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β-Lactoglobulin as nanotransporter for bioactive compounds of garlic (*Allium sativum* L.)

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Abstract

The suitability of the whey protein β-lactoglobulin as nanotransporter for bioactive organosulfur compounds of garlic, i.e. allicin and diallyl disulfide, was investigated. Since allicin is relatively unstable and causes an intensive smell and pungency, a delivery system is necessary to enable its enrichment in a functional food. The interactions of allicin and diallyl disulfide with β-lactoglobulin were comprehensively analyzed, the physico-chemical and organoleptic properties of β-lactoglobulin modified with allicin were evaluated and the bioavailability of the bioactive compound transported by the protein was assessed. The binding reaction was analyzed by fluorescence quenching, high performance liquid chromatography and the spectrophotometric detection of free amino and thiol groups. Allicin and diallyl disulfide were covalently bound to the free thiol group of β-lactoglobulin under alkaline conditions. The binding resulted in moderate conformational changes of the protein structure, primarily on tertiary level. According to mass spectrometric analysis of the intact and hydrolyzed protein, the binding reaction with allicin and diallyl disulfide resulted in the formation of S-allylmercaptocysteine, a stable, non-volatile, bioactive compound. Through the binding of allicin by β-lactoglobulin, the typical smell and taste of garlic was significantly reduced. The food grade production of β-lactoglobulin modified with allicin resulted in a consumable beverage that delivered physiologically relevant amounts of bioactive organosulfur compounds without significant garlic like sensory properties. A double-blind, randomized, diet-controlled cross-over study with nine healthy volunteers showed that the bioavailability of S-allylmercaptocysteine was not impaired by the incorporation in the protein chain. Conclusively, the covalent binding of allicin to β-lactoglobulin provides an innovative approach for the delivery of bioactive compounds.

Kurzdarstellung

In der vorliegenden Arbeit wurde die Eignung des Molkenproteins β-Lactoglobulin als Nanotransporter für die bioaktiven Schwefelverbindungen Allicin und Diallyldisulfid aus Knoblauch untersucht. Allicin ist eine relativ unstabile Verbindung, die bedeutend zu dem typischen Geruch und der Schärfe von Knoblauch beiträgt. Für die Anreicherung von Allicin in einem funktionellen Lebensmittel ist daher ein Transportsystem notwendig. Die Interaktionen zwischen Allicin bzw. Diallyldisulfid und β-Lactoglobulin wurden umfangreich analysiert. Des Weiteren wurden die physikochemischen und organoleptischen Eigenschaften des mit Allicin modifizierten Proteins untersucht. Abschließend wurde die Bioverfügbarkeit der transportierten bioaktiven Verbindung ermittelt. Die Bindungsreaktion wurde mittels Fluoreszenzlöschung, Hochleistungsflüssigchromatographie und Reagenzien zur Bestimmung der freien Amino- und Thiolgruppen erfasst. Allicin und Diallyldisulfid wurden kovalent durch die freie Thiolgruppe von β-Lactoglobulin gezielt unter alkalischen Bedingungen gebunden. Diese Bindung veränderte die Proteinkonformation geringfügig und vorrangig auf tertiärer Strukturebene. Die massenspektrometrische Analyse des intakten und des hydrolysierten modifizierten Proteins zeigte, dass durch die Bindung von Allicin bzw. Diallyldisulfid eine S-Allyl-Gruppe auf die freie Thiolgruppe der Cysteinseitenkette übertragen wurde, wodurch das stabile, nicht-flüchtige, bioaktive S-Allymercaptocystein entstand. Durch die Bindung von Allicin an β-Lactoglobulin wurde der Knoblauch-typische Geruch und Geschmack signifikant reduziert. Die lebensmittelgeeignete Produktion von mit Allicin modifiziertem β-Lactoglobulin ermöglichte die Herstellung eines verzehrfähigen Getränks, das physiologisch relevante Mengen bioaktiver Schwefelverbindungen enthielt, ohne ein deutlich wahrnehmbares Knoblaucharoma aufzuweisen. Durch eine doppelt-blinde, randomisierte, Diät-kontrollierte Cross-Over-Studie mit neun gesunden Probanden wurde gezeigt, dass die Bioverfügbarkeit von S-Allylmercaptocystein durch die Integration in die Polypeptidkette von β-Lactoglobulin nicht beeinträchtigt wurde. Schlussfolgernd erwies sich die kovalente Bindung von Allicin an β-Lactoglobulin als ein innovativer Ansatz zum Transport bioaktiver Verbindungen.

