to allow successful decolourization by subsequent acid precipitation. For large-scale the centrifugation step could be removed by applying EBA (Hjorth, 1997).

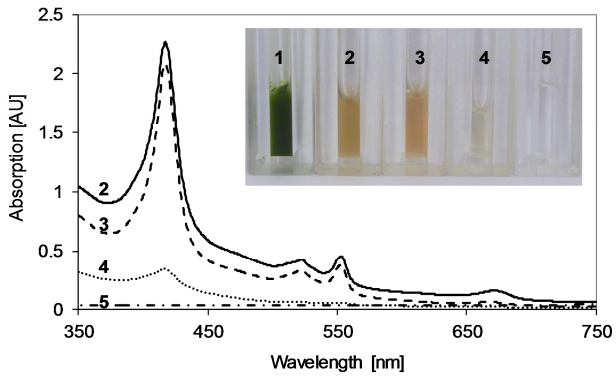

Figure 2.2 - UV spectra of the algae protein solution (5 mg / mL) during the different processing steps, Insert: cuvettes containing 1: Cell suspension before milling (UV spectrum not shown), 2: Algae juice, 3: CASPI, 4: ASPI, 5: Water

2.3.3. Properties of the algae soluble protein isolate

2.3.3.1. Protein solubility

High solubility is generally a prerequisite for successful functional applications of proteins in foods. As shown in figure 2.3, 100% solubility was observed in the pH range from 6.0 – 8.5 for all samples and all ionic strengths tested. Below pH 6, algae protein solubility decreases. The absence of a well-defined minimum solubility is caused by the absence of decisive major protein fractions in the algae soluble protein isolate (Figure 2.1). Seed protein isolates, in contrast, show a well-defined interval of minimum solubility, mostly between pH 4.5 – 6.0 (González-Pérez et al., 2004; Lakemond et al., 2000) primarily due to the solubility behaviour of the major storage proteins present. For example, for soy proteins, the isoelectric points of the major individual protein determine to a great part the point of minimum solubility for the whole isolate (Kuipers et al., 2006). The proteins of the intermediate CASPI and the final ASPI were slightly more soluble at low pH than the proteins of the juice (Figure 2.3), but overall, the solubility profiles were similar.

Two major differences are observed when comparing the solubility profiles for protein from *Tetraselmis sp.* with earlier obtained solubility profiles of food proteins derived from different plants (González-Pérez et al., 2004; Lakemond et al., 2000). Firstly, the solubility of ASPI is independent of ionic strength. The insensitivity of ASPI with regard to ionic strength enables its applications over a broad range of salt concentrations. For plant proteins typically significant effects of ionic strength on the solubility behaviour are

observed. For sunflower proteins the solubility decreases with high ionic strength ($I \geq 0.3$), while for soy glycinin the solubility increases. Secondly, ASPI is completely soluble from pH 5.5 upwards for all tested ionic strengths, whereas plant proteins typically show a clear solubility minimum at low ionic strength ($I \leq 0.3$) in the pH range from 5.5 – 6.5. Due to the strong influence of solubility behaviour on the functional properties of a protein, the differences in solubility behaviour compared to proteins from plant origin could result in unique techno-functional properties for *Tetraselmis* sp. protein isolate.

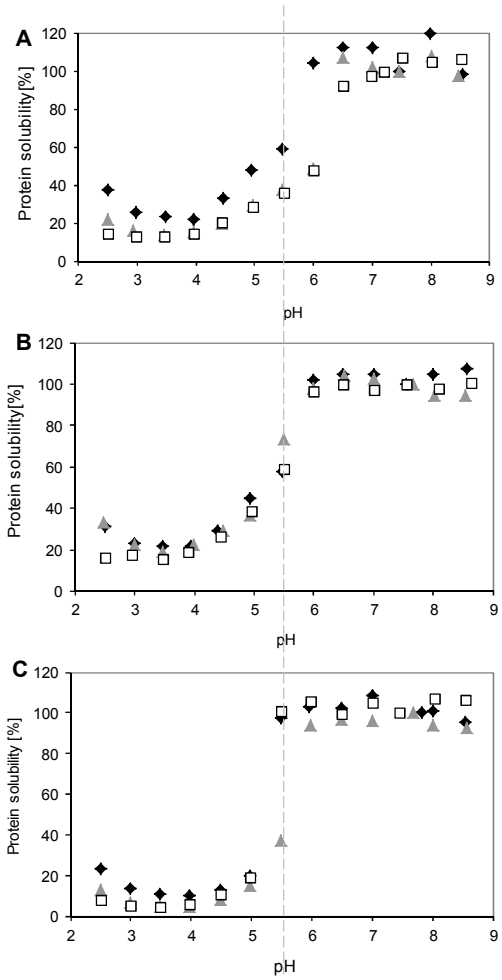

Figure 2.3 - Protein solubility as a function of pH for algae juice (A), crude algae protein isolate (B) and final algae protein isolate (C) at different ionic strengths (I = 0.03 M (♦), 0.2 M (▲) and 0.5 M (□)), 100% = soluble protein concentration at pH 7.6, the dashed line indicates pH 5.5, error bars: according standard deviations

2.3.3.2. Amino acid composition

The overall amino acid profiles of the algae starting material and ASPI (Table 2.3) are comparable to those of egg and soy. With regard to essential amino acids the algae juice and ASPI are comparable to the WHO/FAO reference profile (Becker, 2007). This indicates good nutritional quality of the obtained algae protein isolate. The high similarity in amino acid composition of the algae starting material and ASPI was not unexpected since the protein compositions in both samples were nearly identical (Figure 2.1). Since different microalgae species are known to have similar amino acid composition (Brown, 1991; Fabregas & Herrero, 1985), comparable protein isolates can possibly obtained from other microalgae species.

2.4. Conclusions

By applying a mild and scalable isolation process, it is possible to produce an algae soluble protein isolate from *Tetraselmis sp.*, free from any intense colour. In a pH range where currently used seed protein isolates show low solubility (pH 5.5 -6.5), the isolate obtained shows solubility behaviour independent of ionic strength. Similarities in the amino acid composition and the protein composition of the isolate and those of various green microalgae suggest that other algae might also be useful for food applications requiring protein additions.

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3 Emulsion properties of algae soluble protein isolate from *Tetraselmis* sp.

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Anne Helbig
Peter Wierenga
Harry Gruppen

Abstract

To study possible applications of microalgae proteins in foods, a colourless, protein-rich fraction was isolated from *Tetraselmis* sp. In the present study the emulsion properties of this algae soluble protein isolate (ASPI) were investigated. Droplet size and droplet aggregation of ASPI stabilized oil-in-water emulsions were studied as function of isolate concentration (1.25 – 10.00 mg/mL), pH (3.0 to 7.0), and ionic strength (NaCl 10 to 500 mM; $CaCl_2$ 0 to 50 mM). Whey protein isolate (WPI) and gum arabic (GA) were used as reference emulsifiers. The lowest isolate concentrations needed to reach $d_{32} \leq 1$ μm in 30% oil-in-water emulsions were comparable for ASPI (6 mg/mL) and WPI (4 mg/mL). In contrast to WPI stabilized emulsions ASPI stabilized emulsions were stable around pH 5 at low ionic strength (I = 10 mM). Flocculation only occurred around pH 3, the pH with the smallest net droplet ζ-potential. Due to the charge contribution of the anionic polysaccharide fraction present in ASPI its droplet ζ-potential remained negative over the whole pH range investigated. An increase in ionic strength (≥ 100 mM) led to a broadening of the pH range over which the ASPI stabilized emulsions were unstable. GA emulsions are not prone to droplet aggregation upon changes in pH or ionic strength, but much higher concentrations (50 mg/mL) are needed to produce stable emulsions. Since ASPI allows the formation of stable emulsions in the pH range 5 – 7 at low protein concentrations, it can offer an efficient natural alternative to existing protein-polysaccharide complexes.

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3.1. Introduction

To meet the increasing protein demand of the growing world population in a sustainable way the transition from an animal protein based diet to a plant protein based diet is necessary (Aiking, 2011). As part of this transition, attractive alternatives to the animal-derived techno-functional ingredients currently used in foods need to be provided. Next to different terrestrial crops, aquatic sources, like microalgae, are considered a promising alternative protein source, since they possess high amounts of proteins (up to 50% [w/w]) and well-balanced amino acid profiles (Spolaore et al., 2006). Nevertheless, the structural, biological and techno-functional properties of microalgae proteins are poorly documented. Recently, a protein isolation process has been developed for the isolation of a soluble protein fraction from the green microalgae *Tetraselmis* sp. (Schwenzfeier et al., 2011). The final algae soluble protein isolate (ASPI) contained 64% (w/w) protein, 24% (w/w) polysaccharides, and 12% (w/w) remaining material containing mainly ash and other minor components. ASPI was found to be highly soluble in the pH range where currently used plant food proteins exhibit low solubility (pH 5.5 - 6.5). These results suggest that ASPI can be useful for food applications requiring protein addition in this pH range. To further examine possible applications of ASPI in foods the present study focuses on emulsion properties of ASPI.

Proteins are often used as emulsifiers, since they can stabilize the interface between the oil phase and the aqueous phase. In some cases also polysaccharides are applied as emulsifiers. Their surface activity is often attributed to either naturally occurring proteinaceous moieties or chemically attached hydrophobic groups (Chanamai & McClements, 2001; McClements, 2005; Nakauma et al., 2008). It has been shown that with the use of protein–polysaccharide complexes the beneficial emulsifying attributes of proteins and polysaccharides can be combined (Akhtar & Dickinson, 2007; Bouyer et al., 2011; Neirynck et al., 2004). The more surface active component, normally the protein, primarily adsorbs to the interface. The less surface active component, normally the polysaccharide, becomes associated with the interface by forming a complex with the primarily adsorbed component (Dickinson, 2011). Since the main driving force stabilizing protein-polysaccharide complexes is electrostatic, changes in pH and an increase in ionic strength can influence the formation and stability of the complexes (Guzey & McClements, 2007; Li et al., 2012). One example of a naturally occurring polysaccharide-protein complex used as an emulsifier in food industry is gum arabic (GA), the dried exudate of the acacia tree. GA is a mixture of arabinogalactan (~ 90% [w/w]), glycoprotein (~ 1% [w/w]), and arabinogalactanprotein

(AGP) (~ 10% [w/w]). The AGP fraction is considered to be responsible for the ability of GA to form stable emulsions over a broad pH range and at high ionic strengths (Dickinson, 2003; Islam et al., 1997; McClements, 2005). Although GA emulsions have unique properties (Jayme et al., 1999), problems associated with obtaining a reliable source of consistently high-quality GA require the investigation of alternative natural biopolymers (Chanamai & McClements, 2001). ASPI, also a natural occurring protein-polysaccharide mixture, could offer a natural substitute for GA if its emulsion properties are equal or superior.

The formation and stability of emulsions prepared with ASPI are studied as a function of the isolate concentration, pH, ionic strength and calcium ion concentration. The emulsions are characterized with respect to their average droplet size and their stability against droplet aggregation using microscopy, particle-size distribution analysis using light scattering, and diffusion wave spectrometry (DWS). The first two methods are common experimental methods to monitor the presence and extent of droplet aggregation in emulsions. Both techniques require extensive dilution of the emulsion sample, which can have a large influence on the droplet aggregation behaviour, since it is strongly dependent on extrinsic physico-chemical conditions, such as concentration, pH and ionic strength (Dickinson, 2010). To also quantify droplet aggregation *in situ* DWS was used as alternative third technique. The emulsions prepared with ASPI are compared to whey protein isolate (WPI) and gum arabic (GA) stabilized emulsions. WPI is used as standard protein representing one of the most important classes of proteinaceous emulsifying agents in food applications. GA is used, since it is, like ASPI, a natural occurring polysaccharide-protein conjugate able to stabilize emulsions over a broad pH range and at high ionic strengths.

3.2. Materials and Methods

3.2.1. Materials

Food grade, non-purified sunflower oil was purchased at a local supermarket and stored at 4 °C prior to use. Whey protein isolate (WPI) was purchased from Davisco Foods International (BiPro®, lot JE216-6-440, Le Sueur, MN, USA, 92% [w/w] protein [N x 6.36]) and gum arabic from Caldic Ingredients BV (spray-dried gum acacia 368A, lot 0T060521p, Oudewater, The Netherlands, 2% (w/w) protein [N x 6.60]). Algae soluble protein isolate (ASPI) (64% [w/w] protein [N x 5.60]) was isolated from the commercially available microalgae *Tetraselmis* sp. (Reed Mariculture, Campbell, CA, USA) as described

previously (Schwenzfeier et al., 2011). All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA) if not stated otherwise.

3.2.2. Emulsion preparation

Stock solutions containing 1% (w/w) WPI or ASPI or 5% (w/w) GA were prepared by suspending the respective isolate in 10 mM NaCl solution. The solutions were stirred overnight resulting in solutions of pH 6.8 (WPI), pH 7.0 (ASPI), and pH 4.4 (GA) without any further pH adjustment. Emulsions containing 30% (w/w) sunflower oil were prepared using WPI and ASPI concentrations of 0.125, 0.250, 0.500, 0.800, and 1.000% (w/w) and GA concentrations of 0.25, 0.50, 1.00, 2.50, and 5.00% (w/w) to investigate the influence of the oil-to-protein ratio on the average droplet size. To study the influence of different environmental conditions emulsions containing 10% (w/w) sunflower oil were prepared using WPI and ASPI concentration of 1% (w/w). For GA 5% (w/w) oil-in-water emulsions containing 7.5% (w/w) emulsifier were prepared. All emulsions were made from pre-emulsions (Ultra-Turrax at 9500 rpm for 1 min) further passed 10 times through a laboratory homogenizer (LH-scope HU-3.0, Delta Instruments, Drachten, The Netherlands) at a pressure of 100 bar. The pH of the emulsions was adjusted to pH 7.0 directly after preparation with 10 mM HCl or 10 mM NaOH, if necessary.

3.2.3. Influence of pH and ionic strength on droplet aggregation

Emulsions containing 10% (w/w) sunflower oil and 1% (w/w) WPI or ASPI (pH 7) or 5% (w/w) sunflower oil and 7.5% (w/w) GA (pH 7) were subjected to variations of a number of environmental conditions (pH, ionic strength, calcium concentration). The respective oil and protein concentrations were chosen in order to compare the data obtained to earlier reported values for WPI and GA. After each treatment, emulsions were physico-chemically characterized directly after preparation as described below. The influence of pH on emulsion stability was determined by adjusting the pH of the initial emulsion to values ranging from 3 – 6 with 1 unit intervals using 10 or 100 mM HCl. To study the influence of ionic strength on emulsion stability, the salt concentration of the emulsion was adjusted by adding 1 part of a 2 or 5 M NaCl solution to 9 parts of the initial emulsion (10% [w/w] oil, 1% [w/w] emulsifier). To study the influence of different calcium ion concentrations on emulsion stability 1 part of a 500, 250, 50, 25, or 10 mM $CaCl_2$ solution was added to 9 parts of the initial emulsion.

3.2.4. Droplet size determination

Droplet size distributions and volume surface mean diameters (d_{32}) of the emulsions were determined by static light scattering (Mastersizer Hydro 2000SM, Malvern Instruments Ltd, Malvern, UK) at room temperature. A refractive index ratio of 1.1 was used to calculate the particle size distribution. If droplet aggregation ($d_{32} > d_{32\ \mathrm{pH}\ 7} \sim 1$ µm) was detected, droplet size distribution measurements were repeated after diluting the emulsion 1:1 in a 0.5% (w/v) sodium dodecyl sulphate (SDS) solution to stabilize the droplets and disperse any aggregates present (Van Koningsveld et al., 2006). In addition to static light scattering diffusing wave spectroscopy (DWS) was used to quantify emulsion instability. DWS was carried out in transmission and forward scattering geometry using a HeNe laser (JDS Uniphase, λ = 633 nm) (Ruis et al., 2007). Autocorrelation curves were recorded for 120 seconds in 5 sequential runs. The average over all 5 measurements was normalized by dividing g_2(t) – 1 values obtained by the maximum value measured. Normalized autocorrelation curves were fitted assuming that the equation used by Ruis et al. (2007) to describe the autocorrelation function can be simplified to:

$$g_2(t) - 1 = \left(e^{-<\Delta r^2(t)>}\right)^2 \approx \left(e^{-6Dt^p}\right)^2 = e^{-\alpha t^x} \quad (1)$$

In this equation $g_2(t)$ is the autocorrelation function of multiple scattered light as function of time t and $<\Delta r_2(t)>$ is the mean square displacement of the scatters. For unhindered diffusive colloidal motion $<\Delta r_2(t)> = 6Dt$, with D representing the self-diffusion coefficient. According to Ruis et al. (2007) the short time behavior of $<\Delta r_2(t)>$ can be described as $<\Delta r_2(t)> \approx t^p$. This approximation allows to describe the autocorrelation function using the simplified parameters $\alpha = 12D$ and $x = p$. The decay time $\tau_{1/2}$, which is defined as the time at which $g_2(\tau_{1/2}) - 1$ decayed to half of its initial value, was determined using the fitted equation. An increase of the decay time is related to decreased droplet mobility (Blijdenstein et al., 2003; Vasbinder et al., 2001).

3.2.5. ζ-potential measurements

The ζ-potential of the emulsion droplets was determined using a particle electrophoresis instrument (Zetasizer, Nano series – ZS, Malvern). Prior to analysis emulsions were diluted 500x in 10 mM NaCl at the pH of the respective emulsion and equilibrated for 5 min at 25 °C inside the instrument. Data was collected over at least 5 sequential readings and processed using the Smoluchowski model (Hunter, 2001).

3.2.6. Light microscopy

Qualitative detection of droplet aggregation was carried out by microscopic observation after 100x dilution in 10mM NaCl at the pH of the respective emulsion. If aggregated droplets were observed, microscopic examination was repeated after diluting the emulsion 1:1 in 0.5% (w/v) SDS.

3.2.7. Protein adsorption Γ*

The approximate amount of protein adsorbed to the oil-water interface is calculated as Γ* [mg/m^2] at the intercept of the extrapolated linear trend lines of the droplet size functions in the 'emulsifier-poor' and the 'emulsifier-rich' regimes, introduced elsewhere (Tcholakova et al., 2003). Since at this point most of the protein present is assumed to be adsorbed to the interface, the amount of non-adsorbed protein is negligible, Γ* is calculated from equation (2), where d_{32} is the volume surface mean diameter, c_{INI} the initial protein concentration used during emulsion preparation, and ϕ the volume faction of the oil phase, expressed as ratio of the total emulsion volume.

$$\Gamma^* = \frac{d_{32} * c_{INI} * (1 - \phi)}{6\phi} \qquad (2)$$

3.2.8. Computation of the theoretical isoelectric point of ASPI

The theoretical isoelectric point of ASPI was calculated based on the amino acid composition reported by Schwenzfeier et al. (2011) using the program provided on http://web.expasy.org/compute_pi/. Since no separated data for ASN/GLN and ASP/GLU was available, the isoelectric point was estimated to be between the values calculated for ASX and GLX present as either 100% ASN/GLN or 100% ASP/GLU ($6.2_{\,[100\%\ GLN/ASN]} < pI_{\,ASPI} < 8.0_{\,[100\%\ ASP,\ GLU]}$).

3.3. Results and Discussion

3.3.1. The emulsifying ability of ASPI in comparison to WPI and GA

The emulsifying ability of ASPI, WPI, and GA was determined from the volume-surface average droplet size (d_{32}) of 30% (w/w) oil-in-water emulsions as a function of the isolate concentration at pH 7 (Figure 3.1). For WPI and ASPI stabilized emulsions the average droplet size decreased steeply with increasing isolate concentration up to concentrations of 4 mg/mL and 6 mg/mL, respectively. In this so-called 'emulsifier-poor' regime (Tcholakova et al., 2008) the isolate concentration is insufficient to stabilize the overall surface of the smallest droplets formed. Therefore, the effective bigger average droplet size is strongly affected by droplet-droplet coalescence within the homogenizer valve. Above concentrations of 4 mg/mL for WPI and 6 mg/mL for ASPI, in the 'emulsifier-rich' regime, the final droplet size (~ 1 µm) became independent of the isolate concentration indicating the presence of sufficient protein for the stabilization the smallest droplets formed. For GA stabilized emulsions much higher isolate concentrations are necessary to form stable emulsions. Emulsions containing 10 mg/mL or less GA show immediate macroscopic phase separation after homogenization and could therefore not be measured accurately. Even at isolate concentrations as high as 50 mg/mL on total weight basis, the 'emulsifier-rich' regime has not been reached.

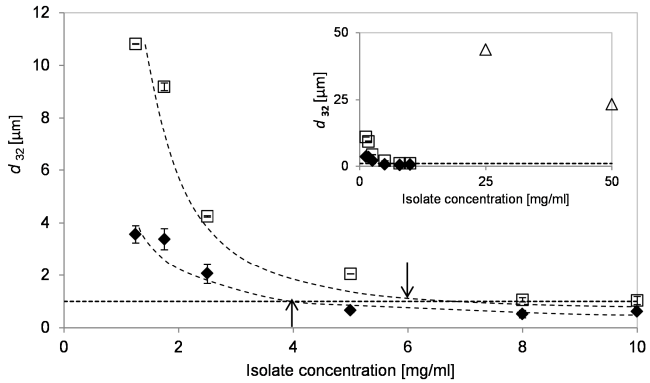

Figure 3.1 – Average droplet diameter (d_{32}) of ASPI (□) and WPI (♦) stabilized emulsions (30% oil, pH 7, I = 10 mM) as function of the isolate concentration (mg/mL), finer dotted line indicates the 'emulsifier-rich' regime with $d_{32} \leq 1$ µm, rough dotted lines and arrows serve to guide the eye, Insert: Average droplet size of GA stabilized (Δ) emulsions (30% oil, pH 7, I = 10 mM) as function of the isolate concentration (mg/mL) in comparison to ASPI and WPI stabilized emulsions, GA concentrations of 10 mg/mL and lower led to immediate macroscopic phase separation and the respective emulsions could therefore not be measured accurately, error bars indicate standard deviations

From the d_{32} function obtained the approximate amount of material adsorbed to the oil-water interface was calculated as Γ* [mg/m^2] at the intercept of the extrapolated linear trend lines of the droplet size functions in the 'emulsifier-poor' and the 'emulsifier-rich' ($d_{32} \leq 1$ μm) regimes. To acknowledge the fact that the protein fraction in ASPI and GA will be more surface active than the polysaccharide fraction present in these samples, Γ* is expressed in isolate as well as protein adsorbed to the interface. The value calculated as Γ_i* is based on the assumption that all material present in the respective isolate adsorbs to the interface. The value calculated as Γ_p* is based on the assumption that only the protein present in the respective isolate adsorbs to the interface. The theoretical isolate adsorption Γ_i* calculated for ASPI and WPI are found to be similar (Table 3.1). This shows that for ASPI, the isolate with the lower protein content, the polysaccharide fraction contributes to emulsion stabilization by co-adsorption to the droplet surface. For GA, which is composed for only 2% (w/w) of protein, a much higher theoretical isolate adsorption (Γ_i* = 75.56 mg isolate/m^2) was calculated based on data reported by Nakauma et al. (2008). Based on protein, however, Γ_p* of GA (1.70 mg protein/m^2) is almost equal to those of WPI (0.97 mg/m^2) and ASPI (0.82 mg/m^2). This shows the importance of the minor protein fraction for the emulsifying ability of GA. It has been postulated that here the predominant polysaccharide fraction does not adsorb directly to droplet surface (Dickinson, 2003; Jayme et al., 1999). To be able to contribute to droplet stabilization by steric hindrance, the polysaccharides need to be linked to the droplet surface via the minor protein residue.

Table 3.1 – Parameters used for calculating Γ* for ASPI, WPI, helianthinin (Hel), protease inhibitor protein from potato (PIP), and GA

	ASPI	WPI	Hel[a]	PIP[b]	GA[c]
Protein content [% (w/w)]	64	92	~ 95	~ 95	2
Oil fraction	0.30	0.30	0.10	0.10	0.15
Isolate conc at intercept [mg/mL]	3.26	2.69	1.58	1.58	80.00
Protein conc at intercept [mg/mL]	2.12	2.50	1.50	1.50	1.80
d_{32} [μm]	1.00	1.00	1.00	0.90	1.00
Homogenization conditions	100 bar, 10x	100 bar, 10x	60 bar, 10x	60 bar, 10x	500 bar, 2x
Γ_p* [mg protein/m^2]	**0.82**	**0.97**	**2.25**	**2.03**	**1.70**
Γ_i* [mg isolate/m^2]	**1.29**	**1.06**	**2.37**	**2.13**	**75.56**

[a] According to González-Pérez et al. (2005)

[b] According to Van Koningsveld et al. (2006)

[c] According to Nakauma et al. (2008)

By calculating Γ* it is also possible to compare the emulsifying abilities of ASPI with the emulsifying abilities reported for plant protein isolates (González-Pérez et al., 2005; Van Koningsveld et al., 2006) even though in those studies different emulsion compositions were used. Based on both, isolate and protein concentrations, the Γ* of ASPI is two times smaller than Γ* of the sunflower protein helianthinin (Hel) and protease inhibitor protein isolate from potato juice (PIP). These numbers suggest a superior emulsifying ability of ASPI compared to those of Hel and PIP.

3.3.2. Comparison of droplet stabilization mechanisms in ASPI, WPI, and GA stabilized emulsions

3.3.2.1. Effect of pH on the droplet ζ-potential in ASPI, WPI, and GA stabilized emulsions

The droplet ζ-potentials of 10% (w/w) oil-in-water WPI and ASPI stabilized emulsions and 5% (w/w) oil-in-water GA stabilized emulsions were determined as function of pH in the pH range 3 - 7 (Figure 3.2). The pH dependent droplet ζ-potential of WPI stabilized emulsion is 0 at pH 4.8 indicating the isoelectric point of the isolate at this pH. This finding is in line with previously reported data for WPI (e.g. Kulmyrzaev & Schubert, 2004). Typically food ingredient proteins (e.g. from milk, soy, egg) have an isoelectric point in the pH range 4 – 6. Therefore, the respective droplet ζ-potentials converge to zero in this pH range to finally change from positive to negative values at pH values above the pI. The droplet ζ-potential of ASPI stabilized emulsions, however, remained negative at all pH values tested. From pH 5 (-45 mV) downwards it increased to reach its maximum (-15 mV) at pH 3. These results suggest an overall isoelectric point for ASPI (including the effect of the polysaccharide fraction) below pH 3, although purely based on its amino acid composition an isoelectric point around pH 7 is calculated ($6.2_{[100\%\ GLN/ASN]} < pI_{ASPI} < 8.0_{[100\%\ ASP,\ GLU]}$).

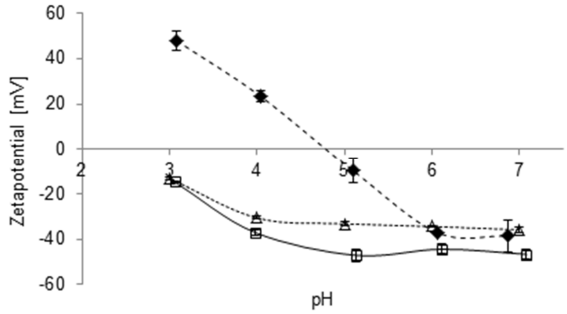

Figure 3.2 – pH dependent droplet zeta potential of 10% oil-in-water emulsions stabilized with 1% ASPI (□) and WPI (♦) and 7.5% oil-in-water emulsions stabilized with 5% GA (Δ) (all I = 10 mM), error bars indicate standard deviations

The pH dependent droplet ζ-potential of ASPI stabilized emulsion is comparable to that of GA. For gum arabic isoelectric points around pH 1.5 – 2.0 have been reported earlier (Jayme et al., 1999; Nakauma et al., 2008). These values are quite similar to the pK_a values of the α-carboxyl groups of the charged polysaccharide fraction of the gum (Jayme et al., 1999). Like gum arabic, also ASPI contains a considerable carbohydrate fraction (24% w/w), composed for approximately one fourth of uronic acids (Schwenzfeier et al., 2011). This anionic fraction seems to contribute sufficient charge to the droplet surface to retain a negative droplet ζ-potential over the pH range 3 - 7, although the polysaccharide-to-protein ratio (1:3 [w/w]) is much smaller than in GA (49:1 [w/w]). For emulsions stabilized by pectin-WPI mixtures a comparable low polysaccharide-to-protein ratio (1:4) was reported to be sufficient to retain a negative electrophoretic mobility in the pH range 4.0 – 5.5 (Neirynck et al., 2004).

3.3.2.2. Effect of pH and ionic strength on droplet aggregation in ASPI, WPI, and GA stabilized emulsions

The volume surface average droplet size (d_{32}) of 10% (w/w) oil-in-water WPI and ASPI stabilized emulsions and 5% (w/w) oil-in-water GA stabilized emulsions was determined in the pH range 3 to 7 at different ionic strengths. At low ionic strength (10 mM NaCl) (Figure 3.3) the average droplet size of WPI and ASPI stabilized emulsions increased significantly around the pH value corresponding to the smallest net droplet ζ-potential of the respective isolate (for WPI ~ pH 5, for ASPI ~ pH 3). The loss of electrostatic repulsion between the protein covered droplets results in flocculation of the individual emulsion droplets. Light

microscopy was used to confirm that the observed increase in d_{32} was indeed caused by flocculation, not by coalescence *(data not shown)*. Varying the pH did not influence the average droplet size of GA stabilized emulsions. Even if the pH is close to the isoelectric point of GA (pH 1.5 - 2.0), no droplet aggregation is observed. This can be explained by the fact that the main contribution to droplet stabilization in GA stabilized emulsions is steric repulsion (Jayme et al., 1999). For ASPI stabilized emulsions macroscopic observation of the prepared emulsions indicated instability already at pH 4 (*data not shown*). However, using the Mastersizer equipment no droplet aggregation was observed at pH 3. To eliminate the effects of dilution and mechanical deformation introduced in the Mastersizer equipment emulsions were also measured using DWS. The DWS results shown in figure 3.4 confirm the macroscopic observations. At 10 mM NaCl ASPI stabilized emulsion are stable in the pH range 5 – 7. Below pH 5 the decay time $\tau_{1/2}$ increased to reach its maximum at pH 3, indicating limited drop mobility due to aggregation at pH 3 and 4. Since the flocculation observed is reversible by adding 0.5% (w/v) SDS to the emulsion, it is concluded that droplet aggregation in ASPI stabilized emulsion is caused by the loss of electrostatic repulsion close to pH 3.

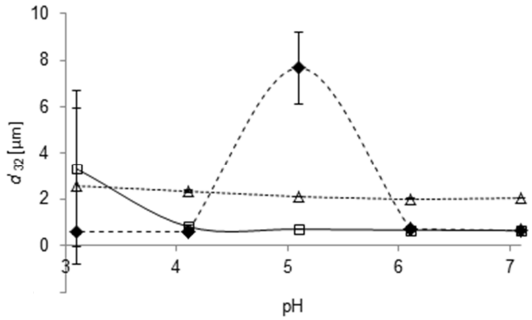

Figure 3.3 – Average droplet diameter (d_{32}) of 10% oil-in-water emulsions stabilized with 1% ASPI (□) and WPI (♦) and 7.5% oil-in-water emulsions stabilized with 5% GA (Δ) (all I = 10 mM) as function of the pH, error bars indicate standard deviations

An increase in ionic strength (100 mM NaCl and higher) did not lead to droplet aggregation of GA stabilized emulsions in the pH range 3 - 7, but it strongly affects the pH dependent droplet aggregation of WPI and ASPI stabilized emulsions (Figure 3.4). For WPI stabilized emulsions an increase in NaCl concentrations up to 100 mM led to a broadening of the pH range over which the emulsions were unstable around the isoelectric point of the isolate. If the salt concentration was increased to 200 mM and higher, also emulsions at pH 3 became unstable, most probably due the shielding effect of the added salt, which is in line with

earlier reported values for WPI (Kulmyrzaev, 2004; Hunt & Dalgleish, 1996). For ASPI stabilized emulsions an increase of the ionic strength to either 100 mM, 200 mM or 500 mM always led to increased decay times over the pH range 3 – 6. This indicates a decreased mobility of the oil droplets due to droplet aggregation. The tendency of the emulsion to flocculate broadens and shifts at the same time closer to the isoelectric point of the proteins present in ASPI ($6.5_{\text{[100\% GLN/ASN]}} < pI_{\text{ASPI}} < 8.0_{\text{[100\% ASP, GLU]}}$). With this the pH dependent aggregation behaviour of ASPI stabilized emulsions is, at high ionic strength, in line with the pH dependent solubility of the isolate reported earlier (Schwenzfeier et al., 2011). As discussed above, for ASPI stabilized emulsions it is assumed, that at low ionic strength the charged polysaccharides present in the isolate contribute to droplet stabilization by forming complexes with the proteins adsorbed to the interface.

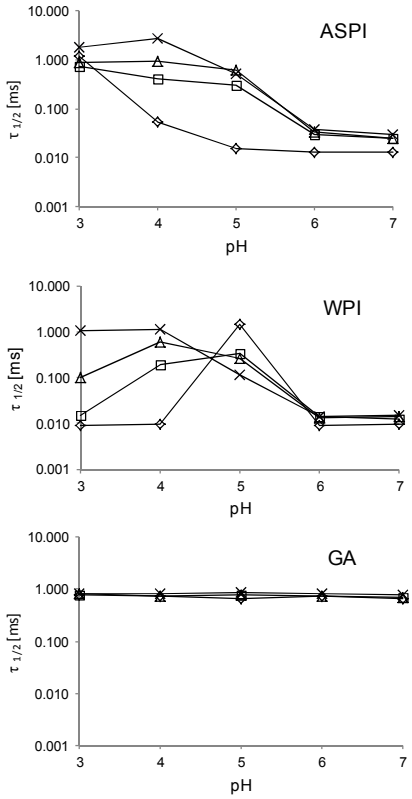

Figure 3.4 – Decay time $\tau_{1/2}$ [ms] as function of pH of 10% oil-in-water emulsions stabilized with 1% ASPI and WPI and 7.5% oil-in-water emulsions stabilized with 5% GA at NaCl concentrations of 10 mM (◊), 100 mM (□), 200 mM (Δ) and 500 mM (x)

An increase in ionic strength above a certain level can cause dissociation of the polysaccharides from the protein coated droplet surface due to weakened electrostatic attraction (Guzey & McClements, 2007; Li et al., 2012). The dissociation of the polysaccharides from the droplet surface at high ionic strength could cause ASPI stabilized emulsions to behave more like purely protein-stabilized emulsions.

Next to the sensitivity of ASPI stabilized emulsion towards monovalent Na^+ ions also the sensitivity towards divalent calcium ions (Ca^{2+}), the major source of multivalent ions in many food emulsions (Keowmaneechai & McClements, 2002), was studied. The average droplet size of ASPI stabilized emulsions increased steeply at Ca^{2+} concentration of 5 mM (Figure 3.5). Above this Ca^{2+} concentration flocculation occurred. With this the calcium sensitivity of ASPI stabilized emulsions is similar to the one of WPI stabilized emulsions. For both emulsions already low levels (≤ 10 mM) of Ca^{2+} ions clearly reduce the stability against droplet aggregation. For WPI stabilized oil droplets it has been suggested that their reduced aggregation stability at low calcium concentrations is caused by two mechanisms. First, divalent Ca^{2+} ions reduce the electrostatic repulsion between the droplets through electrostatic shielding. Due to their double charge this effect is much stronger than for monovalent ions at similar concentrations. Second, it has been reported that Ca^{2+} ions bind to oppositely charged groups on the surface of the emulsion droplet, again resulting in reduced electrostatic repulsion (Keowmaneechai & McClements, 2002). It is expected that the same two mechanisms are responsible for the reduced aggregation stability of ASPI stabilized oil droplets in the presence of 10 mM calcium chloride. It is concluded that the aggregation stability of ASPI is much smaller in the presence of divalent Ca^{2+} ions than in the presence of monovalent Na^+ ions.

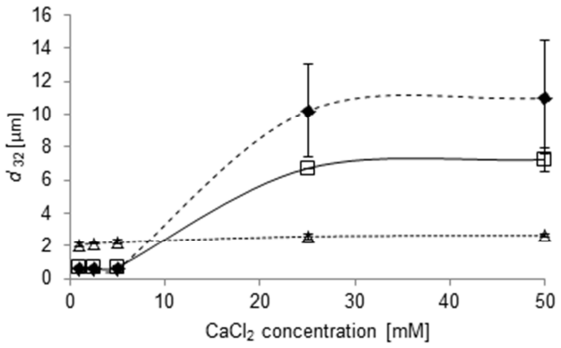

Figure 3.5 – Average droplet diameter (d_{32}) of 10% oil-in-water emulsions stabilized with 1% ASPI (□) and WPI (♦) and 7.5% oil-in-water emulsions stabilized with 5% GA (Δ) (all I = 10 mM, pH 7) as function of the $CaCl_2$ concentration, error bars indicate standard deviations

3.4. Conclusions

Unlike WPI, ASPI stabilized emulsions are stable against droplet aggregation around pH 5 at low ionic strength. It is suggested that the emulsion stability at this pH is caused by the co-adsorption of the polysaccharide fraction of ASPI to the droplet surface. The main stabilization mechanism in ASPI stabilized emulsions is an electrostatic one and, therefore, ASPI stabilized emulsions are sensitive to changes in pH and ionic strength. In comparison to the naturally occurring polysaccharide – protein hybrid GA, which also allows the formation of stable emulsions at pH 5, ASPI shows a higher emulsifying efficiency due to its higher protein content (64% [w/w]). Due to the combination of the positive emulsifying properties of its protein fraction with the broad pH stability of its polysaccharide fraction, ASPI offers a natural alternative to GA and synthetically produced protein-polysaccharide complexes. Further studies are underway to unravel the exact contribution of the polysaccharide fraction to the emulsifying properties of ASPI.

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4 Effect of pH and ionic strength on the stability of foams stabilized with algae soluble protein isolate

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Peter Wierenga
Michel Eppink
Harry Gruppen

Abstract

In this study, the foam properties of algae soluble protein isolate (ASPI) were investigated as function of isolate concentration (0.1 – 1.0 mg/mL) and pH (3.0 – 7.0) at different NaCl concentrations (10 mM and 200 mM). In addition, adsorption kinetics and dilatational elasticity at the air-water interface were studied. Whey protein isolate (WPI) and egg white albumin (EWA) were used as reference proteins. The consistent dilatational behaviour of ASPI at all pH values and ionic strengths tested indicated a similar interfacial composition at all these conditions. Adsorption kinetics, in contrast, were influenced by varying environmental conditions. At increased ionic strength and close to the theoretical isoelectric point calculated based purely on the amino acid composition of ASPI (pH 7) adsorption increased. Since similar adsorption behaviour was also observed for WPI and EWA, the interfacial properties of ASPI are most likely dominated by its protein fraction. This is further confirmed by the fact that ζ-potential measurements suggested an overall isoelectric point of ASPI below pH 3, while adsorption kinetics varied between pH 5 and pH 7 (the theoretical protein-based isoelectric point of ASPI). The overall foam stability of ASPI stabilized foams was superior to those of WPI and EWA at the pH range 5 - 7. In conclusion, the molecular and interfacial properties of ASPI, a complex mixture of a broad variety of proteins and polysaccharides, seems to favour the production of very stable foams in this pH range by the selective adsorption of its protein fraction to the air-water interface.

4.1. Introduction

To support the development of sustainable food products there is interest in replacing currently used animal-derived protein ingredients by plant-derived protein ingredients (Aiking, 2011). These plant-derived protein ingredients should possess excellent techno-functional properties and high solubility in order to be accepted by the food industry. Due to their abundance and well-balanced amino acid profiles proteins from photosynthetic systems, like leaves and microalgae, have long been considered promising alternatives to existing food proteins (e.g. Davies, 1971). Nevertheless, their application did not gain significant importance, since the harsh isolation conditions applied to remove green colour and bitter taste often resulted in loss of solubility (e.g. Kohler & Knuckles, 1977; Wang & Kinsella, 1976). To enable the study of techno-functional properties of microalgae protein a process has recently been developed for the mild isolation of the soluble protein fraction from the green microalgae *Tetraselmis* sp. (Schwenzfeier et al., 2011). The final algae soluble protein isolate (ASPI) was devoid of green colour and composed of 64% (w/w) protein, 24% (w/w) polysaccharides, and 12% (w/w) remaining material, which contained mainly ash and other minor components. ASPI was found to be highly soluble in the pH range, in which currently used plant protein isolates exhibit low solubility (pH 5.5 - 6.5). As solubility is such an important property, ASPI can be useful for food applications in this pH range. To further examine possible functional applications of ASPI in foods, the present study focuses on the investigation the foaming properties of ASPI.

When studying the foaming properties of ASPI, it is important to consider a possible effect of the polysaccharides present in the isolate. Normally, polysaccharides do not adsorb directly to the air-water interface, but they can improve foam stability by decreasing the rate of disproportion and drainage by increasing the bulk viscosity (Yang & Foegeding, 2010). In addition, anionic polysaccharides, which are typically not surface active, can co-adsorb to the air-water interface by the formation of soluble complexes with a surface-active protein above the pK_a of the polysaccharide and below the isoelectric point of the protein (Ganzevles et al., 2006). The interfacial properties and subsequent foam stabilities of such protein-polysaccharide complexes were found to be equal or superior to those of the pure protein (Benichou et al., 2007; Schmitt & Turgeon, 2011; Miquelim et al., 2010). Since these types of protein-polysaccharide complexes are only stable in the case of electrostatic attraction between the two different molecules, changes in pH and ionic strength strongly influence the degree of complexation and by this the adsorption kinetics and foaming properties of the complexes and the free protein (Sperber et al., 2009).

If an interfacial layer is composed of protein-polysaccharide complexes, rather than sole proteins, this difference in composition is typically reflected in changed interfacial properties (e.g. adsorption kinetics, dilatational elasticity) (e.g. Ganzevles et al., 2006). In addition, interfacial properties are often studied as a first indication for the effects of pH and ionic strength on the respective foaming properties. Although it has been suggested that interfacial properties can be related to foam systems, information on the correlation of interfacial properties and foam stability is still limited and not conclusive. To fully understand the effect of parameters like pH and ionic strength on the foaming properties of a protein isolate, also their effect on the molecular properties and the resulting interfacial behaviour needs to be studied.

In the present study the interfacial properties as well as the foaming properties of ASPI are studied as function of isolate concentration, pH, and ionic strength. The obtained data is compared to data of whey protein isolate (WPI) and egg white albumin (EWA) to compare the functional properties of ASPI to those of commonly used food foaming agents.

4.2. Material and Methods

4.2.1. Materials

Whey protein isolate (WPI) was purchased from Davisco Foods International (BiPro®, lot JE216-6-440, Le Sueur, MN, USA, 92% [w/w] protein [N x 6.36]) and albumin from chicken egg white (EWA) from Sigma (St. Louis, MO, USA, 88% (w/w) protein [N x 6.25]). Algae soluble protein isolate (ASPI) (54% [w/w] protein [N x 5.60]) was isolated from the commercially available microalgae *Tetraselmis sp.* (Reed Mariculture, Campbell, CA, USA) as described previously (Schwenzfeier et al., 2011). All chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO, USA) if not stated otherwise.

4.2.2. Chemical analyses

Total nitrogen contents were determined by the Dumas method using a Flash EA 1112 NC analyser (Thermo Fisher Scientific Inc. Waltham, MA, USA) according to the manufacturer's protocol. Nitrogen-to-protein conversion factors named above were used to calculate the protein contents of the respective samples. Total neutral sugar contents were determined with the orcinol-sulfuric acid colour assay (Tollier & Robin, 1979) and uronic acids contents with the *m*-hydroxydiphenyl assay (Ahmed & Labavitch, 1978), both using

an automated colorimetric assay analyser. As standards galactose or glucose (25 – 200 µg/mL) and galacturonic acid (12.5 – 100.0 µg/mL) were used, respectively.

4.2.3. Viscosity of protein isolate solutions

To determine the viscosities of ASPI, WPI, and EWA 1 mg/mL of the respective protein isolate was dissolved in 10 mM NaCl. The viscosities of the solutions were measured using an Ubbelohde viscometer with a gauge constant (C) of 0.004972 in a water bath at 25°C. The dynamic viscosity (η_{dyn}) in Pa*s was calculated from equation (1), in which t is the flow time in seconds and ρ the density of the solution in kg*m^{-3}.

$$\eta_{dyn} = t * C * \rho * 10^{-6} \quad (1)$$

4.2.4. Protein solubility

Protein solubility was determined as described previously (Schwenzfeier et al., 2011). Each protein isolate was dissolved (1 mg/mL) in 10 mM or 200 mM NaCl solution (pH 7). Under these conditions no protein precipitation was observed at the initial pH 7. The pH of the protein isolate solutions was adjusted by adding small amounts of 0.01 M, 0.10 M or 1.00 M HCl to obtain final pH values ranging from 3.0 – 7.0 in 0.5 pH-unit intervals. Protein solubility was defined as protein concentration in the respective supernatant relative to the initial protein concentration of the pH 7 supernatant.