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List of abbreviations

AGE	Aged garlic extract
AITC	Allyl isothiocyanate
AMS	Allyl methyl sulfide
ANS	1-Anilino-8-naphthalenesulfonate
CD	Circular dichroism
DADS	Diallyl disulfide
DATS	Diallyl trisulfide
DLS	Dynamic light scattering
DPPH	1,1-Diphenyl-2-picrylhydarzyl
DSC	Differential scanning calorimetry
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
EGCG	(-)-Epigallocatechin-3-gallate
GSH	Glutathione, reduced
GSSG	Glutathione disulfide
HP-GPC	High performance gel permeation chromatography
HS GC-MS	Headspace gas chromatography-mass spectrometry
IAEDANS	5-((((2-Iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid
MALDI	Matrix assisted laser desorption ionization
MMTS	Methylmethanethiosulfonate
MS	Mass spectrometry
MTSL	(1-Oxy-2,2,5,5-tetramethylpyrrolinyl-3-methyl)-methanethiosulfona
NADP	Nicotinamide adenine dinucleotide phosphate
NASAC	N-acetyl-S-allyl cysteine
OPA	*o*-Phthaldialdehyde
PALP	Pyridoxal-phosphate
PDS	2-Pyridine disulfide
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
RP-HPLC	Reverse phase high performance liquid chromatography
RSH	Reactive thiol groups
SAC	S-allyl cysteine

SAM	S-adenosylmethionine
SAMC	S-allylmercaptocysteine
SAMG	S-allylmercaptoglutathione
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELDI	Surface-enhanced laser desorption ionization
SH	Thiol group
SS	Disulfide group
TEAC	Trolox equivalent antioxidative capacity assay
TNBS	Trinitrobenzenesulfonic acid
TOF	Time-of-flight
WPI	Whey protein isolate
β-LG	β-lactoglobulin

1. Motivation and objectives

During the last decades research revealed valuable insights into the relation of nutrition and disease. Consequently, consumer awareness of their diet influencing their health status and with it the demand for healthy food increased (Siró et al., 2008; Urala & Lahteenmaki, 2007). Further, the progress in nutrition science provided indications for potential health benefits of specific food ingredients, especially of phytochemicals (Benshitrit et al., 2012). Foods with added bioactive compounds, namely functional foods, could improve health and reduce the risk of diseases. Particularly population groups with defined risk factors could profit from functional foods (Kraus, 2015). However, food fortification or enrichment of functional ingredients is a technological challenge because many potential compounds are relatively instable, poorly water-soluble or cause adverse sensory effects. The development of suitable delivery systems could ensure high bioaccessibility and bioavailability by protection of the bioactive compounds during food processing, storage and digestion and their release at the desired absorption site (Benshitrit et al., 2012; Vos et al., 2010). In addition, bioactive compounds can cause bitter or astringent tastes or unpleasant off-flavors. Since consumers are not willing to compromise on taste in favor of health benefits, potential adverse effects on sensory properties need to be eliminated (Verbeke, 2006).

Different delivery systems were developed in order to overcome undesired effects of bioactive ingredients, for instance liposome entrapment, coating, coacervation or inclusion complexation (Fang & Bhandari, 2010). Referring to food products, the usage of delivery systems needs to be simple and cost-efficient (Ezhilarasi et al., 2013). Milk proteins have been reported to meet these needs and are involved in natural transport mechanisms (Livney, 2010). The usage of milk proteins is further favored by the fact that dairy products belong to the best base products for functional foods from the consumer point of view (Kraus, 2015). The whey protein β-lactoglobulin (β-LG) has been suggested as a transporter for small, hydrophobic ingredients. The globular protein is folded into a hydrophobic calyx which functions as the major non-covalent binding site, beside hydrophobic pockets on the surface of the protein (Kuwata et al., 1999; Qin et al., 1998a). Furthermore, β-LG has diverse techno-functional properties, GRAS (generally recognized as safe) status, a high nutritional value, and is soluble over a wide pH range – thus, it is a multifunctional ingredient (de Wit, 1998)

The use of β-LG as a transporter for non-covalently bound ligands was frequently reported, but the targeted covalent binding of bioactive compounds is a more recent approach (Gutierrez-Magdaleno et al., 2013; Teng et al., 2013; Shpigelman et al., 2012; Bello et al., 2012). Thus far, only allyl isothiocyanate (AITC) was investigated as covalent bioactive ligand and reacted with amino and thiol

groups of β-LG (Keppler et al., 2014a; Rade-Kukic et al., 2011). The thiosulfinate allicin from garlic is also an electrophilic phytochemical and could be a potential covalently binding ligand.