4.2.5. Foam preparation and stability

All foams were prepared by sparging N_2 through 40 mL protein isolate solution at a flow rate of 830 mL/min through the metal grid of the foam beaker as described by Wierenga et al. (2009). When the foam height had reached 12 cm, the gas flow was stopped and the foam height was recorded as function of time. One parameter to evaluate foam stability is the foam half-life time ($t_{1/2}$), the time in which the foam decreased to half of its initial height. Since some of the foams prepared showed inconsistent long-term stability due to the formation of holes inside an overall stable foam, in this study $t_{5/6}$, the time in which the foam height decreased from 12 cm to 10 cm is used as a parameter to describe foam stability (Figure 4.1).

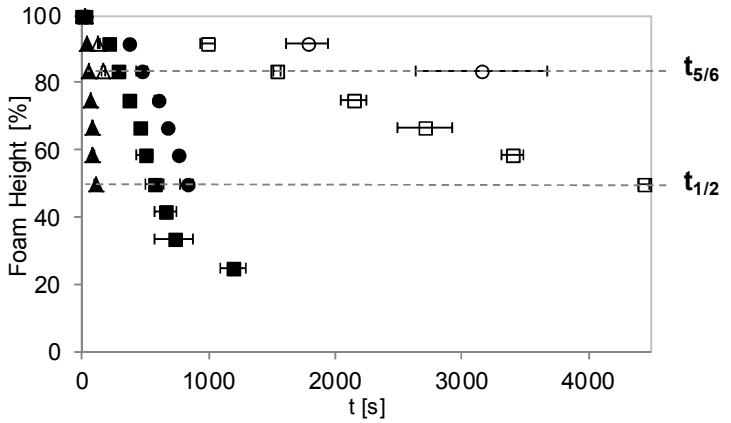

Figure 4.1 – Decrease in foam height of ASPI (●), WPI (■), and EWA (▲) stabilized foams as function of time (s) at pH 7 at NaCl concentrations of 10 mM (full symbols) and 200 mM (open symbols); $t_{1/2}$ indicates the time in which the foam decreased to half of its initial height, $t_{5/6}$, the time in which the foam height decreased from 12 cm to 10 cm; error bars indicate standard deviations

4.2.6. Influence of isolate concentration on foam stability

To study the influence of isolate concentration on foam stability solutions containing 0.10, 0.25, 0.50, 0.75 and 1.00 mg/mL ASPI, WPI and EWA were prepared by suspending the respective isolate in 10 mM NaCl at least one hour before foaming. If necessary, the pH of the protein solution was adjusted to pH 7 by adding small amounts of 5 M NaOH. After one hour the samples were centrifuged (5 min, 4500 x g, 20°C) and the supernatants obtained were foamed as described above.

4.2.7. Influence of pH and ionic strength on foam stability

To study the influence of pH and ionic strength on foam stability solutions containing 0.5 mg/mL ASPI, WPI and EWA were prepared by suspending the respective isolate in either 10 mM or 200 mM NaCl solution. The pH of the initial solutions was adjusted to values ranging from 3 – 7 with 1 unit intervals using small amounts of 0.1 M – 5.0 M HCl or 0.1 M - 5 M NaOH. After one hour the samples were centrifuged (5 min, 4500 x g, 20°C) and the supernatants obtained were foamed as described above.

4.2.8. Automated drop tensiometry

At least one hour before measurements ASPI, WPI and EWA solutions (0.01 mg/mL) were prepared by suspending the respective isolate in 10 mM or 200 mM NaCl solution previously adjusted to pH 7 or pH 5. If necessary, the pH was readjusted to the respective pH after dissolving the isolate by addition of 0.05 M HCl. An automated drop tensiometer (ADT, Teclis ITconcept, Longessaigne, France) was used to measure the interfacial tension and the dilatational modulus of the interface as function of time (0 – 3600 s) as described earlier (Wierenga et al., 2005). The interfacial pressure at time i was calculated by subtracting the interfacial tension of the protein solution at time i from the interfacial tension of the buffer used in the respective experiment. The bubble volume was kept constant at 7 µL. The dilatational rheological properties were measured using a deformation of the interfacial area with 5% at a frequency of 0.1 Hz. The modulus was calculated from the measured changes in surface tension upon the relative area change of the surface area averaged over a sequence of five sinuses. The samples were measured in duplicate using the same protein solution.

4.3. Results

4.3.1. Solubility

High solubility is considered a prerequisite for successful functional applications of protein isolates in foods and can strongly be influenced by changes in pH. Therefore, the pH dependent solubility of ASPI, WPI, and EWA was investigated prior to pH dependent foam stability. At low ionic strength (10 mM NaCl) ASPI is 100% soluble in the pH range 6.0 – 7.0 (Figure 4.2A). Below pH 6 the solubility decreases rapidly (to ≤ 20%) to remain constant in the pH range 3.0 – 4.5, close to the isoelectric point of ASPI (Table 4.1). At high ionic strength (200 mM NaCl) the solubility of ASPI already started to decrease at pH 6. While the solubility of ASPI observed at low ionic strength is similar to a previous batch, the solubility at high ionic strength was found to be slightly different. WPI and EWA show high solubility in the pH range 3 – 7 at both ionic strengths tested. While EWA was 100% soluble at all conditions tested, at low ionic strength the solubility of WPI decreased around its isoelectric point (~ pH 5) to 90%.

Table 4.1 – Physico-chemical properties important for the adsorption kinetics and the foaming properties of ASPI, WPI and EWA

		ASPI	WPI	EWA
Protein content [w/w%]		54	92	88
Sugar content [w/w%]		16	1	4
pI		< 3[a]	~ 5[b]	~5[c]
ζ-potential [mV] (0 – 10 mM NaCl)	pH 7	- 47[a]	- 58[b]	- 2 μm*cm/(V*s)[c]
	pH 5	- 47[a]	0[b]	0[c]
	pH 3	-15[a]	62[b]	2 μm*cm/(V*s)[c]
η_{dyn} [mPa*s] (0.1% protein, pH 7)	10 mM	0.96	0.93	0.92
	200 mM	0.96	0.95	0.97

[a]According to Schwenzfeier et al. (2013), pI estimated based on ζ-potential measurements

[b]Accodring to Kulmyrzaev & Schubert, (2004), pI estimated based on ζ-potential measurements

[c]According to Padala et al. (2009), pI estimated based on electrophoretic mobility measurements

4.3.2. Foam properties

4.3.2.1. Effect of isolate concentration on foam stability

The foam properties of ASPI, WPI and EWA were determined by measuring the foam life time $t_{5/6}$ as function of isolate concentration at pH 7 and 10 mM NaCl (Figure 4.3). Under

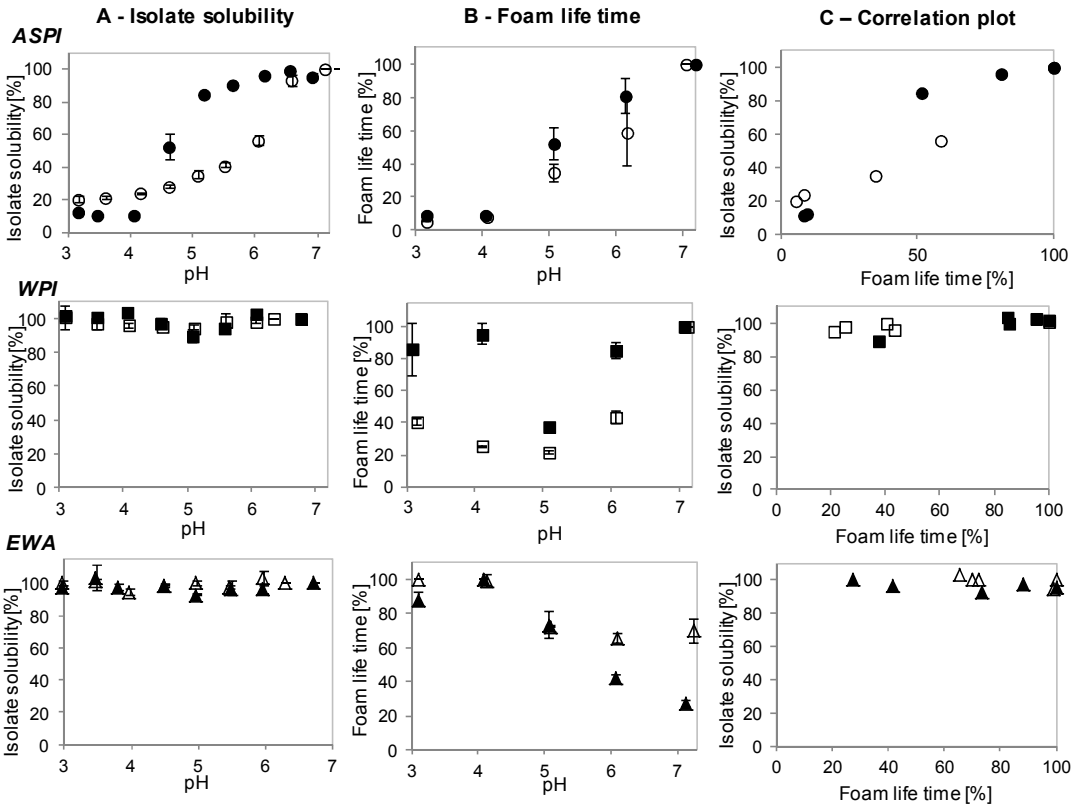

Figure 4.2 – A: Protein solubility as function of pH of 1 mg/mL ASPI, WPI and EWA solutions at NaCl concentration of 10 mM (filled symbols) and 200 mM (open symbols); B: Corresponding foam stabilities of foams stabilized with 0.5 mg/mL ASPI, WPI or EWA; C: Correlation plot of the data shown in part A and B; error bars indicate standard deviations

these conditions all three isolates were 100% soluble at all concentrations used. The foam ability can be characterized by the minimum concentration needed to make stable foam. For ASPI and WPI, this minimum isolate concentration was 0.10 mg/mL, while for EWA 0.25 mg/mL were necessary. Another foam property is foam stability, in this study measured as the foam life time $t_{5/6}$. With increasing isolate concentration $t_{5/6}$ increases for all isolates. Interestingly, ASPI stabilized foams exhibit the highest foam stability at each concentration. At the highest isolate concentration tested (1 mg/mL) $t_{5/6}$ of ASPI stabilized foams is 1.6 times higher than for WPI stabilized foams and 10.2 times higher than for EWA stabilized foams. This high stability of ASPI stabilized foams is unexpected, especially when considering that the actual protein content of ASPI is 38% (w/w) and 34% (w/w) lower than in WPI and EWA, respectively.

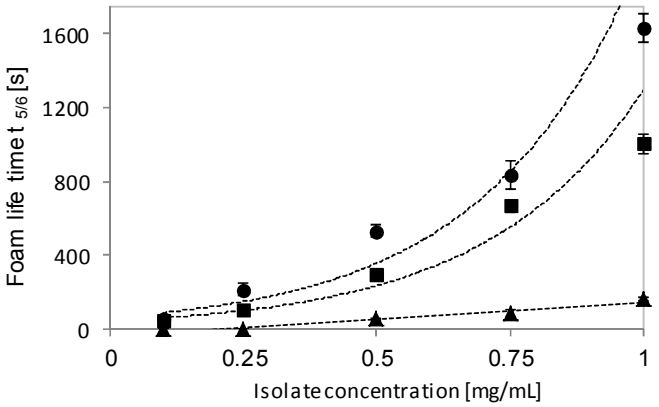

Figure 4.3 – Foam life time ($t_{5/6}$) of ASPI (●), WPI (■), and EWA (▲) stabilized foams (pH 7, I = 10 mM) as function of isolate concentration (mg/mL), dotted lines represent polynomial (2nd order) trendlines, error bars indicate standard deviation

To investigate if the presence of polysaccharides in ASPI (Table 4.1) possibly results in a higher viscosity of the ASPI solutions compared to WPI and EWA solutions, the dynamic viscosities were determined for 1 mg/mL solutions of all three isolates. Since all dynamic viscosities measured were found to be similar to the viscosity of water (0.9 mPa*s < η_{dyn} < 1.0 mPa*s) at both 10 mM and 200 mM NaCl (Table 4.1), it can be concluded that the polysaccharide fraction present in ASPI does not contribute to foam stability by increasing the viscosity of the isolate solution. It still can contribute to foam stability, e.g by the formation of protein-polysaccharide complexes.

4.3.2.2. Effect of pH on foam stability

The influence of pH on the foam properties of ASPI, WPI and EWA was determined by measuring the foam life time $t_{5/6}$ in the pH range 3 - 7 at an initial protein concentration of 0.5 mg/mL and in the presence of 10 mM NaCl (Figure 4.2B – relative values, Figure 4.4 – absolute values). The stability of ASPI stabilized foams is highest at pH 7. With decreasing pH until pH 4, the amount of soluble protein as well as the stability of ASPI stabilized foams decreased. Below pH 4, close to the overall isoelectric point of ASPI (Table 4.1) no stable foams can be formed due to a lack of soluble protein available for the adsorption to the air-water-interface.

For WPI, foam stability decreased around pH 5, the isoelectric point of WPI, by 62% compared to the original foam stability at pH 7. In contrast to ASPI and WPI, which both show minimum foam stability close to their isoelectric points (for ASPI ~ pH 3, for WPI ~ pH 5, Table 4.1), EWA stabilized foams are more stable at the isoelectric point of EWA (pH 5) and below. At both ionic strengths tested, $t_{5/6}$-values of EWA stabilized foams increase from pH 5 downwards to reach their maximum in the pH range 3 – 4. For WPI and EWA the pH hardly affected protein solubility, while it did significantly affect foam stability (Figure 4.2).

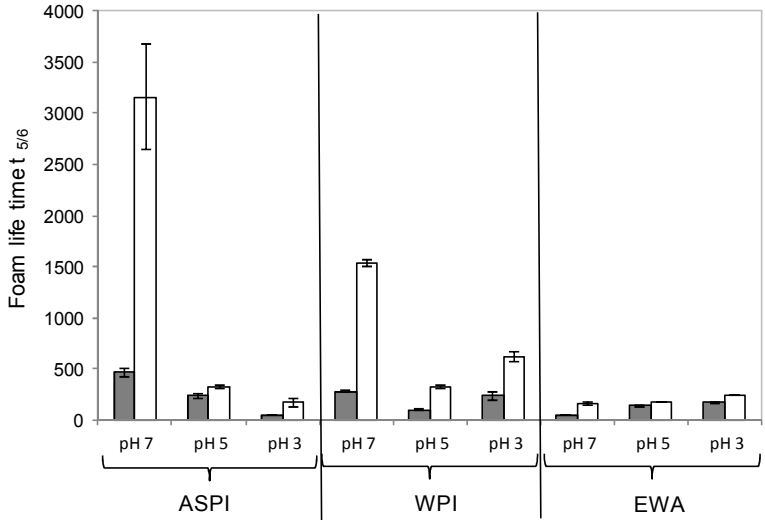

Figure 4.4 – pH dependent foam life times ($t_{5/6}$) of ASPI, WPI, and EWA stabilized foams at 10 mM (filled bars) and 200 mM NaCl (open bars) at initial isolate concentrations of 0.5 mg/mL, error bars indicate standard deviations

4.3.2.3. Effect of ionic strength on foam stability

Next to pH, the effect of ionic strength on the stability of ASPI, WPI and EWA stabilized foams was determined. At both ionic strengths tested (10 mM and 200 mM NaCl) the stability of ASPI and EWA stabilized foams show each a similar trend as function of pH (Figure 4.2B). While for ASPI absolute foam stabilities are much higher at high ionic strength than at low ionic strength, for EWA only a slight increase in foam stability was observed (Figure 4.4). For WPI, foam stability at high ionic strength is still minimal around pH 5. At all pH values tested, however, absolute WPI foam stabilities are higher at high ionic strength than at low ionic strength.

4.3.3. Interfacial properties

4.3.3.1. Effect of pH and ionic strength on adsorption behaviour

To characterize the interfacial properties of ASPI, WPI and EWA at the air-water interface elastic modulus (E_d) and surface pressure (Π) were determined as function of time at two different pH values (pH 5 and pH 7) and 10 mM NaCl and plotted against each other (Figure 4.5). For each protein isolate, this plot (Figure 4.5B) was unaffected by the conditions (pH, I), indicating that the composition of the protein layer was similar at all these conditions. Consequently, increased kinetics of the surface pressure development may be interpreted as an increase in adsorption kinetics. For ASPI at pH 7 the surface pressure only increased after a lag-time of approximately 800 s. After this lag time, Π increased rapidly to a maximum surface pressure (Π_{max}) of 13 mN/m. At pH 5, a lag time of 1400 s was observed, indicating much slower adsorption kinetics. Nevertheless, only a small decrease in Π_{max} (11 mN/m) was observed. While changes in pH influence the adsorption kinetics of ASPI considerably, changes in ionic strength do not. In contrast to ASPI, the adsorption kinetics of WPI were hardly affected by varying pH or ionic strength. Only the Π_{max} at pH 7 and 10 mM NaCl is lower (12 mN/m) than at the other conditions (16 mN/m). This deviation of Π_{max} at pH 7 and 10 mM NaCl was also observed for EWA. Here a lag-time of almost 2500 s and hardly any increase in Π were observed at pH 7 and 10 mM NaCl. At all other conditions the surface pressure already increased after 500 s. Still, the final surface pressure observed for EWA under those conditions is lower (9 mN/m) than for WPI and ASPI (16 mN/m).

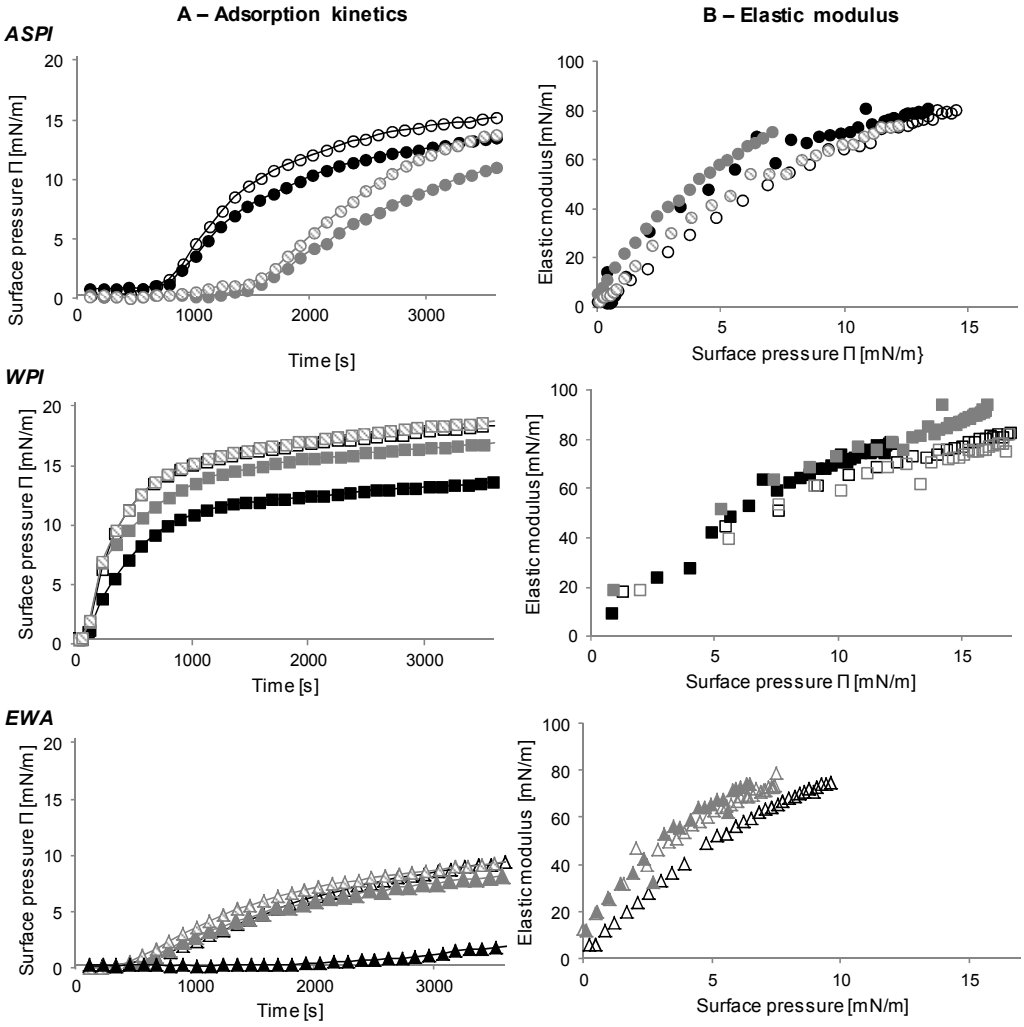

Figure 4.5 – A: Surface pressure at the air/water interface as function of time at NaCl concentrations of 10 mM (filled symbols) and 200 mM (open symbols) at pH 7 (black) and pH 5 (grey) for ASPI, WPI and EWA solutions at a concentration of 0.01 mg/mL; B: Elastic modulus [mN/m] as function of surface pressure [mN/m] of the same samples (EWA sample at 10 mM NaCl, pH 7 not depicted due to low Π – values)

4.4. Discussion

4.4.1. Interfacial composition of ASPI stabilized air-water interfaces

When comparing the foam properties of ASPI with those of WPI and EWA, it is important to consider their different chemical compositions. Unlike WPI and EWA, which are composed for ~90% (w/w) of protein, ASPI contains 54% (w/w) protein and 16% (w/w) polysaccharides, the latter composed for approximately one third of uronic acids. Since typically only proteins are surface active, it might be expected that the foam properties of ASPI compare inferior to the foam properties of WPI and EWA at comparable isolate concentrations. In this study, however, foam ability and foam stability of ASPI stabilized foams were superior to those of WPI and EWA. For ASPI stabilized emulsions it has been shown already that the co-adsorption of charged polysaccharides to the oil-water interface contributes to emulsions stability against droplet aggregation around pH 5 (Schwenzfeier et al., 2013). Similarly, these charged polysaccharides could co-adsorb to the protein-covered air-water interface and with this influence the interfacial properties of the isolate. Typically, changes in interfacial composition by the co-adsorption of a polysaccharide are reflected in changes in the dilatational behaviour. For ASPI, however, the same dilatational behaviour was observed at all pH values and ionic strengths tested and, therefore, the formation of electrostatically stabilized protein-polysaccharide complexes at the air-water interface is not likely. Consequently, a constant, protein-dominated interfacial composition independent of the electrostatics conditions can be assumed.

4.4.2. Effects of pH and ionic strength on interfacial and foam properties of ASPI, WPI and EWA

In order to evaluate the influence of electrostatic interactions between the adsorbing proteins on interfacial properties as well as on foam stability these properties were measured over a range of pH values and at two ionic strengths. For ASPI, decreased foam stability from pH 5 downwards was found to be correlated to decreased solubility at these conditions (Figure 4.2). For WPI and EWA, protein solubility is hardly affected by changes in pH and ionic strength and, therefore, the effect of electrostatic interactions on their adsorption behaviour can be studied more directly. Close to the isoelectric point (pH 5) of the two isolates and at high ionic strength (pH 7, 200 mM NaCl), the decreased electrostatic repulsion between the proteins present at the air-water-interface results in higher Π_{max} values than at pH 7 and low ionic strength, respectively. The addition of salt at pH 5 did not increase Π_{max} considerably, since electrostatic repulsion around the isoelectric point is

already minimal at low ionic strength. Similar observations have been described for the proteins BSA (Cho et al., 1997) and ovalbumin (Wierenga et al., 2005). In those studies the increase in Π_{max} was confirmed to correspond to an increased surface load (Γ) using ellipsometry. As described above, based on the dilatational behaviour of ASPI it is expected that only the proteins present in the isolate adsorb to the air-water-interface. In this case changing the electrostatic conditions in the pH range, where ASPI is highly soluble (pH 5 – 7), should influence the adsorption behaviour of ASPI similarly to that of WPI and EWA. Indeed, the adsorption of ASPI increased close to the isoelectric point calculated based purely on the amino acid composition of the isolate (pH 7) (Schwenzfeier et al., 2013).

Despite the fact that pH and ionic strength are considered important parameters for techno-functional applications of protein isolates and their effects on gelation and aggregation have often been studied, only a limited number of studies are available focusing on their effect on adsorption kinetics and respective foam stability. So far, increased intermolecular interactions under conditions of decreased electrostatic repulsion have been linked to increased foam stability (e.g. Engelhardt et al., 2012; Kuropatwa et al., 2009). In this study, however, the increased adsorption at high ionic strength always resulted in an increase in foam stability, while the increased adsorption around the isoelectric point of the respective protein isolate had diverging effects on foam stability. According to expectations, the increased adsorption of EWA at pH 5 resulted in increased foam stability. Maximum foam stability, however, was reached in the pH range 3 – 4, where pH dependent ζ-potential measurements (Table 4.1) suggest that the electrostatic repulsion between the proteins is high. In contrast to EWA, the increased adsorption of WPI at pH 5 corresponded to a decrease in foam stability compared to pH 7. Kuropatwa et al. (2009), who also report minimal foam stability for WPI around pH 5, have suggested that the formation of β-lactoglobulin octamers at this pH changes the interfacial properties of the major protein present in WPI and with this influences foam stability of the isolate. The interfacial properties observed in this study, however, did not change accordingly.

For ASPI, adsorption as well as foam stability were found to be maximal at pH 7, close to the isoelectric point calculated purely based on the amino acid composition of ASPI. Together with the consistent dilatational behaviour, this correlation between adsorption behaviour and foam stability confirms the adsorption of only proteins to the air-water interface at all pH values and ionic strength tested. So, in the case of ASPI, an increased adsorption of the proteins present in the isolate close to their isoelectric point is reflected in higher foam stability. The overall foam stabilities of ASPI stabilized foams are superior to

those of WPI and EWA in the pH range 5 - 7. In this pH range the molecular and interfacial properties of the protein fraction of ASPI, seems to favour the production of very stable foams. A further study to unravel the exact contribution of the polysaccharide fraction to the foaming properties of ASPI is described in Chapter 5.

4.5. Conclusions

In the pH range 5 – 7 the stability of ASPI stabilized foams is superior to the stabilities of WPI and EWA stabilized foams. Based on the dilatational measurements of this study, it is assumed that solely the proteins present in ASPI contribute to its superior foam stability. The stability of ASPI stabilized foams increases close to the isoelectric point of its protein fraction and at increased ionic strength. For WPI and EWA, in contrast, this correlation between increased intermolecular interactions under conditions of decreased electrostatic repulsion and increased foam stability could not be observed.

Acknowledgements

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5 Effect of charged polysaccharides on the techno-functional properties of fractions obtained from algae soluble protein isolate

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Abstract

Algae soluble protein isolate (ASPI) contains proteins, glycoproteins and charged polysaccharides. To determine the effect of the charged polysaccharide on the foam and emulsion properties of the isolate, ASPI was fractionated over a 50 kDa MWCO membrane. The fractions obtained, a filtrate (ASPI-F), a retentate (ASPI-R) and a pellet (ASPI-P), were analysed with regard to their chemical composition and their emulsifying and foaming properties. While the fraction enriched in uronic acids (ASPI-R) showed increased emulsion stability over the whole pH range investigated (pH 3 – 7), the fractions with decreased uronic acid content (ASPI-F, ASPI-P) showed decreased emulsion stability around pH 5. This indicates the importance of the charged polysaccharide present in ASPI for its emulsion stability around pH 5. The effect of charged carbohydrates in ASPI-R was also reflected in the ζ-potential of the emulsion droplets, which remain negative over the whole pH range. Only for the protein fraction enriched in protein (ASPI-P), the ζ-potential becomes positive at pH 3. Detailed studies of the air-water interfacial properties, however, did not show significant differences between the fractions. This indicates that at the charged polysaccharides do not contribute to the air-water interfacial layer. Interestingly, F-ASPI, the fraction containing small molecular weight proteins and glycoproteins (≤ 50 kDa), showed considerably higher foam stabilities in the pH range 5 – 7 than the other fractions at comparable isolate concentrations. Based on the results of this study it is suggested that the charged carbohydrates present in ASPI contribute considerably to high emulsion stability in the pH range 5 – 7, while foam stability is mainly influenced by dissociated proteins and small glycoproteins.

5.1. Introduction

Microalgae have been considered a promising alternative protein source in foods already since the early 1950's. Initially, the interest in microalgae protein was based purely on its high nutritional quality. Recently, the combined cost-efficient production of high-value protein isolates and biofuels from microalgae biomass has triggered renewed interest in the isolation of proteins from microalgae for food applications (e.g. Wijffels et al., 2010; Williams & Laurens, 2010). Despite the increased interest, the structural and techno-functional properties of microalgae proteins are poorly documented. To enable the study of techno-functional properties of microalgae protein a process has recently been developed for the mild isolation of the soluble protein fraction from the green microalgae *Tetraselmis* sp. (Schwenzfeier et al., 2011). The final algae soluble protein isolate (ASPI) was free from green colour and contained 64% (w/w) protein, 24% (w/w) carbohydrates, composed for one fourth of uronic acids, and 12% (w/w) remaining material containing mainly ash and other minor components. Part of the carbohydrates in ASPI was found to be present in form of glycoproteins. Since carbohydrate moieties in glycoproteins are typically composed of neutral sugars, the uronic acids present in ASPI are expected to represent building blocks of charged polysaccharides. This charged polysaccharide fraction could form soluble, electrostatically stabilized complexes with a surface-active protein. These complexes promote the co-adsorption of the polysaccharides to the interface (e.g. Ganzevles et al., 2006), thereby influencing the foam and emulsion properties of ASPI.

For ASPI stabilized emulsions, the presence of charged carbohydrates on the emulsion droplet surfaces was confirmed by measuring their ζ-potentials, which remained negative over the whole pH range investigated (pH 3 – 7) (Schwenzfeier et al., 2013). The co-adsorption of the charged polysaccharide fraction resulted in increased stability against droplet aggregation around pH 5, the pH where emulsions stabilized with currently used food protein isolates (e.g. whey protein isolate) often do aggregate. An increase in ionic strength (≥ 100 mM NaCl) caused the ASPI stabilized emulsion to flocculate at pH 5, presumably due to the dissociation of protein-polysaccharide complexes. In contrast to emulsion properties, the foam properties of ASPI were not found to be affected by the contribution of polysaccharides (Chapter 4). Similar dilatational moduli at varying pH and ionic strength suggested the sole adsorption of protein to the air-water interface. Still, the stability of ASPI stabilized foams compared superior to the stabilities of foams stabilized with whey protein isolate or egg white albumin in the pH range 5 – 7 at comparable isolate concentrations, despite the lower protein content of ASPI.

In the present study the influence of the polysaccharides present in ASPI on the techno-functional properties of the isolate is investigated in more depth. For this purpose the isolate was further fractionated by centrifugal fractionation via a 50 kDa MWCO membrane. The fractions obtained were characterized with regard to their chemical composition. Subsequently, their foaming and emulsion properties were studied as function of pH.

5.2. Material and Methods

5.2.1. Materials

Algae soluble protein isolate (ASPI) (53% [w/w] protein) was isolated from the commercially available microalgae *Tetraselmis* sp. (Reed Mariculture, Campbell, CA, USA) as described previously (Schwenzfeier et al., 2011). All chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany) if not stated otherwise. Proteinase K (order no. 92905, 825 U/mg) from Fluka was used for hydrolysis experiments.

5.2.2. Fractionation of ASPI

Fractionation of ASPI was carried out using Amicon Ultra-15 centrifugal filter units equipped with 50 kDa MWCO regenerated cellulose membranes (Merck Millipore, Darmstadt, Germany). Prior to fractionation, 2.5 mg ASPI were dissolved in 250 mL demineralized water, stirred overnight at 4 °C and centrifuged (20 °C, 20 min, 4,500 x g). ASPI solution (10 mL) was added to a filter unit previously washed with 5 mL demineralized water. Subsequently, fractionation was carried out by centrifugation (20 °C, 20 min, 4,500 x g). The fractionation step was repeated three times by adding 10 mL ASPI solution to the remaining retentate in the filter unit. Next to a filtrate (ASPI-F) and a retentate (ASPI-R), a pellet (ASPI-P) developed during the repeated addition of ASPI solution onto the already obtained retentate. The pellet most probably developed due to protein precipitation from the intensely concentrated, protein-saturated ASPI(-R). After the actual fractionation the material remaining in the filter units was washed three times with 7 mL demineralized water (20 °C, 20 min, 4,500 x g). The wash filtrate obtained was combined with the actual filtrate obtained during fractionation. To obtain sufficient material for subsequent experiments, the fractionation was performed in parallel using eight filter units. The three final fractions obtained from each filter were pooled, freeze dried and weighed.

5.2.3. Compositional analysis

All samples used for compositional analysis were freeze dried prior to analysis and stored at 50 °C until further analysis to prevent adsorption of additional water. Chloroform/methanol (2:1 v/v) extractable material was determined gravimetrically after exhaustive extraction of freeze-dried samples by Soxhlet extraction. All analyses mentioned were carried out at least in duplicate.

5.2.3.1. Amino acid composition and protein content

Amino acid composition analysis was carried out in duplicate using samples from independent fraction runs by Ansynth Service BV (Berkel en Roodenrijs, The Netherlands) according to the method of Moore & Stein (1963). Standard deviations were found to be on average < 1% of the mean. In the worst case the standard deviation was 19%. Protein contents of ASPI and its fractions were calculated as the sum of amino acid residue weights. For soluble fractions, concentrations were also determined with the Pierce® BCA protein assay according to the producer's instructions (Pierce, Rockford, IL, USA). BSA was used as standard. All protein content determinations were at least carried out in duplicate.

5.2.3.2. Total neutral sugar and total uronic acid content

Total neutral sugar contents were determined with the orcinol-sulfuric acid colour assay (Tollier & Robin, 1979) and uronic acids contents with the *m*-hydroxydiphenyl assay (Ahmed & Labavitch, 1978), both using an automated colorimetric assay analyser. As standards galactose or glucose (25 – 200 µg/mL) and galacturonic acid (12.5 – 100.0 µg/mL) were used, respectively.

5.2.3.3. Size exclusion chromatography

Size exclusion chromatography (SEC) was performed on a ÄKTAmicro liquid chromatography system equipped with a Superose 6 column (both GE Healthcare, Uppsala, Sweden). The samples (50 µL, ~10 mg/mL protein) were eluted with 50 mM Tris/HCl buffer (pH 8) containing 150 mM NaCl at a flow rate of 0.5 mL/min at room temperature. Elution was monitored by UV detection at 280 nm (internal detector) and by refractive index detection (Shodex RI150, Showa Denko K.K., Tokyo, Japan). Apparent molecular weights were estimated with the help of a high molecular weight gel filtration calibration kit (GE Healthcare).

5.2.3.4. SDS-PAGE

The protein composition of the different ASPI fractions was visualized using SDS-PAGE analysis under reducing conditions (10 mM 2-mercaptoethanol) on a Mini-protean II system (Bio-Rad Laboratories, Hercules, CA, USA) according to the instructions of the manufacturer. Gels were stained with either Coomassie brilliant blue (CBB) or Periodic acid - Schiff's reagent (PAS). Precision Plus Protein Dual Colors Standard (Biorad) was used as molecular weight marker in all gels.

5.2.4. Protein hydrolysis

Hydrolysis of the proteins present in ASPI was performed by Proteinase K using a pH-stat. For this, 10 mL ASPI solution (5 mg/mL) were preheated to 40 °C in the temperature controlled pH-stat. Subsequently, the pH was adjusted to pH 8 with 0.2 M NaOH and 10 µL of a 10 mg/mL proteinase K solution was added. The pH was kept constant by the pH-stat (pH 8.0) during 3 hours of Proteinase K incubation. The obtained hydrolysate was analysed with SEC as described above without any further dilution.

5.2.5. Automated drop tensiometry

Automated drop tensiometry was carried out as described in Chapter 4. In this study 0.1 mg/mL ASPI, ASPI-R, ASPI-F and ASPI-P solutions were prepared by suspending the respective isolate in 10 mM NaCl solution and adjusted to pH 7 or pH 5 at least one hour before measurements. If necessary, the pH was readjusted to the respective pH after dissolving the isolate by addition of 0.05 M HCl. All samples were measured in duplicate using the same protein solution.

5.2.6. Foam preparation and stability

To study the influence of pH on foam stability, the pH of solutions of ASPI and its fractions (0.5 mg/mL in 10 mM NaCl) was adjusted to values ranging from 3 – 7 with 1 unit intervals using small amounts of 1 M HCl or 5 M NaOH. After stirring for at least one hour at 20 °C the solutions were centrifuged (5 min, 4500 x g, 20°C). Foams were prepared by sparging N_2 through 40 mL of the solution at a flow rate of 830 mL/min through the metal grid of the foam beaker as described by Wierenga et al. (2009). When the foam height had reached 12 cm, the gas flow was stopped and the foam height was recorded as function of time. In order to compare the data obtained to the data reported in Chapter 4, in this study

$t_{5/6}$, the time in which the foam height decreased from 12 cm to 10 cm, is used as a parameter to describe foam stability.

5.2.7. Emulsion preparation and stability

Prior to emulsion preparation 0.2 g ASPI, ASPI-F, ASPI-R or ASPI-P were each suspended in 18 g of 10 mM NaCl solution. The solutions were stirred for at least one hour at 20 °C resulting in solutions of pH 7.6 (ASPI), pH 7.9 (ASPI-F), pH 7.0 (ASPI-R) and pH 7.2 (ASPI-P), which were used without any further pH adjustment. To obtain emulsions containing 10% (w/w) sunflower oil and 1% (w/w) of the respective ASPI fraction, 2 g sunflower oil was added to all solutions. All emulsions were made from pre-emulsions (Ultra-Turrax at 9500 rpm for 1 min) further passed 10 times through a laboratory homogenizer (LH-scope HU-3.0, Delta Instruments, Drachten, The Netherlands) at a pressure of 100 bar. The pH of the emulsions was adjusted to pH 7.0 directly after preparation with 0.1 M or 1.0 M HCl, if necessary. The influence of pH on emulsion stability was determined by adjusting the pH of the initial emulsion to values ranging from 3 – 6 with 1 unit intervals using 0.1 or 1.0 M HCl. To study the influence of ionic strength on emulsion stability, the salt concentration of the emulsion was adjusted by adding 1 part of a 2 M NaCl solution to 9 parts of the initial emulsion.

5.2.7.1. Droplet size determination

Droplet size distributions and volume surface mean diameters (d_{32}) of the emulsions were determined by static light scattering (DLS) (Mastersizer Hydro 2000SM, Malvern Instruments Ltd, Malvern, UK) at room temperature. A refractive index ratio of 1.1 was used to calculate the particle size distribution. In addition to static light scattering, diffusing wave spectroscopy (DWS) was used to quantify emulsion instability independent of the effects of dilution and mechanical deformation introduced in the Mastersizer. DWS was carried out as described earlier (Schwenzfeier et al., 2013).

5.2.7.2. ζ-potential measurements

The ζ-potential of the emulsion droplets was determined using a particle electrophoresis instrument (Zetasizer, Nano series – ZS, Malvern). Prior to analysis emulsions were diluted 500x in 10 mM NaCl at the pH of the respective emulsion and equilibrated for 5 min at 25 °C inside the instrument. Data was collected over at least 5 sequential readings and processed using the Smoluchowski model (Hunter, 2001).

5.2.7.3. Light microscopy

Qualitative detection of droplet aggregation was carried out by microscopic observation after 100x dilution in 10 mM NaCl at the pH of the respective emulsion. If aggregated droplets were observed, microscopic examination was repeated after diluting the emulsion 1:1 in 0.5% (w/v) SDS.

5.3. Results and discussion

5.3.1. Preparation and characterization of the ASPI fractions

5.3.1.1. Chemical composition

The ASPI batch used in this study contained 53% (w/w) proteins, 16% (w/w) carbohydrates, composed for approximately one third of uronic acids, and 17% (w/w) chloroform/methanol ($CHCl_3$/MeOH) extractable material (Table 5.1). The remaining material is expected to contain mainly ash and other minor components. After centrifugal fractionation via a 50 kDa MWCO membrane 92% of proteins, 88% of the total sugars and 85% of the uronic acids originally present in ASPI were recovered in the fractions obtained. Next to a filtrate (ASPI-F) and a retentate (ASPI-R), a pellet (ASPI-P) developed during the repeated addition of ASPI solution onto the already obtained retentate. The pellet most probably developed due to protein precipitation from the intensely concentrated, protein-saturated ASPI solution and that of the retentate (ASPI-R). Consequently, the protein content of this pellet fraction (72% w/w) is higher than that of the original ASPI (53% [w/w]). In contrast to ASPI-P, the protein contents of ASPI-F (44% [w/w]) and ASPI-R (31% [w/w]) are lower than in the original ASPI. The fraction with the highest carbohydrate content was ASPI-R (34% [w/w]). In combination with its low protein content, the high amounts of carbohydrates resulted in a total sugar-to-protein ratio almost four times higher than in ASPI (1.10 and 0.30, respectively) (Table 5.1). In addition, also the uronic acid-to-protein ratio (UA/Pro ratio) of ASPI-R (0.39) was higher than that of ASPI (0.09). This indicates that in ASPI-R charged polysaccharides accumulated. Therefore, the other two fractions, ASPI-F and ASPI-P, had very low UA/Pro ratios (0.02 and 0.04, respectively).

Table 5.1 - Total protein and sugar contents and protein and sugar yields of ASPI and its fractions obtained after centrifugal fractionation, parentheses: according standard deviations

Fraction	Total mass recovered [%][a]	Proteinaceous material [w/w%]	Neutral sugars [w/w%]	Uronic acids [w/w%]	$CHCl_3$ / MeOH extractables [w/w%]
ASPI	100	53 (<1)	11 (1)	5 (<1)	17 (n.d.)
ASPI-R	24	31 (1)	22 (<1)	12 (<1)	5 (n.d.)
ASPI-F	14	44 (1)	8 (1)	1 (<1)	4 (n.d.)
ASPI-P	53	72 (1)	9 (<1)	3 (<1)	2 (n.d.)
Fraction	**Proteinaceous yield [%]**	**Neutral sugar + uronic acid yield [%]**	**Total uronic acid yield [%]**	**Neutral sugar + Uronic acids / protein ratio**	**Uronic acids / protein ratio**
ASPI	100	100	100	0.30	0.09
ASPI-R	13	47	53	1.10	0.39
ASPI-F	10	7	3	0.20	0.02
ASPI-P	65	34	29	0.17	0.04

[a] Assuming 10% residual water in inital ASPI
n.d = not determined

5.3.1.2. Protein composition

For all samples tested, SDS-PAGE analysis under reducing conditions indicated that most of the proteins present are assembled of polypeptide chains with apparent molecular masses smaller than 50 kDa (Figure 5.1). In agreement with previous results (Schwenzfeier et al., 2011), the bands with the highest intensities represented polypeptide chains with a molecular masses of about 50, 40, 25 and 15 kDa.

Since the SDS PAGE analysis indicated very similar polypeptide patterns for all fractions, SEC experiments were performed to determine the molecular mass distribution of the proteins under non-dissociative, non-reducing conditions (Figure 5.2). For ASPI, the UV 280 nm elution profile showed an intense void peak after 7 mL elution volume, followed by a broad pattern of eluting material with only one other distinct peak around 18 mL elution volume. This UV 280 nm elution profile suggests a highly diverse protein composition of ASPI with molecular masses ranging from < 50 kDa to > 440 kDa.

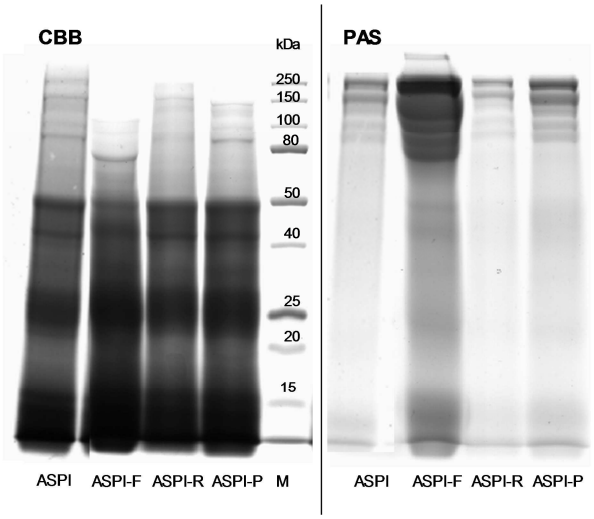

Figure 5.1 – SDS-PAGE gels stained by Coomassie brilliant blue (CBB) or Peroidic acid – Schiffs's reagent; protein concentration for all samples is 5 mg/mL ; ASPI = algae soluble protein isolate, ASPI-F = filtrate obtained after centrifugal fractionation of ASPI, ASPI-R = retentate obtained after centrifugal fractionation of ASPI, ASPI-P = pellet obtained after centrifugal fractionation of ASPI, M = molecular weight marker

Since the UV 280 elution profiles of ASPI-R and ASPI-P resembled the elution profile of ASPI, a protein composition similar to that of ASPI is expected. The elution profile of ASPI-F, in contrast, differs considerably. Protein elution is only detected after an elution volume of 15 mL, confirming that all proteins present in ASPI-F are smaller than 50 kDa. In combination with the SDS PAGE analysis these observations suggest that the polypeptides present in ASPI-R and ASPI-P, the two fractions retained by the 50 kDa MWCO membrane, are associated in quaternary protein structures bigger than 50 kDa, while in ASPI-F the same polypeptides are present in a dissociated form. In addition, clear differences in the RI signal during SEC analysis were observed between the different fractions (Figure 5.2, B-D). They will be discussed in detail in the following paragraph.