The organosulfur compound contributes largely to the typical smell and taste of garlic and has been shown to be mainly responsible for its health benefits, such as the risk reduction of certain cancers and of cardiovascular diseases (Butt et al., 2009; Fleischauer et al., 2000). With respect to functional foods, these effects are two of the most important health related properties for consumers which classifie allicin as a potential functional ingredient (Kraus, 2015). However, due to its reactive character allicin is relatively unstable and its chemical half-life is ranging from several hours to some days, depending on the conditions (Fujisawa et al., 2008a; Hunter et al., 2005; Lawson & Gardner, 2005). Furthermore, allicin is a pungent compound and can lead to gastrointestinal disturbances if ingested in high amounts (Salazar et al., 2008; Taucher et al., 1996). Many people avoid garlic because of its pungent taste and the malodorous breath after garlic consumption (Rosin et al., 1992). In short, these drawbacks necessitate a technological solution. The covalent binding of allicin to β-LG could offer an innovative approach to stabilize the organosulfur compound, mask the strong flavor and pungency and finally enable the enrichment in a functional food.

The aim of the present thesis is to investigate the suitability of β-LG as a transporter for covalently bound bioactive ingredients. This work comprises the whole approach from studying the interaction between the protein and the ligand, characterization of the physico-chemical properties of the modified protein and finally the transfer to the food level by pilot plant production of a functional food enriched with the modified protein for sensory analysis and bioavailability assessment *in vivo*.

Hypothesis 1: β-lactoglobulin can covalently bind allicin and diallyl disulfide at its free thiol group. The binding reaction is dependent on reaction conditions.

Background

The targeted covalent binding of bioactive compounds to β-LG is a new approach. Only allyl isothiocyanate (AITC) was investigated as a covalent bioactive ligand at β-LG so far (Keppler et al., 2014a; Rade-Kukic et al., 2011). The reaction between allicin or diallyl disulfide and this protein has not been analyzed before. According to previous studies, allicin is generally able to react with thiol groups by thiol disulfide exchange reaction, where the reaction rate depends on the pH value (Miron et al., 2010; Rabinkov et al., 2000).

Experimental approach

The overall binding kinetics of allicin and diallyl disulfide (DADS) to native and thermally denatured β-LG will be investigated by fluorescence quenching and reverse phase high performance liquid chromatography (RP-HPLC). Ellman's reagent (determination of free thiol groups, RSH) and *o*-phthaldialdehyde reagent (determination of free amino groups, OPA) will be used to identify the functional groups involved. In order to localize the binding sites more precisely the intact protein and the peptides after protein digestion will be analyzed by liquid chromatography-mass spectrometry (LC-MS). The influence of the pH value on the binding reaction will be studied (**chapter 3 and 4**).

Hypothesis 2: The binding of allicin to β-LG provides a stable bioactive derivate which does not smell or taste like garlic.

Background

Allicin contributes largely to the characteristic odor and taste of garlic. Due to the strong sensory impression, its fortification in food is limited to a relatively low concentration and to consumers who like the garlic flavor. If allicin should be used as a health-promoting ingredient in functional foods, the absence of its sensory characteristics would be advantageous. β-LG showed the potential to mask flavors in previous studies (Bohin et al., 2013; Reiners et al., 2000; Guichard & Langourieux, 2000). The enrichment of allicin in processed foods is also restricted by its low stability (Fujisawa et al., 2008b). It has been shown that cysteine forms a stable S-allylthio-derivate after reacting with allicin (Miron et al., 2010; Hunter et al., 2005).

Experimental approach

Quantitative descriptive sensory analysis of garlic powder mixed with whey protein isolate (β-LG source) with and without prior binding process will be conducted. Additionally, sensory properties of a beverage containing the modified protein in a physiological relevant concentration will be assessed. The concentrations of volatile sulfur compounds in garlic powder with and without β-LG will be determined after binding and drying processes by headspace gas chromatography-mass spectrometry (HS GC-MS). To reduce the garlic flavor to a minimal level, different protein-ligand-ratios and drying methods shall be tested (**chapter 5**).

Hypothesis 3: The allicin derivate S-allylmercaptocysteine integrated in the polypeptide chain of β-LG provides the same bioavailability as allicin in vivo. Allyl methyl sulfide is a suitable metabolite to assess the bioavailability of allicin and S-allylmercaptocysteine.

Background

Although the bioavailability of allicin is high (> 90%), its intake is limited due to its low stability (Fujisawa et al., 2008b; Lawson & Wang, 2005; Lachmann et al., 1994). S-allylmercaptocysteine is the stable reaction product of allicin and cysteine and was suggested as one of its metabolites (Rabinkov et al., 1998; Hunter et al., 2005). Additionally, it is present in garlic products, especially in aged garlic extract, even in very low amounts. The metabolism and the bioavailability of S-allylmercaptocysteine have not been investigated in humans before. However, studies indicate that the transfer of the thiol allyl group, as in form of S-allylmercaptocysteine, seems to play a crucial role in the metabolism of allicin and in the mediation of its bioactive effects (Rabinkov et al., 1998). Allyl methyl sulfide (AMS) in breath gas was identified as a suitable parameter of the bioavailability of allicin (Lawson & Wang, 2005).

Experimental approach

Garlic powder will be produced and the allicin yield shall be determined. Food grade, allicin-modified β-LG will be manufactured in a pilot plant scale. A consumable beverage will be developed, in which the modified β-LG shall be incorporated.

A bioavailability study with 9 healthy, male subjects will be conducted in a double-blinded, diet-controlled cross-over design. The both test products will comprise the beverage containing modified β-LG and also garlic powder in capsules providing allicin. The bioavailability will be assessed by quantification of allyl methyl sulfide in breath gas. The corresponding analytical methods have to be developed (**chapter 6**).

2. General introduction

2.1 β-Lactoglobulin

β-Lactoglobulin (β-LG) is a globular protein present in the milk of ruminants and many other mammalian species, but not in rodent, lagomorph and human milk (Sawyer & Kontopidis, 2000a). In bovine whey it is the major protein (concentration of ≈ 3 g/L) and accounts for about 50% of the total proteins (Wit, 1998). β-LG occurs in a number of genetically different variants, at least 12 were identified by now (Rachagani et al., 2006). Most common variants in western bovine milk are A and B, which differ in two amino acids (A: Asp^{64}, Val^{118}; B: Gly^{64}, Ala^{118}). The small differences in the primary structure have a significant effect on their properties, like solubility and thermal stability (Keppler et al., 2014c; Qin et al., 1999).

Historically, whey was considered as waste by dairy industry. But since the value of the whey components has been recognized, it has become a valuable dairy stream. Due to its special structural characteristics, β-LG contributes essentially to the properties of whey protein concentrates and isolates, which are increasingly used in food industry (Wit, 1998). The protein is rich in essential amino acids and provides a high nutritional value (Chatterton et al., 2006). Furthermore β-LG has multiple techno-functional properties. It can form interfacial films, that stabilize emulsions and foams, can associate to networks and build gels or edible films, binds water and contributes to viscosity and texture (Foegeding et al., 2002).

However, the functionality of the protein causes also some drawbacks. Due to its aggregation behavior, β-LG contributes to the fouling of heat exchangers, which reduces the efficiency of the process and deteriorates product quality (Bansal & Chen, 2006). During thermal treatment of milk, β-LG tends to aggregate via disulfide bonds with κ-casein which dramatically reduces the rennetability of milk and impairs its quality for cheese making (Livney & Dalgleish, 2004). With respect to health effects, β-LG can also be problematically for some consumers. Due to its compact globular structure and its resistance to gastric conditions, it is the main allergen in bovine milk. A variety of methods has been tested to reduce the allergenicity. Through the reduction of disulfide bonds and protein hydrolysis, β-LG becomes sensitive to peptic digestion and loses its allergenicity partly till completely (del Val et al., 1999; Pecquet et al., 2000).