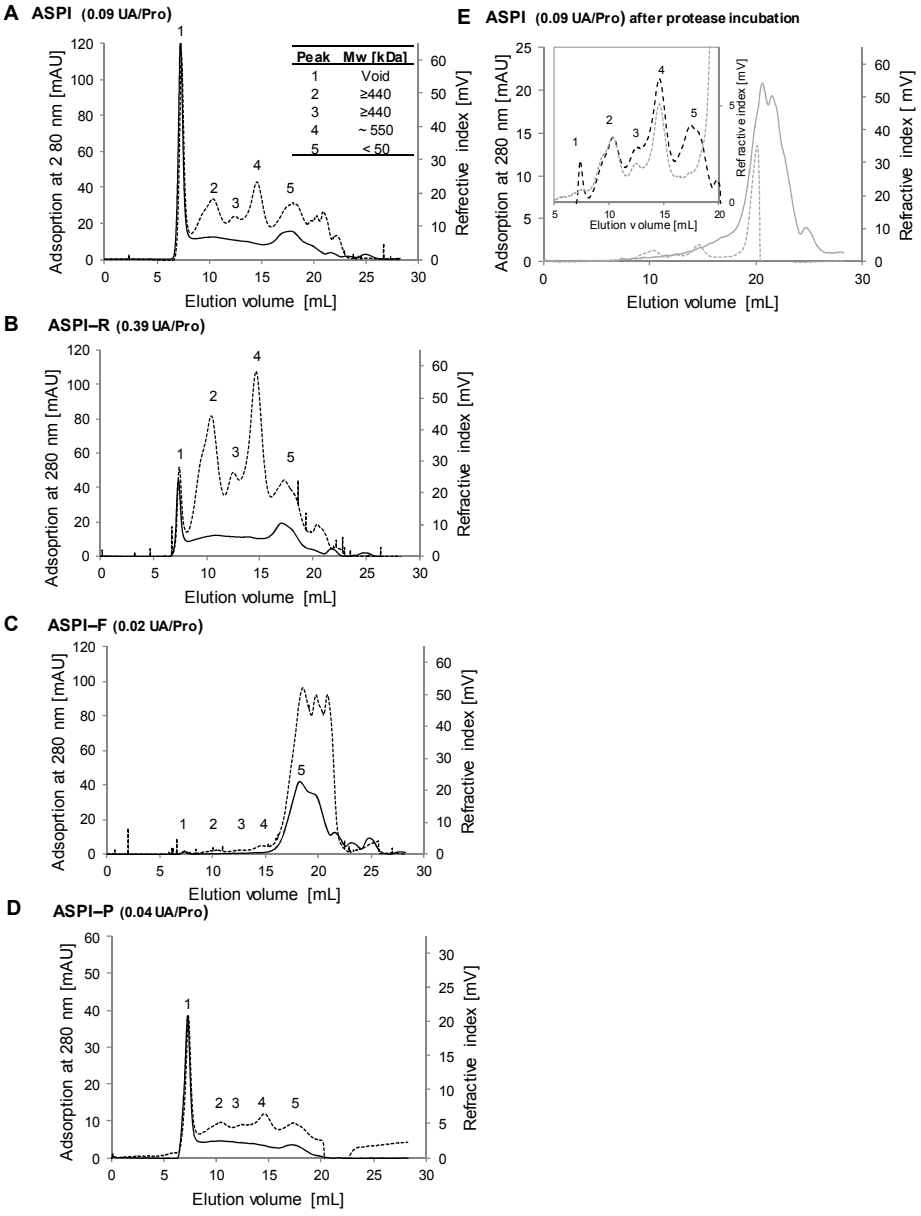

Figure 5.2 – SEC elution pattern of ASPI (before [A] and after protease incubation [E]), ASPI-R (B), ASPI-F (C) and ASPI-P (D) (continuous line: UV 280 nm signal; dashed line: RI signal); Insert table: Estimated molecular weights of five distinct RI peaks (1 - 5) observed; Insert figure: RI signal detected for ASPI before and after protease incubation between 5 and 20 mL elution volume; UA/Prot = uronic acid-to-protein ratio of the respective samples

5.3.1.2.1. Glycoproteins

As shown by PAS staining after SDS PAGE, part of the proteins present in ASPI and its fractions are present in the form of glycoproteins (Figure 5.1). In each fraction, similar glycoprotein bands were observed at comparable protein concentrations (5 mg/mL). However, ASPI-F showed a more pronounced stain intensity, despite its relatively low carbohydrate content. This suggests, that the glycoprotein fraction was accumulated in the filtrate passing through the 50 kDa MWCO membrane.

To confirm the presence of glycoproteins in ASPI, it was hydrolysed by Proteinase K. This protein has a broad specificity and has been used before for the degradation and analysis of glycoproteins (Bedi, 1997; Zauner et al., 2010). After hydrolysis, ASPI and the hydrolysate were analysed by SEC, with refractive index (RI) as well as UV 280 nm detection. Prior to enzyme incubation, the UV elution profile showed the broad pattern described above. For the RI elution profile, five distinct peaks with molecular masses ranging from > 440 kDa to < 50 kDa (peaks 1 – 5, Figure 5.2) were identified. After enzyme incubation, successful protein hydrolysis was shown by the presence of one single peak in the UV 280 nm elution profile at an elution volume of 20 mL. The RI elution profile of the hydrolysate, however, resembled the elution profile of the untreated ASPI except for the peak with the smallest molecular mass (peak 5, < 50 kDa). These observations show, that ASPI contains carbohydrates in form of polysaccharides (peaks 1 – 4) as well as glycoproteins (peak 5). The enrichment of ASPI-R in charged polysaccharides, already shown by sugar analysis, is also visible in the intense RI signal of peaks 1 - 4. The presence of peak 5 in RI elution profile of ASPI-F strengthens the hypothesis, that in this fractions a small glycoprotein fraction with a molecular mass > 50 kDa is accumulated. Since the 9% (w/w) carbohydrates remaining in ASPI-F are mainly composed of neutral sugars, it is suggested that the polysaccharide moieties of this glycoprotein fraction are most probably composed of neutral monosugars with galactose as main building block (*sugar composition data not shown*).

5.3.2. Physico-chemical properties of the ASPI fractions

5.3.2.1. Effect of pH on adsorption behaviour of the different ASPI fractions to the air-water interface

To characterize the interfacial properties of ASPI, ASPI-R, ASPI-F and ASPI-P at the air-water interface at two different pH values (pH 5, pH 7), the surface pressure (Π) and the elastic modulus (E_d) were measured as function of time and plotted against each other (Figure 5.3). For ASPI and its fractions, this plot was not affected by varying pH, indicating that the composition of the protein layer was similar at both pH values tested. Consequently, an increase in kinetics of surface pressure development may be interpreted as an increase in adsorption kinetics.

For almost all samples, at both pH 5 and 7, the surface pressure (Π) increases directly from the start of the experiment. Only for ASPI-R, the fraction enriched in carbohydrates, a short lag-time of approximately 80 s is observed. At pH 7, Π increases rapidly to a maximum surface pressure of 16 mN/m at approximately t = 1000 s (Π_{max}). At pH 5, the adsorption of ASPI and ASPI-F results in an increased Π_{max} (21 mN/m), while for ASPI-R and ASPI-P, Π_{max} remained constant (16 mN/m). The interfacial properties of the different ASPI fractions suggest a significant contribution of the small molecular weight proteins and glycoproteins (≤ 50 kDa) accumulated in ASPI-F to the overall adsorption kinetics of ASPI.

5.3.2.2. Effect of pH on the stability of foams stabilized with the different ASPI fractions

The pH dependent foam stability of ASPI, ASPI-R, ASPI-F and ASPI-P stabilized foams (protein solution concentration 0.5 mg/mL) was determined from the foam life time $t_{5/6}$ in the pH range 3 – 7 at a NaCl concentration of 10 mM (Figure 5.4). While ASPI-F stabilized foams were considerably more stable in the pH range 5 – 7 than ASPI stabilized foams, foams stabilized with ASPI-R and ASPI-P were less stable. This observation was quite surprising, since the ASPI-F fraction contains less of the charged polysaccharides, which was previously shown to positively affect emulsion stability (Chapter 3). The high foam stability of ASPI-F may be due to the fact that it consists of dissociated subunits of the proteins present in the other fraction. Similar positive effects of dissociation of multi-subunit proteins has been described for soy and pea proteins (Subirade et al., 1992; Wagner & Guéguen, 1995). From pH 6 downwards, foam stabilities decreased for all fractions and

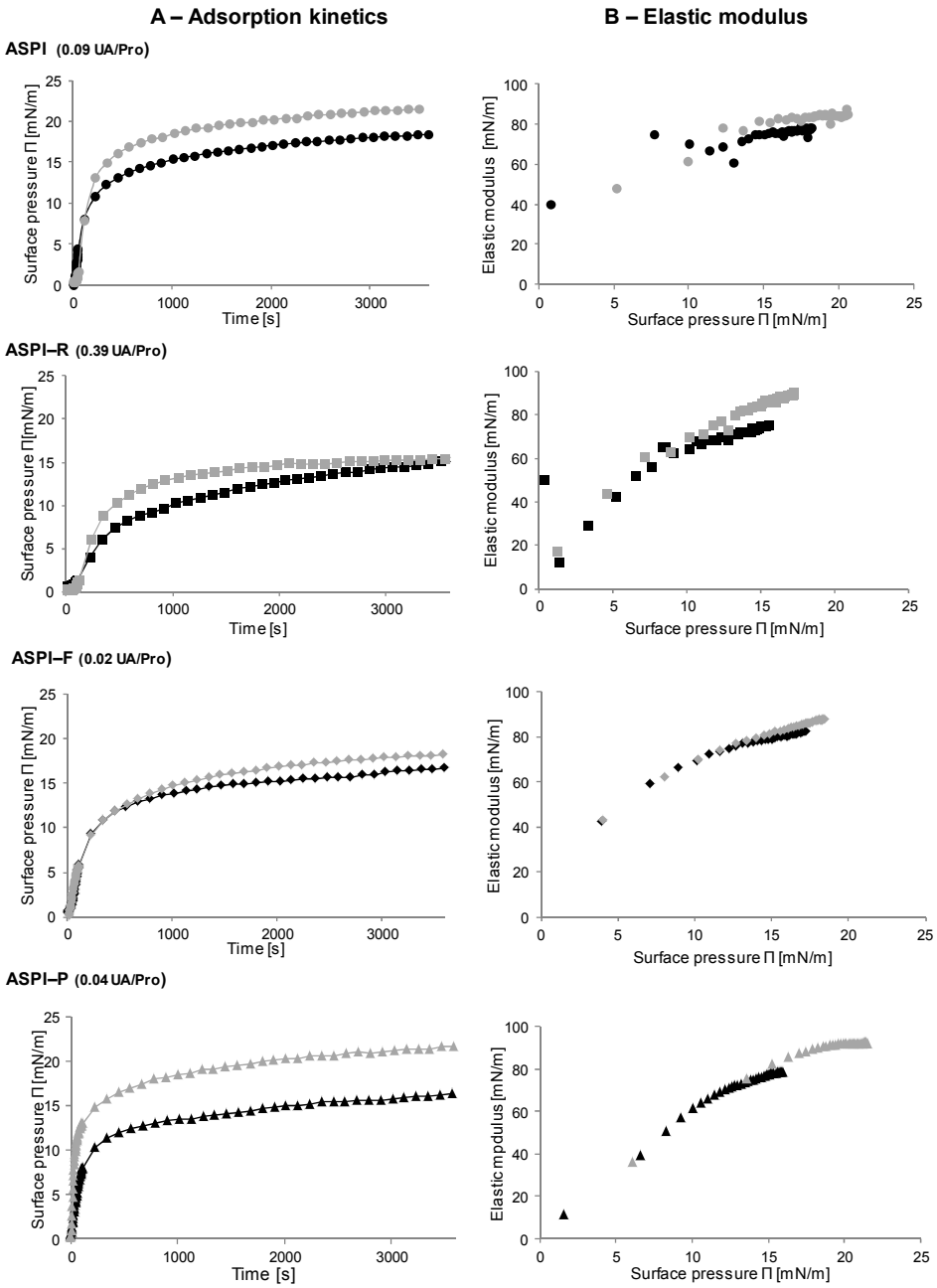

Figure 5.3 – A: Surface pressure at the air/water interface as function of time at pH 7 (black) and pH 5 (grey) (I = 10 mM NaCl) for ASPI, ASPI-R, ASPI-F and ASPI-P solutions at a concentration of 0.1 mg/mL; B: Elastic modulus [mN/m] as function of surface pressure [mN/m] of the same samples; UA/Prot = uronic acid-to-protein ratio of the respective samples

in the pH range 3 – 4 no stable foams can be produced with any of the three fractions. As suggested earlier (Chapter 4), the observed pH dependent foam stabilities are expected to correlate to pH dependent isolate solubility. Since ASPI-R, the fraction enriched in polysaccharides, showed the lowest foam stabilities at all pH values tested, it can be assumed that the presence of polysaccharides at least partly corrupts the foam stability of ASPI.

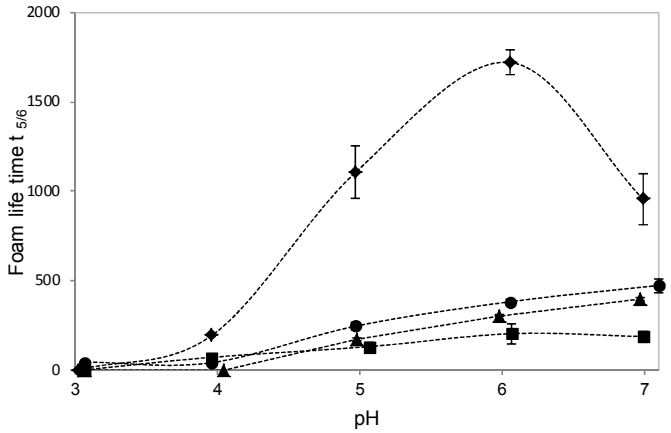

Figure 5.4 – pH dependent foam life time ($t_{5/6}$) of ASPI (●), ASPI-R (■), ASPI-F (♦) and ASPI-P (▲) stabilized foams (I = 10 mM) at an initial isolation concentration of 0.5 mg/mL, error bars indicate standard deviation

5.3.2.3. Emulsifying ability

The emulsifying ability of the different ASPI fractions was evaluated by comparing the volume-surface-average droplet size (d_{32}) of their respective 10% (w/w) oil-in-water-emulsions at isolate concentrations of 10 mg/mL at pH 7. At the given conditions the average droplet sizes of all tested emulsions are smaller than 1 µm and the emulsions stable against droplet aggregation (Figure 5.5B).

5.3.2.4. Effect of pH on the droplet ζ-potential in emulsion stabilized with the different ASPI fractions

The droplet ζ-potentials of 10% (w/w) oil-in-water emulsions stabilized with 1% (w/w) ASPI, ASPI-R, ASPI-F or ASPI-P were determined as function of pH in the pH range 3 – 7 (Figure 5.5A). At pH 7, all samples showed negative droplet ζ-potentials between -58 mV

and –33 mV. At lower pH values the droplet ζ-potentials of the different fractions diverge. ASPI-R stabilized emulsions maintained their negative droplet ζ-potential over the whole pH range investigated. For ASPI and ASPI-F stabilized emulsions, the droplet ζ-potentials converge to zero around pH 3. Lastly, for ASPI-P stabilized emulsions the droplet ζ-potential changes from negative to positive values at pH.

Based on the high uronic acid contents of ASPI and ASPI-R, the negative droplet ζ-potential in the pH range 3 – 7 can be explained by the presence of anionic polysaccharides fractions. Above the pK_a of their uronic acid building blocks (~ pH 3) the anionic polysaccharides contribute sufficient charge to the droplet surface to retain a negative droplet ζ-potential. Indeed, the influence is most pronounced in ASPI-R, the fraction with the highest uronic acid content. The different droplet ζ-potentials of ASPI-P and ASPI-F, two fractions containing hardly any uronic acids, are most probably caused by the different protein contents of the two fractions (72% [w/w] and 44% [w/w], respectively). In addition, ASPI-P contained higher amounts of the two charged amino acids arginine and histidine compared to ASPI-F (Table 5.2). Therefore, the droplet ζ-potentials of ASPI-P showed a behaviour more typical for proteins.

5.3.2.5. Effect of pH on droplet aggregation in emulsions stabilized with the different ASPI fractions

Occurrence and extent of pH induced droplet aggregation in ASPI, ASPI-R, ASPI-F and ASPI-P stabilized emulsions were examined in the pH range 3 – 7 at a NaCl concentration of 10 mM. Droplet aggregation was characterized by the volume surface average droplet size (d_{32}) using DLS and by the decay time $\tau_{1/2}$, an indication for droplet mobility obtained from DWS (Figure 5.5A). For all samples tested, these two parameters showed the same pH dependent behaviour. In ASPI stabilized emulsions, the d_{32} as well as $\tau_{1/2}$ increased significantly around the isoelectric point of the isolate (~ pH 3), which agrees with results obtained in a previously isolated ASPI (Schwenzfeier et al., 2013). Light microscopy was used to confirm that the observed increase in d_{32} and $\tau_{1/2}$ was caused by flocculation, not by coalescence (*data not shown*).

Table 5.2 –Amino acid profile of ASPI and derived fractions obtained by centrifugal membrane filtration (g per 100 g protein), parentheses: according standard deviations

	ILE	LEU	VAL	LYS	PHE	TYR	MET	CYS	THR
ASPI	4.98 (0.02)	8.43 (0.08)	6.22 (0.09)	5.66 (0.06)	5.33 (0.01)	4.37 (0.06)	2.66 (0.08)	1.95 (0.04)	5.86 (0.02)
ASPI-R	4.82 (0.08)	8.05 (0.02)	5.94 (0.14)	5.72 (0.04)	5.02 (0.03)	3.49 (0.04)	2.73 (0.02)	1.86 (0.08)	8.17 (0.04)
ASPI-F	5.21 (0.02)	8.68 (0.12)	6.99 (0.04)	5.56 (0.21)	4.36 (0.01)	3.85 (0.06)	2.85 (0.02)	1.51 (0.06)	5.84 (0.02)
ASPI-P	4.72 (0.17)	8.35 (0.01)	5.94 (0.14)	5.47 (0.07)	5.73 (0.01)	4.87 (0.08)	3.08 (0.01)	2.15 (0.00)	5.47 (0.08)
	ALA	**ARG**	**ASX[a]**	**GLX[a]**	**GLY**	**HIS**	**PRO**	**SER**	**TRP**
ASPI	6.80 (0.01)	6.06 (0.01)	10.68 (0.01)	12.42 (0.05)	5.51 (0.01)	2.23 (0.02)	4.24 (0.02)	4.47 (0.05)	2.12 (0.21)
ASPI-R	7.30 (0.02)	5.21 (0.08)	10.57 (0.13)	13.18 (0.15)	5.25 (0.01)	1.92 (0.03)	4.72 (0.01)	4.60 (0.01)	1.44 (0.27)
ASPI-F	7.63 (0.11)	3.24 (0.17)	11.72 (0.01)	13.95 (0.06)	5.76 (0.05)	1.77 (0.02)	4.61 (0.02)	5.01 (0.10)	1.47 (0.08)
ASPI-P	6.60 (0.00)	6.70 (0.02)	10.40 (0.10)	11.76 (0.02)	5.52 (0.01)	2.32 (0.02)	4.12 (0.02)	4.52 (0.13)	2.27 (0.06)

[a]No separate analysis of ASP/ASN and GLU/GLN

For ASPI-R stabilized emulsions, even at pH 4 no droplet aggregation was observed and at pH 3 d_{32} and $\tau_{1/2}$ only increased slightly. This corresponds with the high negative ζ-potentials observed for ASPI-R at these pH values. Like ASPI-R, the use of the naturally occurring protein-polysaccharide hybrid GA also enabled the production of stable emulsions over broad pH range (Chapter 3). For GA, however, it was shown that a 5 times higher concentration (50 mg/mL) was needed to obtain stable emulsions (Chapter 3). Even then, the droplet size of GA emulsions was 25 μm, while at 10 mg/mL concentration ASPI-R already made stable emulsions with droplet sizes of 1 μm. The better emulsifying capacity of ASPI-R is attributed to its higher protein content (31% [w/w]). For emulsions stabilized with ASPI-F and ASPI-P, the two fractions with decreased uronic acid-to-protein ratios compared to ASPI, droplet aggregation was observed in the pH range 3 – 6. For ASPI-F as well as for ASPI-P stabilized emulsions, d_{32} and $\tau_{1/2}$ clearly increased from pH 6 downwards. For both isolates, maximum d_{32} and $\tau_{1/2}$ values were observed at the pH values corresponding to a zero droplet ζ-potential (for ASPI-F ~ pH 3, for ASPI-P ~ pH 4).

For ASPI and derived fractions, stability against droplet aggregation was always observed at absolute droplet ζ-potentials ≥ 33 mV (indicated in grey in Figure 5.5B). In contrast, for emulsions stabilized with whey protein isolate (WPI) stability against droplet aggregation was already observed at absolute droplet ζ-potentials of ≥ 20 (e.g. Kulmyrzaev & Schubert, 2004; Schwenzfeier et al., 2013). For emulsions stabilized by β-lactoglobulin – pectin adsorbed layers, it was shown, that droplet ζ-potentials of -20 mV and lower were also insufficient to stabilize the individual droplets against aggregation in the pH range 4 – 6 (Moreau et al., 2003). The authors suggested, that in this pH range the interactions of the pectin molecules with the protein covered droplet surfaces became weaker due to a reduced positive charge of the proteins. The resulting partial desorption of pectin from the droplet surface caused the emulsions to flocculate at net droplet ζ-potentials ≤ -20 mV. A similar explanation might apply for ASPI and ASPI-R, the two fractions containing charged polysaccharides, which can associate with the proteins at emulsions droplets. Since for F-ASPI and P-ASPI, the two fractions hardly containing any uronic acids, droplet aggregation was also observed at ζ-potentials ≤ -20 mV, the co-adsorption of polysaccharide moieties in form of glycoproteins is likely.