2.1.1 Structure

β-LG consists of 162 amino acid residues with a molecular weight of approximately 18.3 kDa (Eigel et al.). The molecule contains **five cysteine residues**, where four of them are involved in two disulfide bridges (Figure 2-1). Cys^{66} forms a disulfide bridge with Cys^{160} that is located at the surface, close to the C-terminus. The second bridge between Cys^{106}-Cys^{119} is in the inner of the protein (Papitz et al., 1986). The disulfide bonds stabilize the tertiary structure of the protein, which contributes to the high resistance against peptic digestion and hence to its allergenicity (del Val et al., 1999). The thiol group of Cys^{121} is free and buried between β-strand H and the α-helix (Burova et al., 1998). Contradictory results indicate, that the disulfide bond of Cys^{106} exists in equilibrium between Cys^{119} and Cys^{121} (Ferranti et al., 2011; Brownlow et al., 1997). Due to the limited accessibility of the free thiol group its reactivity is low, but it is strongly influenced by the pH value (Burova et al., 1998). It has been shown, that the reactivity of thiol group increases with increasing pH from 4 to 8.5 (Kehoe et al., 2007). A remarkable increase in reactivity was observed above pH 6.7 (Dunnill & Green, 1966). The dissociation degree and the accessibility of the thiol group cause the change in its reactivity. Since the pK value of the group is 8.5, the fraction of the thiolate anion increases until pH 8.5 and thus the reactivity of the group which is strongly nucleophilic (Fernandes & Ramos, 2004; Thurlkill et al., 2006). The accessibility of Cys^{121} is increased at a pH above 7.4 due to the Tanford transition (Qin et al., 1998a).

The **secondary structure** of β-LG comprises 15% α-helix, 50% β-sheet and 30% random coil according to spectroscopic methods (Sawyer & Kontopidis, 2000b). The **tertiary structure** is dominated by the β-barrel, the hydrophobic cavity inside the protein. This calyx is made of the antiparallel β-strands A-D forming one sheet, and strands E-H forming a second. A three-turn α-helix is located on the outer surface. The ninth β-strand (I) flanking the first strand forms together with the AB loop the dimer interface. The dimeric structure is stabilized by twelve hydrogen bonds and two ion pairs (Brownlow et al., 1997; Papitz et al., 1986). Between pH 6 and 8 the structure undergoes a pH-induced conformational change, known as Tanford transition, which involves mainly the EF loop acting as a gate of the calyx. At a pH value below 7 it blocks the entrance of the calyx, at a pH value above 7.4 the loop is folded back and reveals the cavity of the protein (Qin et al., 1998a; Tanford et al., 1959). As mentioned above the accessibility of the free thiol group is also influenced by the Tanford transition and becomes more available if the EF loop flips into the “open” position.

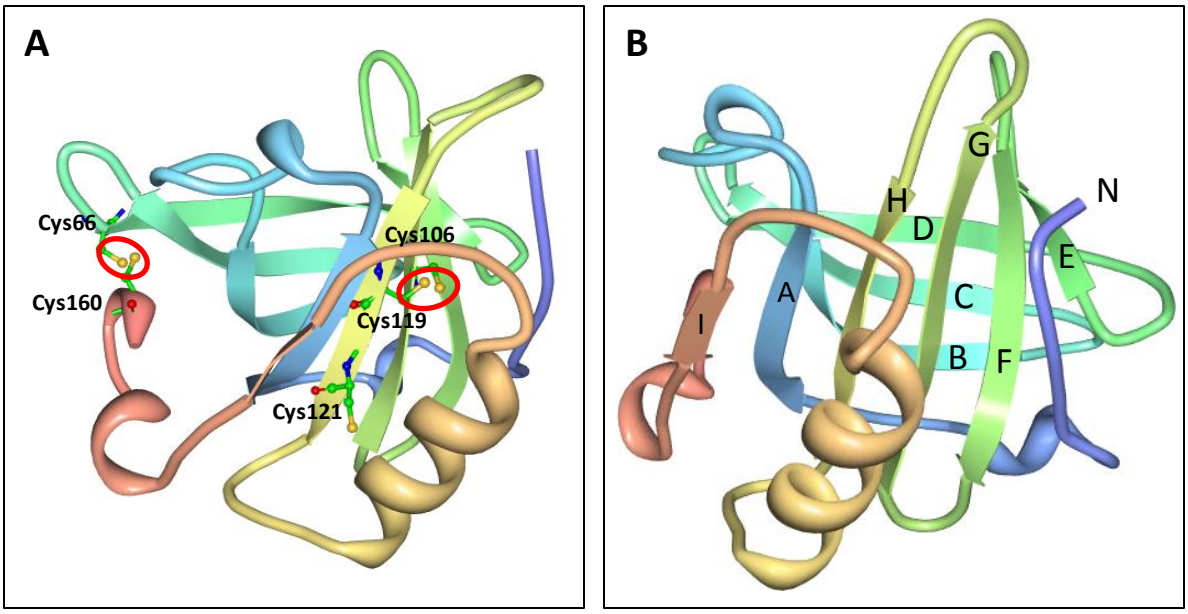

Figure 2-1: Schematic structure of β-lactoglobulin. A) The five cysteine residues and the two disulfide bonds (Cys^{66}-Cys^{160}, Cys^{106}-Cys^{119}) are indicated. B) β-strands A-I are indicated. Diagram was drawn by PDB Protein Workshop 4.2.0 with file 3NPO of Protein Data Bank RCSB provided by Loch et al. (2011) (Moreland et al., 2005).

In its **quaternary structure** β-LG is mainly present in monomeric or dimeric form, but the equilibrium is influenced by various parameters, as pH value, ionic strength, temperature and protein concentration (Figure 2-2). At physiological conditions the dimer is predominant (Aymard et al., 1996). In the pH range 3.7-5.2, just below the isoelectric point, β-LG associates to larger oligomers, like octamers, which is enhanced by a decrease in ionic strength and temperature (Verheul et al., 1999). With increasing distance from the isoelectric point (pI = 5.2) the repulsive forces get stronger and shift the equilibrium towards the monomeric form, which is prevalent at pH 2 and above 7.5 (Yan et al., 2013). At a basic pH (pH > 8.0) intermolecular disulfide bonds are formed and induce the irreversible formation of larger aggregates (Verheul et al., 1999). Beside the pH range 3.7-5.2, the self-association of β-LG to dimers increases with increasing ionic strength, because ions screen the electrostatic repulsion (Gottschalk et al., 2003; Renard et al., 1998).

At a neutral pH, the monomer-dimer equilibrium can also be shifted by covalent modifications of β-LG. The modification of the free thiol group of Cys^{121} by a reagent enhances the dissociation into monomers (Kontopidis et al., 2004). It was suggested that the introduced group destabilizes the rigid hydrophobic core and the nearby dimer interface (Sakai et al., 2000; Burova et al., 1998). The extent of the destabilizing effect seems to be dependent on the properties of the thiol reagent. Larger molecules, like 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), or charged groups can induce a molten globule-like tertiary structure, beside the dissociation into monomers (Sakai et al., 2000; Cupo & Pace, 1983).

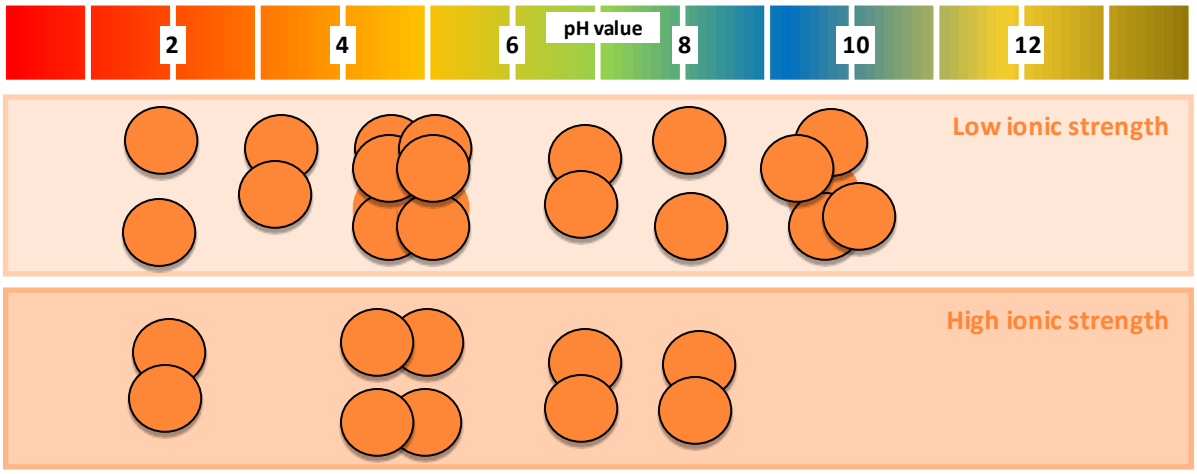

Figure 2-2: Schema of quaternary structure of β-lactoglobulin as a function of pH value and ionic strength.