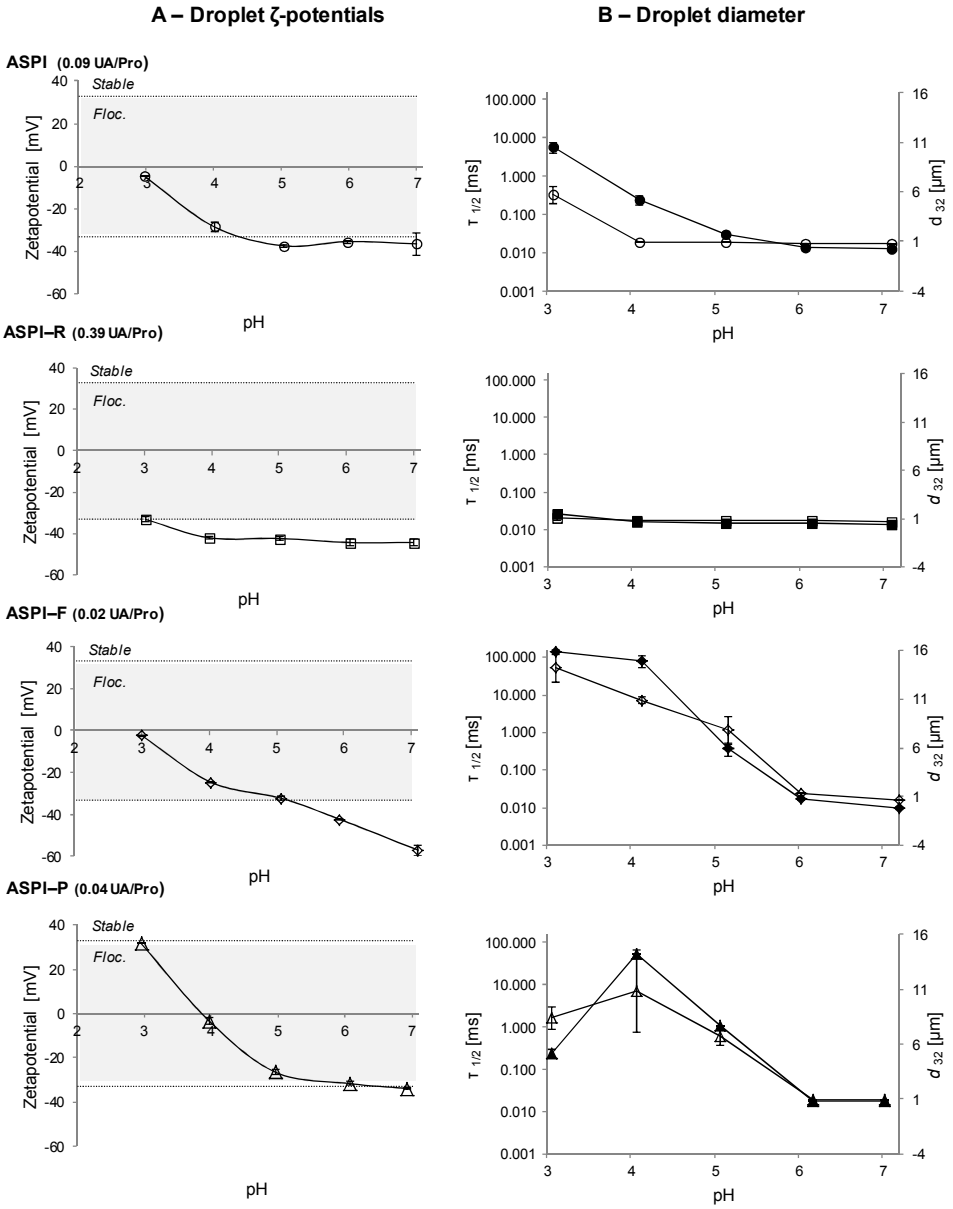

Figure 5.5 – A: pH dependent droplet ζ-potential of 10% (w/w) oil-in-water emulsions stabilized with 1% (w/w) ASPI (●),ASPI-R (■), ASPI-F (♦) and ASPI-P (▲) (all I = 10 mM), dashed lines indicate droplet ζ-potentials of -33 mV and 33 mV, within this interval flocculation was observed (grey area); B: Decay time $\tau_{1/2}$ [ms] (filled symbols) and average droplet diameter d_{32} (open symbols) of the same samples as function of pH, error bars indicate standard deviations; UA/Prot = uronic acid-to-protein ratio of the respective samples

While foams stabilized with ASPI-F are more stable than foams stabilized with the original ASPI at pH 5, it is not possible to produce stable emulsions at this pH using F-ASPI as emulsifier instead of ASPI, most probably due to the low content of charged polysaccharide moieties in this fraction. Hereby, the results of this study confirm, that the emulsion properties of ASPI are determined by the co-adsorption of a charged polysaccharide fraction to the droplet surface. Based on droplet ζ-potential measurements and corresponding particle size distributions, it is likely that at least part of charged polysaccharide moieties co-adsorb in form of glycoproteins. The foam properties of ASPI, in contrast, seems to be determined by the fast adsorption of the dissociated proteins and small glycoproteins containing mainly neutral sugar moieties. The highly heterogeneous composition of ASPI seems to support the production of very stable emulsions and foams in the pH range 5 – 7.

5.4. Conclusions

Compared to ASPI, increased amounts of charged polysaccharide moieties ensured electrostatic repulsion between the individual emulsion droplets and, therefore, emulsion stability over a broader pH range (4 – 7). Based on droplet ζ-potential measurements and corresponding particle size distributions, it is likely that at least part of these charged polysaccharide moieties co-adsorb in form of glycoproteins. The foam properties of ASPI, in contrast, are determined by the fast adsorption of the dissociated proteins and small glycoproteins containing mainly neutral sugar moieties. The involvement of different glycoproteins as part of the highly heterogeneous composition of ASPI seem to contribute considerably to high emulsions and foam stability in the pH range 5 – 7.

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6 General Discussion

As described in the General Introduction of this thesis, the extraction of high-value proteins for techno-functional applications in foods is expected to contribute considerably to the commercial value of microalgae biomass. This expectation is based on the assumption that 10% (w/w) of the algae biomass could be utilized as high-value protein fraction for techno-functional applications in foods. In spite of the indicated importance of high-value proteins within an algae biorefinery concept, no adequate studies of protein compositions and resulting techno-functional properties were available at the start of this project. Therefore, the main aim of this research was to provide knowledge about the physico-chemical and techno-functional properties of microalgae proteins. As a first step towards achieving this aim an isolation protocol for algae proteins was developed using the organism *Tetraselmis* sp. Subsequently, the techno-functional quality of the algae soluble protein isolate (ASPI) obtained was assessed in reference to the techno-functional properties of currently used food proteins (e.g. whey protein isolate [WPI]).

In Chapter 2 of this thesis it was shown, that ASPI was readily isolated from *Tetraselmis* sp. free from any intense colour by the use of a simple, mild and scalable isolation process. In its final form, the techno-functional properties of ASPI were comparable or superior to those of currently used animal-derived proteins (e.g. WPI, egg white protein) with respect to emulsion and foam stability. As described in Chapters 3 – 5, the complex, heterogeneous composition of ASPI contributes positively to its techno-functional properties. Due to the combination of the positive interfacial properties of its protein fraction with the broad pH stability of its charged polysaccharide fraction, ASPI possesses the positive attributes of two types of techno-functional ingredients. The results of Chapters 2 - 5 indicated the large potential of ASPI obtained from *Tetraselmis* sp. as techno-functional ingredient in foods. Still, the questions remain if *Tetraselmis* sp. can be considered a representative of green microalgae in general and in which way the insoluble proteins present in the pellet fraction after bead milling could be utilized. These questions, as well as a discussion on the compositional analysis of microalgae protein isolates and the study of their techno-functional properties will be covered in this chapter.

6.1. *Tetraselmis* sp. as representative of green microalgae

For the research described in this study, ASPI needed to be produced on a preparative scale. Since the production capacities of available research photobioreactors were insufficient to produce microalgae biomass in adequate amounts, the commercially available green microalgae *Tetraselmis* sp. was used for the preparative isolation of microalgae protein.

With its strong cell wall and high chlorophyll content (Brown, 1991) *Tetraselmis* sp. possesses two main physiological characteristics typically hindering the isolation of proteins from green microalgae (Becker, 2007; Davies, 1971; Doucha & Lívanský, 2008). Therefore, it is expected that the mild isolation protocol developed for the isolation of soluble protein from *Tetraselmis* sp. (Chapter 2) can also be applied for other green microalgae. This was tested by bead milling (as described in Chapter 2) the cells of *Chlorella vulgaris*. Intact *Chlorella* cells are considerably smaller (4 µm) than *Tetraselmis* sp. cells (10 µm) (Figure 6.1). Typically, smaller cells are more difficult to disintegrate. Still, by using particle size analysis and light microscopy it could be shown (Figure 6.1), that the cells of *Chlorella vulgaris* were successfully disintegrated by bead milling.

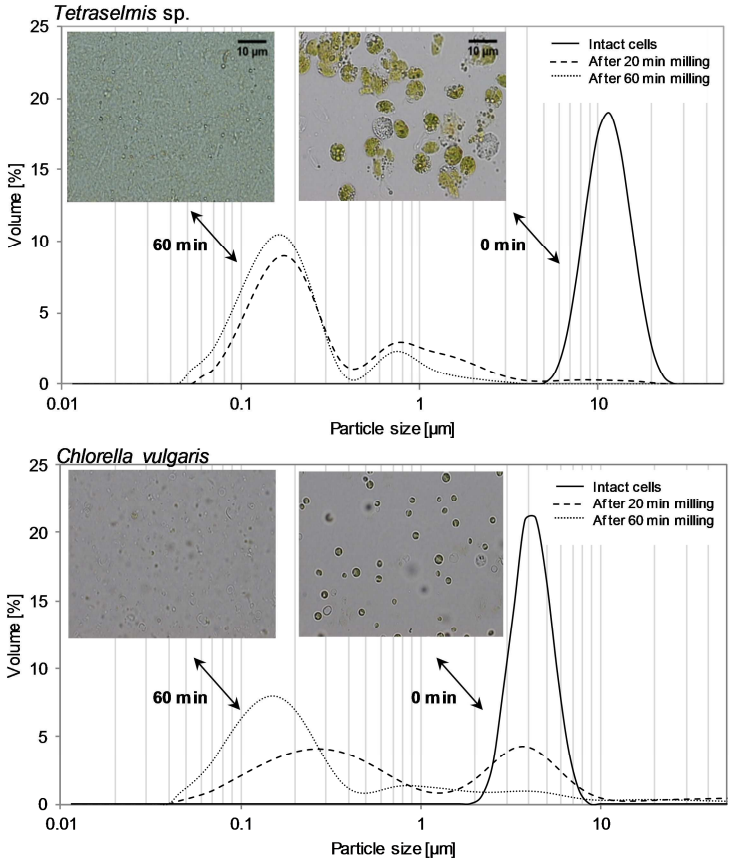

Figure 6.1 – Particle size distribution by dynamic light scattering of *Tetraselmis* sp. and *Chlorella vulgaris* suspensions (~100 g/L) after 0 (-), 20 (----) and 60 (···) min bead milling with the Dynomill equipment; Insert: Microscopic images of the same samples before (0 min) and after bead milling (60 min)

In addition, the protein composition of ASPI obtained from *Tetraselmis* sp. as determined with SDS-PAGE analysis (Figure 2.1) was found to be similar to those of cellular extracts obtained after physical cell disintegration from *Chlamydomonas reinhardtii* (Kuchitsu et al., 1988), *Chlorella vulgaris* (Villarejo et al., 1998) and *Haematococcus pluvialis* (Wang et al., 2004). While the molecular details of these proteins may differ, it is expected that they still share common properties. For protein isolates from different leaf species (tobacco, alfalfa, soy, sugar beet), for example, it has already been shown that they possess similar techno-functional properties (Sheen, 1991). A similar situation is observed for legumin seeds. In those seeds, typically an albumin and a globulin protein fraction are found. These proteins, obtained from a wide range of legumin seeds are found to have similar chemical and structural properties (Derbyshire et al., 1976; Marcone et al., 1998). Based on the above, it is concluded that the results presented for ASPI from *Tetraselmis* sp. give a good indication of the properties of protein isolates obtained from other green microalgae.

6.2. Extraction of microalgae protein

As described in Chapter 2, 21% of the total proteinaceous material present in *Tetraselmis* sp. was readily extractable in a soluble form after successful cell disintegration (Table 2.2). Half of this material consisted of low molecular weight components (e.g. peptides or free amino acids, inorganic nitrogen, nucleic acids) and could be removed by dialysis. The remaining fraction, defined as algae soluble protein isolate (ASPI), contained high molecular weight proteins. The 79% protein that remained in the pellet fraction after bead milling and subsequent centrifugation are expected to contain only a small fraction of proteins insoluble by nature (e.g. membrane proteins). The majority of those proteins, however, are expected to be associated with the insoluble cell wall debris or to be entrapped in cell organelles still intact after cell disintegration. For Rubisco, for example, it is known that in microalgae this soluble protein is stored in pyrenoids (starch covered cell organelles).

6.2.1. Extraction of microalgal Rubisco

Ribulose biphosphate carboxylase/oxygenase (Rubisco) is the most abundant protein in leaves (30 - 50% of the soluble cellular protein) and, most probably, also in algae (Ellis, 1979). While Rubisco is easily identified as the major protein fractions in cytoplasmic leaf extracts (e.g. Ranty & Cavalie, 1982), it could not be identified as the major fraction in ASPI. Comparison of SDS-PAGE results of ASPI (Figure 2.1) and other microalgae cell

extracts (e.g. Kuchitsu et al., 1988; Wang et al., 2004) showed, that next to the two polypeptide bands representing Rubisco (55 and 15 kDa), also other intense polypeptide bands occurred. The reason for the moderate concentration of microalgal Rubisco in soluble cell extracts is its localization in pyrenoids. Under all growth conditions, those starch granule-like cell organelles contain the majority of the Rubisco present in microalgae (Borkhsenious et al., 1998; Kuchitsu et al., 1988). If the starch sheath surrounding the pyrenoid (Griffiths, 1970) is not affected by the applied cell disintegration method, the pyrenoid and the associated Rubisco are discarded in the insoluble cell debris fraction.

The yield of Rubisco may be improved by inducing release of Rubisco from the pyrenoid in the living microalgae. During cell growth, the release of pyrenoid-bound Rubisco into the cytoplasm could be achieved by applying specific culture conditions. The development of pyrenoids and their starch sheaths in green microalgae is more pronounced at low environmental CO_2 concentrations than at high ones (Tsuzuki et al., 1986). In addition, the structure of pyrenoids can be influenced by applying an alternating dark/light regime. Typically, the decrease in total starch content in algae kept in the dark is associated with a decreased starch sheath around the pyrenoid (Griffiths, 1970). Since algae from the genus *Tetraselmis* are known to contain pyrenoids (Hori et al., 1982), in this study the influence of light intensity on the protein extractability was tested. The chemical compositions of *Tetraselmis* sp. harvested after continuous illumination (55 µmol/[s*m^2] inside the reactor) and harvested after a dark period of an alternating light/dark regime of 16 and 8 hours, respectively, were compared. In both cultures, CO_2 was applied continuously to control the pH.

Table 6.1 – Total and extractable protein content (w/w%), soluble protein yield (%) and starch content (w/w%) of *Tetraselmis* sp. biomass harvested after continues illumination and after a dark period of an alternating of an alternating light/dark regime; parentheses according standard deviation

Cultivation conditions	Total protein content (w/w%)	Extractable soluble protein (w/w%)	Soluble protein yield (%)	Starch content (w/w%)
Continuous illumination	29 (6)	6 (n.d.)	21	11(2)
After dark period	29 (4)	4 (n.d.)	14	13 (1)

n.d. = not determined

As shown in table 6.1, comparable amounts of protein (29% [w/w]) and starch (11 - 13% [w/w]) were found in both cell cultures after harvesting. From the algae cells grown under continuous illumination 21% of the total protein present in the microalgae were present in a

soluble form after cell disintegration. From the cells harvested after a dark period only 14% of the total protein could be isolated in a soluble form. So, although illumination has been identified as important parameter for pyrenoid development, the implementation of dark period during cell growth does not increase extractability of protein in general or Rubisco in particular. In contrast, a lower soluble protein yield was observed after a dark period.

Another possible approach for the release of pyrenoid-bound Rubisco is the enzymatic degradation of the pyrenoid surrounding starch sheath. In a preliminary study during this thesis, the pellet fraction obtained after bead milling (Chapter 2) was incubated with α-amylase (Termamyl, Novozymes) for 30 min at 80°C. This temperature was expected to be sufficient for starch gelatinization, since for the stroma and the pyrenoid starch of *Chlorella kessleri* gelatinization temperatures of 75 °C and 67 °C have been reported, respectively (Izumo et al., 2007). Nevertheless, after incubation of the *Tetraselmis* sp. pellet fraction neither an increase in reducing sugars (analysed by PAHBAH assay) nor in soluble protein (analysed by BCA assay) was observed, indicating that the starch granules and pyrenoids present were not affect by the α-amylase-treatment.

6.2.2. Extraction of cell wall debris bound protein

Next to the pyrenoid-embedded Rubisco, part of the protein present in the pellet fraction might be unavailable for extraction, because it is part of the insoluble cell wall debris. For some red and green seaweeds the use of polysaccharidases has been shown to facilitate protein extraction (Fleurence, 1999). In this thesis, a mild heat treatment (30 min, 80°C) and subsequent incubation with a broad spectrum of cell wall degrading enzymes (pectinases: Rapidase Liq^{+} [DSM], Pectinex Ultra SP-L [Novozymes]; xylanases: Shearzyme 500L, Viscozyme Rye [both Novozymes]; β-glucanases: Ultraflo L [Novozymes]; cellulases: Accelerase [Danisco], Celluclast [Novozymes]) was applied for the pellet fraction obtained after bead milling described in Chapter 2. After the initial heat treatment 16% of the proteins initially present in the insoluble pellet fraction were solubilized. The subsequent incubation with commercially available pectinases or xylanases resulted in a two to three times higher release of soluble proteins (31 - 53% of the total insoluble protein) compared to the sole heat treatment. Without the initial heat treatment, however, no considerable pectinase or xylanase activity could be observed. Even after the heat treatment, the application of cellulases and β-glucanases showed no significant increase in soluble protein. These results indicate that the application of the tested commercial pectinases and xylanases preparations during the isolation process described in

Chapter 2 could potentially increase the yield of soluble protein. However, the preliminary experiments described above need to be repeated in order to valid the high yields of soluble protein after a heat treatment and subsequent pectinase or xylanase incubation. In addition, it needs to be investigated to what extend the increase in soluble protein after enzyme incubation is caused by possible proteolytic side activities of the commercial enzyme preparations.

Nevertheless, the application of specific polysaccharidases provided some preliminary information about the cell wall composition of *Tetraselmis* sp. The successful solubilisation of initially bound protein by the use of pectinases indicates the partial breakdown of a pectin-like structure present in the pellet fraction. This finding is consistent with the molar sugar composition of the pellet fraction (Table 2.2), which shows that the pellet is composed for 38 mol% of uronic acids, the building blocks of charged polysaccharides. In agreement with earlier reported neutral sugar compositions of different *Tetraselmis* species (Brown, 1991), the sugar composition of the pellet fraction given in Table 2.2 suggests that glucose (23 mol%) and galactose (30 mol%) provide the main building blocks for neutral parts of the cell wall. Nevertheless, an incubation of the pellet fraction with cellulases did not result in an increase in soluble protein.

Although interesting from an analytical point of view, the recovery of the soluble, but not readily extractable protein fraction from microalgae might not be profitable. Firstly, additional energy is needed to liberate the extracted soluble proteins. Secondly, the techniques needed to release them may in fact require conditions that negatively affect the protein quality. Lastly, if microalgae are produced on a large enough scale, e.g. for biofuel production, the recovery of the 10% easily extractable soluble proteins as ASPI may already yield sufficient material to cover the current and future demands for high-value food protein isolates.

6.3. Fractionation of ASPI

As described in Chapter 2 most of the bulk impurities (e.g. residual chlorophyll, neutral carbohydrates) present in the *Tetraselmis* sp. cell lysate after bead milling were removed by a batch anion-exchange process and acid precipitation. Due to the binding of the proteins to the anion-exchange material a chromatographic fractionation of ASPI rather than a fractionation by membrane filtration (Chapter 5) might have been expected. Indeed, gradient anion exchange chromatography was tested as a possible method for the

fractionation of proteins present in the crude ASPI. As shown in figure 6.3, however, the UV 280 nm elution profile showed a broad pattern of eluting material without any distinct individual peaks. This result shows, that the large heterogeneity of proteins present in ASPI (as indicated in Chapter 5) hinders effective chromatographic fractionation. In order to obtain pure protein fractions, multiply chromatographic steps would have to be applied. Hence, filtration was used to obtain fractions of ASPI.

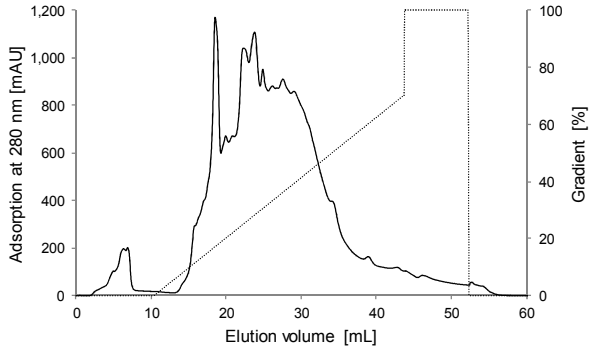

Figure 6.3 – Anion exchange chromatography elution pattern of crude ASPI (continuous line: UV 280 nm signal; dashed line: Applied gradient of 1M NaCl in 50 mM Tris/HCl buffer, pH 8)

6.4. The chemical analysis of algae biomass, ASPI and its fractions

In order to evaluate the isolation process described in Chapter 2, a complete mass balance (of dry matter and of individual component classes) was made. Prior to isolation, proteinaceous material, carbohydrates and lipids (determined as $CHCl_3$/MeOH soluble material) accounted together for 79% of the *Tetraselmis* sp. biomass (Table 6.2). Additional 15% (w/w) of the biomass were identified as ash (Table 2.1), which agrees with previously reported ash contents of ~15% (w/w) for different green microalgae (Renaud et al., 1999). From the total mass, however, still a residual 6% (w/w) of the dry matter of the *Tetraselmis* sp. biomass remained unidentified.

Also in earlier reported gross chemical compositional analyses of different microalgae, a part of the dry weight of the samples could not be identified. Even after correction for the high contents of ash in the microalgae biomass the residual unidentified mass was 20 - 50% (w/w) in the reported cases (Table 6.2). Despite this common observation, hardly any suggestions have been made for the origin or composition of the residual material. The results of the studies summarized in table 6.2 suggest that the analytical methods applied

could potentially be insufficient for a complete determination of the gross chemical composition (protein, carbohydrates, lipids) of microalgae biomass.

Table 6.2 – Gross chemical composition of different microalgae species and ASPI (in w/w%)

Species	Reference	Protein	Carbohydrates	Lipids	Ash	Residual
Tetraselmis sp.	This thesis	36[a]	24	19	15	6
Tetraselmis chui	Brown, 1990	31[a]	12	17	n.d	40
Tetraselmis sp.	Renoud, 1999	30[b]	8	13	17	32
Dunaliella tetriolecta	Brown, 1990	20[a]	12	15	n.d.	53
Scendesmus obliquus	Becker, 2007	53[b]	14	13	n.d.	20
Nannochloris salina	Ben-Amotz, 1987	23[c]	24	15	n.d.	38[d]
Chlorella stigmatophora	Ben Amotz, 1987	31[c]	21	20	n.d.	28[d]
ASPI (dialysed)	This thesis	54[a]	16	17	n.d.	13

[a] Determined from amino acid composition
[b] Determined with N*6.25
[c] Determined with Lowry method after incubation with 1 N NaOH (1h, 100 °C)
[d] Residual determined from ash free dry weight after ashing at 540°C
n.d. = not determined

Moreover, even the isolated and dialysed soluble proteins (ASPI) still contained 13% w/w residual unidentified material. Further fractionation of ASPI by centrifugal membrane filtration (Chapter 5) seems to result in an accumulation of these unidentified components in both the retentate (ASPI-R) and the filtrate (ASPI-F). Here 31% (w/w) and 41% (w/w) of total mass of ASPI-R and ASPI-F, respectively, remained unidentified (Table 5.1).

To obtain an indication of the origin of the residual unidentified components, the protein contents of ASPI and derived fractions, determined from their respective amino acid contents, were compared to the respective total nitrogen contents, obtained from DUMAS. As explained in Chapter 2, this approach provides an indication on the amount of non-proteinaceous nitrogen. As shown by the high proteinaceous-to-total nitrogen ratios (N_{AA}/N_T) (Table 6.3), almost all nitrogen present in ASPI can be assigned to protein. In addition, the absence of non-protein nitrogen (NPN) substances, such as DNA and chlorophyll, is indicated by the fact, that k_p is located within the calculated interval for k_a. The subsequent fractionation of ASPI resulted in a slight decrease of proteinaceous nitrogen for ASPI-R and ASPI-F indicating the accumulation of residual NPN present in ASPI in these two fractions. It is, however, unlikely that this NPN fraction accounts for 30 - 40% (w/w) of the total mass of ASPI-R and ASPI-F.

Table 6.3 – Proteinaceous nitrogen and nitrogen-to-protein conversion factors k_a and k_p for ASPI and subsequently obtained fractions by centrifugal membrane filtration

Fraction	N_{AA}/N_T[a,b] [%]	N-Prot factor k_p	N-Prot factor k_a[c]
ASPI	$88 < x < 103$	5.58	$5.40 < y < 6.33$
ASPI-R	$85 < x < 100$	5.43	$5.43 < y < 6.41$
ASPI-F	$80 < x < 96$	5.26	$5.50 < y < 6.60$
ASPI-P	$91 < x < 106$	5.69	$5.40 < y < 6.28$

[a] Proteinaceous nitrogen (N_{AA}) as proportion of total nitrogen (N_T)

[b] Lower limit represents theoretical value calculated with ASX/GLX = 100% ASP/GLU, upper limit calculated with ASX/GLX = 100% ASN/GLN

[c] Lower limit represents theoretical value calculated with ASX/GLX = 100% ASN/GLN, upper limit calculated with ASX/GLX = 100% ASP/GLU

Another possibility is, that not all carbohydrate material present could be identified and quantified completely during compositional analysis. Especially for ASPI-R, the fraction enriched in carbohydrates (Table 5.1), HPAEC-PAD analysis after acid hydrolysis (Figure 6.4) revealed the presence of peaks that could not be assigned to one of the used mono sugar, uronic acid or oligomer standards. Nevertheless, based on the elution profiles of these standards the unidentified peaks could be grouped into three classes (Figure 6.4, 1 – monomers, 2 oligomers or uronic acids, 3 – oligomers). The unidentified peaks classified as monomers (region 1, Figure 6.4) could possibly represent sugar-like moieties typically not detected in plant-derived material, but in some marine organisms (e.g mannitol in brown seaweeds, Dittami et al., 2011). The unidentified peaks classified as oligomers or uronic acids (regions 2 and 3, Figure 6.4) are expected to result from incomplete acid hydrolysis of the uronic acid and glycoprotein structures present in ASPI (as indicated in Chapter 5). For the animal-derived glycosaminoglycan heparin containing iduronic acid and glucuronic acid, it has been shown that both methanolysis with subsequent TFA hydrolysis and hydrochloric acid hydrolysis (1 M HCl) were not sufficient for the complete hydrolysis to monomeric residues (De Ruiter et al., 1992; Raedts, 2011). In addition, it has been shown that especially the *O*-glycosidic linkages of glycoproteins present in microalgae are difficult to cleave (Miller et al., 1972; Sommer-Knudsen et al., 1998). Although those hydroxyproline-rich glycoproteins have been assigned as part of the insoluble cell wall, it is still likely that also glycoproteins present in ASPI contribute to the incomplete chemical analysis.

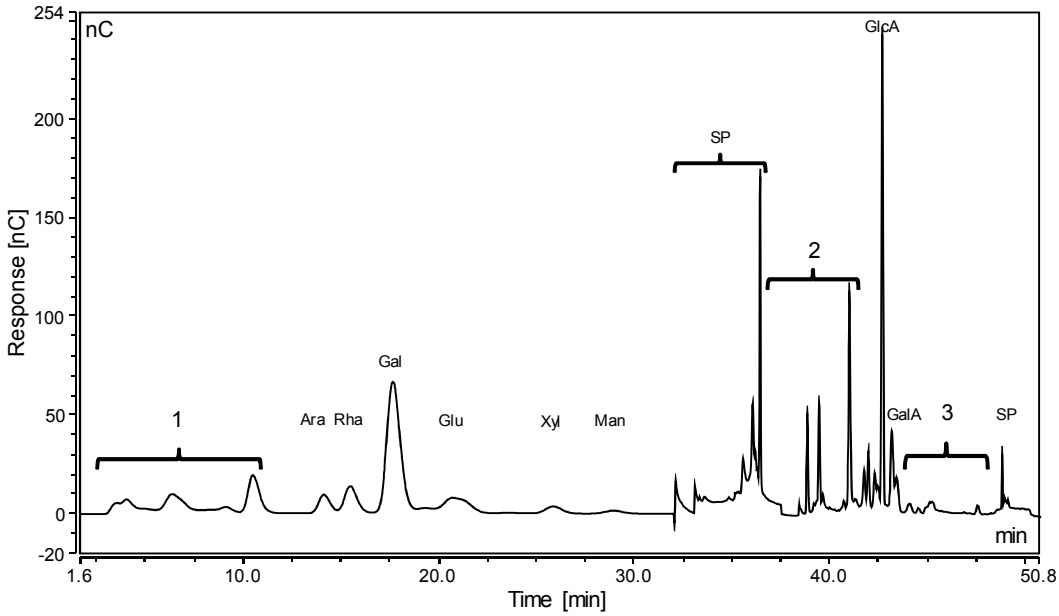

Figure 6.4 – HPAEC PAD elution pattern of ASPI-R after methanolysis with subsequent TFA hydrolysis, 1 - unidentified peaks of monomeric characteristic, 2 - unidentified peaks of oligomeric or uronic acid characteristics, 3- unidentified peaks of uronic acid characteristic, SP – system peaks

6.5. Techno-functional properties of ASPI

To assess the quality of ASPI, its techno-functional properties were investigated in reference to the currently used high-value protein isolates gum arabic (GA), whey protein isolate (WPI) and egg white albumin (EWA) (Chapters 3 and 4). Except for GA those reference isolates were highly purified protein products composed mainly of a single dominant protein (WPI ≥ 75% (w/w) β-lactoglobulin, EWA ~ 60% (w/w) ovalbumin). The results of this study have shown, that the emulsion and foaming properties of ASPI, a complex, heterogeneous mixture of soluble proteins, polysaccharides and glycoproteins, are comparable or superior to those of the previously mentioned more homogenous food protein isolates (Chapters 3 and 4).

6.5.1. Interfacial composition in ASPI stabilized foams and emulsions

While in the homogenously composed WPI and EWA mainly one protein is available for the adsorption to both the oil-water interface (o/w interface) and the air-water (a/w interface), ASPI provides several types of molecules for the occupation of the two types of

interfaces. From dilatational elasticity measurements of ASPI stabilized a/w-interfaces (Figure 4.5), it was concluded that the adsorbed layer is built up of proteins. From ζ-potential measurements of ASPI stabilized emulsion droplets (Figure 3.2), however, an indication was obtained that the negatively charged polysaccharide fraction present in ASPI co-adsorbs to the oil-water interface. In the emulsions, this formation of protein-polysaccharide complexes at the o/w-interface supports emulsion stability over a broad pH range and, with this, most probably adds to the emulsifying quality of the isolate. Although o/w-interfaces are not directly comparable to a/w-interfaces, it is still surprising that their interfacial compositions seem to differ so significantly under the same environmental conditions.

The major difference between the adsorbed layers in the emulsions (o/w-interfaces) and foams (a/w-interfaces) investigated in this research is the way in which the systems were produced. All emulsions described in Chapters 3 and 5 were produced at pH 7, close to the theoretical isoelectric point calculated for the protein fraction of ASPI (Chapter 3). At this pH, it is likely that mainly proteins adsorb to the newly formed o/w-interface (Figure 6.5A). After emulsion formation the pH was adjusted. Adjusting the pH of the mainly protein-stabilized emulsions to pH values below the protein-based isoelectric point of ASPI, but above the pK_a value of the charged polysaccharide present, results in the electrostatic complexation of the charged polysaccharides with the initially protein-covered o/w-interface (Figure 6.5B). All foams described in this thesis (Chapters 4 and 5) were formed from centrifuged solutions previously adjusted to the pH of interest. In this case, protein-polysaccharide complexes were already present during the formation of the a/w interfaces in the pH range 6 – 3. Nevertheless, the dilatational behaviour of ASPI stabilized a/w interfaces formed at pH 5 and pH 7 suggested the selective adsorption of protein at both pH values (Figures 4.5 and 5.3). Unlike in ASPI stabilized emulsions, the protein-polysaccharide complexes present in the bulk at pH 5 do not seem not interact with the adsorbed protein layer at the a/w interface (Figure 6.5D).

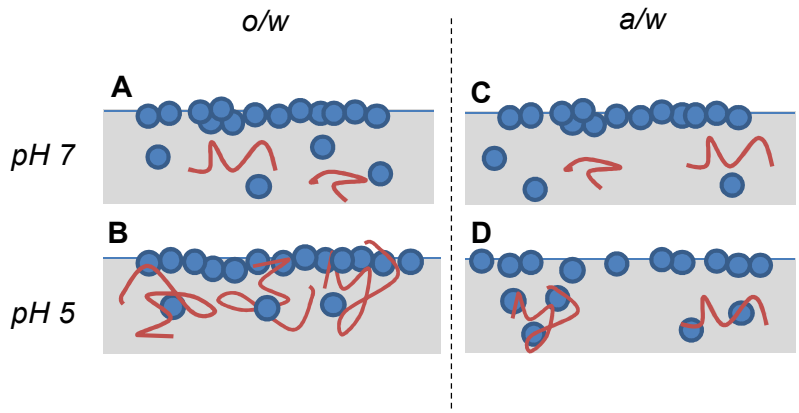

Figure 6.5 – Concept for the ASPI stabilized oil-water (o/w) and air-water (a/w) interfaces at pH 5 and pH 7 at low ionic strength, (A, C) Adsorbed protein layer, protein and charged polysaccharides in bulk at pH 7; (B) Initially adsorbed protein layer at o/w interacts with protein-polysaccharide complexes formed in bulk at pH 5, (D) Selective protein adsorption of protein to a/w from bulk containing protein-polysaccharide complexes at pH 5

In conclusion, the composition of the adsorbed layer of the initial o/w and of the a/w interfaces investigated in this study seem to be qualitatively the same and dominated by the presence of proteins. The interaction of this adsorbed protein layer with the molecules present in the bulk, however, seem to be different for ASPI stabilized emulsions and foams. Further studies on the mechanisms controlling the adsorption of the molecules present in ASPI to a/w and o/w interfaces could contribute to a better control of ASPI stabilized foams and emulsions.

6.6. Concluding remarks

In conclusion, this thesis shows that from a techno-functional point of view ASPI represents an attractive substitute for currently used high-value food protein isolates. It is readily isolated after disintegration of the microalgae cells by the application of a simple and scalable isolation process. In its final form, ASPI can compete with currently used animal-derived proteins with respect to emulsion and foam stability. The complex, heterogeneous composition of ASPI contributes positively to its techno-functional properties. Due to the combination of the positive interfacial properties of its protein fraction with the broad pH stability of its charged polysaccharide fraction, ASPI possesses the positive attributes of two types of techno-functional ingredients.

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Summary
Samenvatting
Zusammenfassung

Microalgae have long been considered a promising alternative protein source in foods, since they possess high amount of proteins (up to 50% [w/w]) and well-balanced amino acid profiles. Nevertheless, the application of microalgae proteins has not gained significant importance. The isolation of microalgae protein is typically hindered by the strong cellulosic cell wall and high contents of chlorophyll characteristic for many microalgae. At the start of this thesis, no adequate isolation procedures had been reported that enable subsequent studies of the techno-functional properties of microalgae protein isolates. Therefore, in this thesis, first a mild isolation process was developed to obtain a soluble protein isolate free from any intense colour from the green microalgae *Tetraselmis* sp. Subsequently, the physico-chemical and techno-functional properties of protein isolate obtained were studied.

Chapter 1 provides an overview of the available information on microalgae protein. Most information is based on proteome analysis of algae extracts, indicating that microalgae contain a wide variety of proteins. Different isolation strategies have been reported for the isolation of microalgae protein. Most published studies used harsh methods (e.g. high temperature, high pH), and did not characterize the obtained protein extract (e.g. protein composition or techno-functional properties). In this thesis, harsh conditions could not be used, as this might result in decreased techno-functionality. The techno-functional properties of a protein are affected by system conditions (i.e. pH and ionic strength) and by sample composition (e.g. the presence of polysaccharides). To study the effects of these properties, the proteins need to be isolated using a strategy that is mild and avoids negative side-reactions.

Chapter 2 describes the mild isolation process used to obtain an algae soluble protein isolate (ASPI) from the green microalgae *Tetraselmis* sp. The isolation process includes an initial cell disintegration step by bead milling (at ≤ 20 °C) and further purification of the soluble protein fraction obtained by anion exchange adsorption and acid precipitation. The chemical composition of the final protein isolate (ASPI) and intermediate fractions was determined. Since the conventional nitrogen-to-protein conversion factor (N-Prot factor) of 6.25 is unsuitable for microalgae due to the high concentrations of non-protein nitrogen (NPN), specific nitrogen-to-protein conversion factors were determined at every processing step. In this way it was determined that 7% of the protein present in *Tetraselmis* sp. were present in the obtained ASPI, which was free from any intense colour. ASPI was composed of 59 ± 7% (w/w) protein and 20 ± 6% (w/w) carbohydrates, the latter consisted for approximately one fourth of uronic acids (4.8 ± 0.4% [w/w]). In the pH range 5.5 – 6.5, where currently used legume seed protein isolates show low solubility, ASPI retains high solubility independent of ionic strength.

Chapter 3 describes the effects of ASPI concentration, pH, ionic strength and calcium ion concentration on the formation and stability of emulsions prepared using ASPI as emulsifier. Whey protein isolate (WPI) and gum arabic (GA) are used as reference emulsifiers. All emulsions were characterized with respect to their average droplet size and their stability against droplet aggregation using microscopy, particle-size distribution analysis using light scattering, and diffusion wave spectrometry (DWS). Unlike WPI, ASPI stabilized emulsions are stable against droplet aggregation around pH 5 at low ionic strength. Based on droplet ζ-potential measurements, the emulsion stability at this pH is suggested to be caused by the co-adsorption of the charged polysaccharide fraction present in ASPI. The use of the naturally occurring protein-polysaccharide hybrid GA also enabled the production of stable emulsions at pH 5. However, for GA a 5 times higher concentration (50 mg/mL) was needed to obtain stable emulsions. Even then, the droplet size of GA emulsions was 25 μm, while at 10 mg/mL concentration ASPI already made stable emulsions with droplet sizes of 1 μm. The better emulsifying capacity of ASPI, compared to GA, is attributed to its higher protein content.

In **Chapter 4** the effects of isolate concentration, pH and ionic strength on the interfacial properties as well as the foam properties of ASPI are discussed. WPI and egg white albumin (EWA), two foaming agents commonly applied in food industry, were used as reference. In the pH range from 5 – 7 the stability of ASPI stabilized foams is superior to the stabilities of WPI and EWA stabilized foams. At pH 3 – 5, the solubility of ASPI decreases and, therefore, no foam can be formed at those pH values. For ASPI, the foam stability and the adsorption to the air-water interface were found to be maximal at pH 7, close to the isoelectric point, which was calculated from the amino acid composition of ASPI. Together with the similar correlation between dilatational modulus and surface pressure of ASPI at pH 5 and pH 7, this observation confirms that the adsorption to the air-water interface is dominated by the protein fraction of ASPI at all pH values and ionic strength tested. So, in contrast to ASPI stabilized emulsions, it is suggested that the superior foam stability of ASPI is due to the protein fraction.

In **Chapter 5** the influence of the polysaccharides present in ASPI on the techno-functional properties of the isolate is investigated in more depth. Therefore, ASPI was fractionated using centrifugal membrane filtration. The three fractions obtained - a filtrate (ASPI-F), a retentate (ASPI-R) and a pellet (ASPI-R) - differed considerably in carbohydrate and uronic acid content. While the fraction enriched in uronic acids (ASPI-R) showed increased emulsion stability over the whole pH range investigated (pH 3 – 7), the fractions with decreased uronic acid content (ASPI-F, ASPI-P) showed decreased emulsion stability around pH 5. This indicates the importance of the charged polysaccharide present in ASPI

for its emulsion stability around pH 5. Detailed studies of the air-water interfacial properties, however, did not show significant differences between the fractions. This indicates that the charged polysaccharides present in ASPI do not contribute to the air-water interfacial layer. Still, ASPI-F, the fraction dominated by dissociated proteins and glycoproteins, showed considerably higher foam stabilities in the pH range 5 - 7 than the other fractions at comparable isolate concentration. Based on the results of this study it is suggested that the charged carbohydrates present in ASPI contribute considerably to high emulsion stability in the pH range 5 – 7, while foam stability was mainly influenced by dissociated proteins and small glycoproteins.

Chapter 6 combines the information described in the previous chapters into a more general overview in order to address points important for the future production of protein isolates from microalgae and the study of their techno-functional properties. In addition, possible strategies are discussed for the extraction of the proteins present in the insoluble pellet fraction after bead milling (in the case of *Tetraselmis* 79% of the total protein present in the starting material). Furthermore, it was found that the gross compositional analysis of microalgae does not explain 100% of the dry matter. This may be due to the incomplete hydrolysis of the uronic acid and glycoprotein structures. The remaining oligomeric structures are typically not included in standard compositional analysis methods. Finally, the role of the charged carbohydrate fraction on the adsorption and interfacial properties of ASPI proteins is discussed.

Summary
Samenvatting
Zusammenfassung

Microalgen worden al lange tijd gezien als een veelbelovende alternatieve bron van voedingseiwitten. Dit vanwege hun hoge eiwitgehalte (tot 50% [w/w]) en gebalanceerde aminozuurprofiel. Desalniettemin spelen microalgeiwitten vandaag de dag geen significante rol in voedingstoepassingen. De isolatie van microalgeiwitten wordt meestal verhinderd door de sterke celwand en het hoge chlorofylgehalte, welke karakteristiek zijn voor veel microalgen. Aan het begin van het onderhavige onderzoek waren er nog geen adequate isolatieprocedures gerapporteerd, welke de daaropvolgende bestudering van de techno-functionele eigenschappen van microalgeiwitisolaten mogelijk maakten. Daarom is er in dit onderzoek eerst een milde isolatieprocedure ontwikkeld om een oplosbaar eiwitisolaat vrij van intense kleur uit de groene microalg *Tetraselmis* sp. te verkrijgen. Vervolgens werden de fysisch-chemische en techno-functionele eigenschappen van het verkregen eiwitisolaat bestudeerd.

Hoofdstuk 1 geeft een overzicht van de beschikbare informatie over microalgeiwit. De meeste informatie is gebaseerd op een proteoomanalyse van algenextracten. Het overzicht geeft aan dat microalgen een brede variëteit aan eiwitten bevatten. Verschillende strategieën voor de isolatie van microalgeiwitten zijn geraporteerd. De meeste gepubliceerde onderzoeken maakten gebruik van ruwe methoden (b.v. hoge temperatuur, hoge pH), en hebben het verkregen eiwitextract niet gekarakteriseerd qua eiwitsamenstelling of techno-functionele eigenschappen. In dit onderzoek kon geen gebruik gemaakt worden van ruwe methoden, omdat dat zou kunnen leiden tot verminderde techno-functionaliteit. De techno-functionele eigenschappen van een eiwit worden beïnvloed door de condities van het systeem (b.v. pH en ionische sterkte) en door de monstersamenstelling (b.v. de aanwezigheid van polysacchariden). Om de effecten van deze eigenschappen te bestuderen moeten de eiwitten geïsoleerd worden volgens een milde strategie, die negatieve nevenreacties voorkomt.

In **hoofdstuk 2** wordt het milde isolatieproces waarmee een oplosbaar algeiwitisolaat (ASPI [algae soluble protein isolate]) wordt verkregen van de groene microalg *Tetraselmis* sp. beschreven. Het isolatieproces omvat een initiële celdisruptie stap door middel van kogelmalen (bij temperaturen $\leq$ 20 °C), en verdere zuivering van de verkregen oplosbare eiwitfractie door middel van anionwisselingsadsorptie en zuurprecipitatie. De chemische samenstelling van het uiteindelijke eiwitisolaat (ASPI) en de intermediaire fracties werd bepaald. Omdat de conventionele stikstof-eiwit conversiefactor (N-Prot factor) van 6,25 ongeschikt is voor microalgen vanwege hun hoge concentraties van niet-eiwit stikstof (NPN), werden specifieke stikstof-eiwit conversiefactoren bepaald bij elke stap in het isolatieproces. Op deze manier is vastgesteld dat 7% van het eiwit aanwezig in *Tetraselmis* sp. ook aanwezig was in het verkregen ASPI, hetgeen niet sterk gekleurd was. ASPI

bestond uit 59 ± 7%(w/w) eiwit en 20 ± 6% (w/w) koolhydraten, welke voor ongeveer een kwart uit uronzuren bestaan (4,8 ± 0,4% [w/w]). In de pH range 5,5 – 6,5, waarin momenteel toegepaste legumine eiwitisolaten een lage oplosbaarheid hebben, behoudt ASPI hoge oplosbaarheid, onafhankelijk van de ionsterkte.

Hoofdstuk 3 beschrijft de effecten van ASPI concentratie, pH, ionsterkte en concentratie calcium ionen op de vorming en stabiliteit van emulsies bereid met ASPI als emulgator. Wei eiwit concentraat (WPI) en arabische gom (GA) werden gebruikt als referentieemulgatoren. Van alle emulsies is de gemiddelde druppelgrootte en de stabiliteit van de druppels tegen aggregatie met behulp van microscopie, deeltjes-grootte verdeling met behulp van lichtverstrooiing en diffusion wave spectrometry (DWS) gekarakteriseerd. In tegenstelling tot WPI, zijn emulsies gestabiliseerd met ASPI stabiel tegen druppelaggregatie rond pH 5 bij lage ionsterkte. Gebaseerd op metingen van de druppel ζ-potentiaal, wordt gesuggereerd dat de stabiliteit van de emulsie bij deze pH wordt veroorzaakt door co-adsorptie van de geladen polysaccharidenfractie die in ASPI aanwezig is. Gebruik van de natuurlijk voorkomende eiwit-polysaccharide hybride GA maakt de vorming van stabiele emulsies bij pH 5 ook mogelijk. Van GA is echter wel een 5 maal hogere concentratie (50 mg/mL) nodig om stabiele emulsies te krijgen. Zelfs dan was de druppelgrootte van GA emulsies 25 µm, terwijl een concentratie van 10 mg/mL ASPI al stabiele emulsies met een druppelgrootte van 1 µm oplevert. De betere emulgerende werking van ASPI ten opzichte van GA wordt toegeschreven aan het hogere eiwitgehalte.

In **hoofdstuk 4** worden de effecten van isolaatconcentratie, pH en ionsterkte op de grensvlakeigenschappen en het schuimgedrag van ASPI besproken. WPI en kippenei-eiwit albumine (EWA), twee algemeen gebruikte ingrediënten in de levensmiddelenindustrie, werden als referentie gebruikt. In het pH bereik van 5-7 is de stabiliteit van ASPI-gestabiliseerde schuimen beter dan die van WPI- en EWA-gestabiliseerde schuimen. Bij pH 3-5 neemt de oplosbaarheid van ASPI af, waardoor geen schuim kan worden gevormd bij die pH waarden. De schuimstabilitiet en de adsorptie aan het lucht-water grensvlak waren voor ASPI het hoogst bij pH 7, dicht bij het isoelektrisch punt, hetgeen berekend is aan de hand van de aminozuursamenstelling van ASPI. Samen met de vergelijkbare correlatie tussen dilatatie modulus en oppervlaktedruk van ASPI bij pH 5 en pH 7 bevestigt dit dat de adsorptie aan het lucht-water grensvlak wordt gedomineerd door de eiwitfractie van ASPI bij alle pH waarden en ionsterktes die zijn getest. Dus, in tegenstelling tot door ASPI gestabiliseerde emulsies, wordt gesuggereerd dat de superieure schuimstabiliteit van ASPI te wijten is aan enkel de eiwitfractie.

In **hoofdstuk 5** wordt de invloed van de polysacchariden aanwezig in ASPI op de techno-functionele eigenschappen van het isolaat verder onderzocht. Daarvoor is ASPI

gefractioneerd met behulp van centrifugale membraanfiltratie. De drie verkregen fracties, een filtraat (ASPI-F), een retentaat (ASPI-R) en een pellet (ASPI-P), verschilden aanzienlijk in koolhydraat- en uronzuurgehalte. Terwijl de in uronzuur verrijkte fractie (ASPI-R) hogere emulsiestabiliteit over het gehele onderzochte pH bereik (pH 3 - 7) liet zien, lieten de fracties met een verminderd uronzuur gehalte (ASPI-F, ASPI-P) een verminderde emulsiestabiliteit rond pH 5 zien. Dit geeft het belang van de geladen polysacchariden in ASPI voor de emulsiestabiliteit rond pH 5 aan. Gedetailleerde studies van de lucht-water grensvlakeigenschappen daarentegen lieten geen significante verschillen tussen de fracties zien. Dit geeft aan dat de geladen polysacchariden aanwezig in ASPI niet bijdragen aan de lucht-water grensvlaklaag. Toch liet ASPI-F, de fractie die gedomineerd wordt door gedissocieerde eiwitten en glycoproteïnen, een aanmerkelijk hogere schuimstabiliteit zien dan de andere fracties in het pH bereik van 5-7, bij vergelijkbare isolaatconcentraties. Gebaseerd op de resultaten van deze studie wordt gesuggereerd dat de geladen koolhydraten aanwezig in ASPI aanmerkelijk bijdragen aan de hoge emulsiestabiliteit in het pH bereik 5 – 7, terwijl schuimstabiliteit vooral werd beïnvloed door gedissocieerde eiwitten en kleine glycoproteïnen.

Hoofdstuk 6 combineert de in de voorgaande hoofdstukken beschreven informatie in een meer algemeen overzicht, om punten die van belang zijn voor de toekomstige productie van eiwitisolaten van microalgen en het bestuderen van hun techno-functionele eigenschappen aan te stippen. Daarnaast worden mogelijke strategiën voor de extractie van eiwitten uit de onoplosbare pelletfractie na het kogelmalen (in het geval van *Tetraselmis* sp. 79% van het totaal aanwezige eiwit in het startmateriaal) besproken. Verder werd gevonden dat de totale samenstellingsanalyse van microalgen niet 100% van de droge stof kan verklaren. Dit zou kunnen komen door de incomplete hydrolyse van de uronzuur- en glycoproteïnenstructuren. De overgebleven oligomere structuren worden normaal gesproken niet meegenomen in standaard samenstellingsanalyse methoden. Tot slot wordt de rol van de geladen koolhydraatfractie op de adsorptie- en grensvlakeigenschappen van ASPI eiwitten besproken.

Summary
Samenvatting
Zusammenfassung

Mikroalgen gelten aufgrund ihres hohen Eiweißgehaltes (bis zu 50% in der Trockenmasse [w/w]) und ihres ausgeglichenen Aminosäureprofils schon lange als aussichtsreiche alternative Eiweißquelle in Lebensmitteln. Dennoch spielt ihre Anwendung im Lebensmittelbereich bis heute keine bedeutende Rolle. Die Gewinnung von Mikroalgeneiweißen wird häufig durch die für Mikroalgen typische starke zellulosehaltige Zellwand und den hohen Chlorophyllgehalt behindert. Zu Beginn der vorliegenden Forschungsarbeit waren keine geeigneten Isolationsmethoden bekannt, die die angemessene Studie der techno-funktionellen Eigenschaften von Mikroalgeneiweißen zugelassen hätten. Aus diesem Grund war der erste Schritt der vorliegenden Forschungsarbeit die Entwicklung eines milden Isolationsprozesses zur Gewinnung eines löslichen Eiweißisolates frei von jeglicher intensiven Farbe aus der grünen Mikroalge *Tetraselmis* sp. Anschließend konnten die physico-chemischen und techno-funktionellen Eigenschaften des gewonnenen Eiweißisolates untersucht werden.

Im **ersten Kapitel** wird eine Übersicht über die vorhandenen Informationen über Mikroalgeneiweiße gegeben. Zum größten Teil basieren diese Informationen auf der Proteomanalyse von Algenextrakten und zeigen, dass Mikroalgen ein breites Spektrum verschiedener Eiweiße enthalten. Für die Gewinnung von Eiweißisolaten aus Mikroalgen wurden bereits verschiedene Methoden angewandt. In den meisten veröffentlichten Studien wurden aggressive Isolationsmethoden (z.B. hohe Temperaturen, hohe pH-Werte) verwendet und die gewonnenen Eiweißextrakte nicht genau beschrieben (z.B. in Bezug auf ihre Eiweißzusammenstellung oder techno-funktionelle Eigenschaften). In der vorliegenden Studie wurden keine vergleichbar aggressiven Isolationsmethoden verwendet, da diese möglicherweise einen negativen Einfluss auf die techno-funktionellen Eigenschaften des endgültigen Isolates hätten haben können. Die techno-funktionellen Eigenschaften eines Eiweißes werden durch Systembedingungen (z.B. pH-Wert und Ionenstärke) und Probeneigenschaften (z.B. die Anwesenheit von Polysacchariden) beeinflusst. Um die genauen Effekte dieser Eigenschaften zu untersuchen, ist es notwendig, die Eiweiße auf milde Art und Weise zu isolieren, um so negative Nebenwirkungen zu vermeiden.

Im **zweiten Kapitel** wird der milde Isolationsprozess beschrieben, der die Gewinnung eines löslichen Algeneiweißisolates (ASPI [algae soluble protein isolate]) aus der grünen Mikroalge *Tetraselmis* sp. ermöglicht. Der Isolationsprozess umfasst einen Zellaufschlussschritt, bei dem eine Kugelmühle verwendet wird (bei $\leq$ 20 °C), und die anschließende Aufreinigung der löslichen Eiweißfraktion mit Hilfe von Anionaustauschadsorption und Säurefällung erfolgt. Die chemische Zusammensetzung des endgültigen Eiweißisolates (ASPI) und aller Zwischenfraktionen wurde bestimmt. Da der konventionelle Stickstoff-zu-Eiweiß-Umrechnungsfaktor (N-Pro-Faktor) von 6,25 für

Mikroalgen aufgrund ihres hohen Nicht-Eiweiß-Stickstoffes (NPN) nicht geeignet ist, wurden spezielle N-Prot-Faktoren für jeden Prozessschritt bestimmt. Auf diese Weise ließ sich feststellen, dass 7% des Gesamteiweißgehaltes von *Tetraselmis* sp. in Form von ASPI gewonnen werden konnten, welches frei von jeglicher intensiven Farbe ist. Das gewonnene ASPI enthielt 59 ± 7% (w/w) Eiweiß und 20 ± 6% (w/w) Kohlenhydrate, die für ungefähr ein Viertel aus Uronsäuren aufgebaut waren (4,8 ± 0,4% [w/w]). In dem pH-Bereich 5,5 - 6,5, dem Gebiet, in dem derzeit verwendete Leguminsaateiweiße schwer löslich sind, ist ASPI gut löslich, unabhängig von der Ionenstärke.

Im **dritten Kapitel** wird der Einfluss der Parameter ASPI-Konzentration, pH, Ionenstärke und Kalziumionenkonzentration auf die Ausbildung und Stabilität von Emulsionen, die unter der Verwendung von ASPI als Emulgiermittel hergestellt wurden, beschrieben. Als Referenzemulgiermittel wurden Molkeneiweißisolate (WPI) und Gummi arabicum (GA) verwendet. Für alle hergestellten Emulsionen wurden die Durchschnittströpfchengröße und Stabilität der individuellen Öltröpfchen gegenüber einer möglichen Aggregation mit Hilfe von Mikroskopie und Partikelgrößeverteilungsanalysis unter der Verwendung von sowohl statischer Lichtbrechung als auch Diffusion Wave Spectrometry (DWS) bestimmt. Im Gegensatz zu WPI-stabilisierten Emulsionen aggregieren ASPI-stabilisierte Emulsionen bei niedriger Ionenstärke nicht im pH-Bereich um pH 5. Basierend auf Tröpfchen-ζ-Potential-Messungen kann angenommen werden, dass ASPI-stabilisierte Emulsionen um pH 5 herum nicht aggregieren, da in diesem pH-Bereich die in ASPI enthaltene geladene Polysaccharidfraktion gemeinsam mit den Eiweißen an die Tröpfchenoberfläche ko-adsorbiert. Auch die Verwendung des natürlich vorkommenden Eiweiß-Polysaccharid-Hybriden GA ermöglicht die Herstellung von stabilen Emulsionen um pH 5 herum. Jedoch muss im Falle von GA die fünffache Isolatmenge (50 mg/mL) verwendet werden, um eine stabile Emulsion zu erzeugen. Selbst bei dieser hohen Isolatkonzentration beträgt die durchschnittliche Tröpfchengröße der GA-Emulsionen 25 µm, während bei einer ASPI-Konzentration von nur 10 mg/mL stabile Emulsionen mit Tröpfchengrößen von ungefähr 1 µm hergestellt werden können. Die höhere Emulgierfähigkeit von ASPI ist auf seinen höheren Eiweißgehalt zurückzuführen.

Im **vierten Kaptiel** wird der Einfluss der Parameter Isolatkonzentration, pH und Ionenstärke auf die Schaumeigenschaften von ASPI diskutiert. WPI und Hühnerei-Eiweißalbumin (EWA), zwei häufig in Lebensmitteln eingesetzte Schaummittel, wurden als Referenzschaummittel verwendet. Im pH-Bereich 5 - 7 sind ASPI-stabilisierte Schäume stabiler als WPI- und EWA-stabilisierte Schäume. Im pH-Bereich 3 - 5 nimmt die Löslichkeit von ASPI stark ab, wodurch in diesem Bereich keine stabilen Schäume geformt werden können. Sowohl die Schaumstabilität von ASPI als auch seine Adsorption an die

Luft-Wasser-Grenzfläche sind maximal bei pH 7, in der Nähe des isoelektrischen Punktes, der basierend auf die Aminosäurezusammensetzung des Isolates berechnet wurde. In Kombination mit der gleichbleibenden Korrelation von Dilatationsmodul und Oberflächendruck von ASPI bei pH 5 und pH 7 bestätigt dies, dass das Adsorptionsverhalten von ASPI an der Luft-Wasser-Grenzfläche bei allen pH-Werten und Ionenstärken von seinen Eiweißen dominiert wird. Dies bedeutet, dass, im Gegensatz zu ASPI-stabilisierten Emulsionen, die ausgezeichneten Schaumeigenschaften des Isolates beinahe ausschließlich von seiner Eiweißfraktion bestimmt werden.

Im **fünften Kapitel** wird der Einfluss der in ASPI anwesenden Polysaccharide auf die techno-funktionellen Eigenschaften des Isolats genauer untersucht. Hierfür wurde ASPI mit Hilfe von Zentrifugalmembranfiltration fraktioniert. Die Kohlenhydrat- und Uronsäuregehalte der drei resultierenden Fraktionen, einem Filtrat (ASPI-F), einem Retentat (ASPI-R) und einem Pellet (ASPI-P), wichen entscheidend voneinander ab. Während die Fraktion mit dem höchsten Uronsäuregehalt (ASPI-R) eine deutlich verbesserte Emulsionsstabilität über den gesamten untersuchten pH-Bereich (pH 3 - 7) aufwies, zeigten die Fraktionen mit einem reduzierten Uronsäuregehalt (ASPI-F, ASPI-P) eine deutliche Abnahme der Emulsionsstabilität um pH 5. Diese Beobachtungen zeigen die große Bedeutung der geladenen Polysaccharide für die Emulsionsstabilität von ASPI bei pH 5. Detaillierte Studien der Luft-Wasser-Grenzflächeneigenschaften zeigten jedoch keine deutlichen Unterschiede zwischen den verschiedenen ASPI-Fraktionen. Dies deutet darauf hin, dass die in ASPI anwesenden geladenen Polysaccharide nicht an die Luft-Wasser-Grenzfläche absorbieren. Trotzdem weist ASPI-F – die Fraktion dominiert durch die Anwesenheit dissoziierter Eiweiße und Glykoproteine – bei vergleichbaren Isolatkonzentrationen im pH-Bereich 5 - 7 deutlich höhere Schaumstabilitäten auf als die anderen Fraktionen. Basierend auf den Resultaten dieser Studie kann angenommen werden, dass die in ASPI anwesenden geladenen Kohlenhydrate bedeutend zu hohen Emulsionsstabilitäten im pH-Bereich 5 - 7 beitragen, Schaumstabilitäten jedoch hauptsächlich durch die Anwesenheit dissoziierter Eiweiße und kleiner Glykoproteine beeinflusst werden.

Abschließend werden in **Kapitel 6** die Informationen der vorhergehenden Kapitel zu einer allgemeineren Übersicht zusammengefasst, um so wichtige Punkte für die zukünftige Produktion von Eiweißisolaten aus Mikroalgen und die Studien ihrer techno-funktionellen Eigenschaften hervorzuheben. Zusätzlich werden mögliche Strategien für die Extraktion von den nach dem Zellaufschluss noch stets unlöslichen Eiweißen (im Falle von *Tetraselmis* sp. 79% des Gesamteinweißgehaltes) diskutiert. Darüber hinaus wird festgestellt, dass die allgemeine chemische Zusammensetzungsanalyse von Mikroalgen

keine 100% des Trockenmassegehaltes liefert. Eine mögliche Erklärung hierfür ist die unvollständige Hydrolyse der vorhandenen Uronsäure- und Glykoproteinstrukturen. Die verbleibenden Oligomerstrukturen werden in Standardanalyseverfahren häufig nicht detektiert. Am Ende des sechsten Kapitels wird die Rolle der geladenen Kohlenhydratfraktion während der Adsorption von ASPI an verschiedenen Grenzflächen und die daraus resultierenden Grenzflächeneigenschaften diskutiert.

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Anja

About the author

Curriculum Vitae

Anja Schwenzfeier was born on December 16th 1982 in Berlin, Germany. After graduating from high school (Herder-Oberschule-Berlin [Gymnasium]) in 2002, she started studying Food Technology at the Technical University Berlin. In 2004, she completed two internships, one at the Max-Planck-Institute for Molecular Genetics and another one at the Herta-Salami-Factory (Nestlé Germany), both located in Berlin. After obtaining her Vordiplom in 2005, she left to the Netherlands to participate in the Erasmus student exchange program of the European Union at Wageningen University. After having finished all courses required for the MSc Food Technology, she returned to Germany for an internship at the Unilever Global Capability Centre in Heilbronn. Afterwards she continued her study of Food Technology in Berlin. In 2007, she returned to Wageningen for her MSc thesis entitled "Enzymatic synthesis of oligosaccharide derivatives containing hydroxyl-phenyl groups" at the Laboratory of Food Chemistry under the supervision of Dr. Ben van den Broek and Dr. Henk Schols. In 2008, she graduated in Food Technology at the Technical University Berlin as well as at Wageningen University. Afterwards, she obtained the opportunity to continue working at the Laboratory of Food Chemistry as a PhD student under the supervision of Prof. dr. ir. Harry Gruppen, Dr. ir. Peter Wierenga and Dr. ing. Michel Eppink. The results of her PhD research are presented in this thesis. Currently, Anja continues working at the Laboratory of Food Chemistry as researcher.

List of publications

Schwenzfeier, A., Wierenga P.A., Gruppen, H. (2013) Emulsion properties of algae soluble protein isolate from *Tetraselmis* sp. *Food Hydrocolloids*, **30**(1), 258-263.

Schwenzfeier, A., Helbig, A., Wierenga P.A., Gruppen, H. (2011) Isolation and characterization of soluble protein from the green microalgae *Tetraselmis* sp. *Bioresource Technology*, **102**(19), 9121-9127.

Schwenzfeier, A., Lech, F., Wierenga, P.A, M.H.M. Eppink, Gruppen, H. (2013) Foam properties of algae soluble protein isolate: Effect of pH and ionic strength, *submitted*

Schwenzfeier, A., Wierenga, P.A, Eppink, M.H.M., Gruppen, H., Effect of charged polysaccharides on the techno-functional properties of fractions obtained from algae soluble protein isolate, *to be submitted*

Overview of completed training activities

Discipline specific activities

Conferences and meetings

- International Algae Congress†, Amsterdam, The Netherlands, 2008
- Food Colloids Conference†, Granada, Spain, 2010
- 8th European Workshop Biotechnology of Microalgae†, Nuthetal, Germany, 2010
- 4. Bundesalgenstammtisch† (DECHEMA), Hamburg, Germany, 2011
- International Conference on Applied Phycology†, Halifax, Canada, 2011
- Food Colloids Conference†, Copenhagen, Denmark, 2012

Courses

- Food Hydrocolloids (VLAG), Wageningen, The Netherlands, 2009
- Intensive Program in Biorenewables, ISU, Ames, Iowa, USA, 2009
- Proteomics (VLAG), Wageningen, The Netherlands, 2011

General courses

- Ph.D. introduction week (VLAG), Bilthoven, The Netherlands, 2009
- Ph.D. competence assessment (WGS), Wageningen, The Netherlands,2009
- Basic statistics (SENSE), Wageningen, The Netherlands, 2010
- Techniques in writing and presenting a scientific paper (WGS), Wageningen, The Netherlands, 2010
- Teaching and supervising thesis students (WU), Wageningen, The Netherlands, 2009
- Career assessment (WGS), Wageningen, The Netherlands, 2011
- Career orientation (WGS), Wageningen, The Netherlands, 2012

Optionals

- Ph.D. trip FCH to China, WU (FCH), 2008
- Food chemistry study trip to Ghent, Belgium, WU (FCH), 2009
- Ph.D. trip FCH to Switzerland and Italy‡, WU (FCH), 2010
- Organization FCH Ph.D. trip to Switzerland and Italy, ’09-‘10
- BSc / MSc thesis students presentations and colloquia, WU (FCH), ’08-’12
- Ph.D. presentations, WU (FCH), ’08-’12
- Ph.D. Research proposal

† Poster presentation
‡ Oral presentation

Used abbreviations:

DECHEMA	= Gesellschaft für Chemische Technik und Biotechnologie e.V.
VLAG	= Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Science
WGS	= Wageningen Graduates Schools
SENSE	= Research School for Socio-Economic and Natural Sciences of the Environment
WU	= Wageningen University
FCH	= Laboratory of Food Chemistry

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