2.1.2 Denaturation

The denaturation of β-LG can be induced by different triggers, where temperature, pressure, pH value and chemical denaturants are the most important ones. With respect to food processing, thermal denaturation is the most relevant process and will be discussed in detail at first.

The thermal denaturation of β-LG is a stepwise process and strongly influenced by reaction conditions like pH value, temperature, protein concentration and ionic strength. Different models have been developed to describe the process, like the two-state model and the dissociation coupled unfolding model (Busti et al., 2005; Roefs & Dekruif, 1994). But no model meets the requirements of a universal validity, all are limited to specific reaction conditions. Under natural conditions, like in milk, the process comprises of three main steps: dissociation into monomers, structural unfolding and aggregation (Figure 2-3).

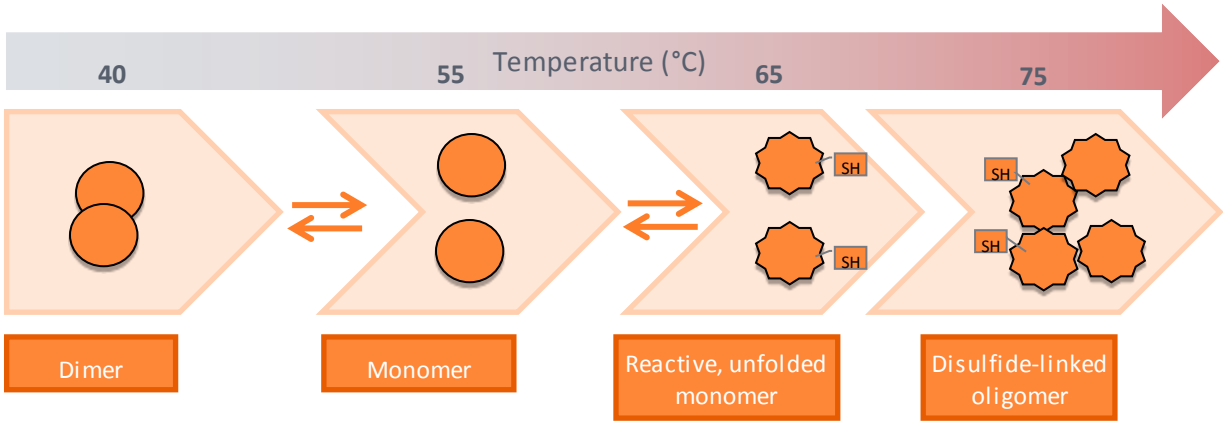

Figure 2-3: Scheme of thermal denaturation of β-lactoglobulin under physiological conditions.

With increasing temperature the monomer-dimer equilibrium is shifted to the **monomeric form** and at 55 °C dimers are completely dissociated (Aymard et al., 1996). At temperatures between 60 °C and 70 °C the protein structure starts to partially unfold. This state is called **molten globule** state and describes a nearly native-like secondary structure with a partially disordered tertiary structure. The free thiol group of Cys^{121} becomes exposed and reactive and hydrophobic residues are revealed (Iametti et al., 1996). The unfolding is considered to be a first-order reaction at neutral pH and relatively low temperatures (65-75 °C) (de Wit & Swinkels, 1980; Relkin, 1996). With respect to reversibility of denaturation, temperatures of 65-70 °C seem to be a threshold for short term heat treatments. Between 70 °C and 85 °C small aggregates are formed via thiol/disulfide exchange reactions (**chemical aggregation**) and the denaturation becomes **irreversible** (Iametti et al., 1996; de Wit, 2009). It has been suggested that the exposed free thiol group reacts intramolecularly with Cys^{106}-Cys^{119} resulting in a free Cys^{119}. This free thiol group reacts with the Cys^{66}-Cys^{160} disulfide bond of another molecule, which is located at the surface of the protein (Creamer et al., 2004; Kehoe et al., 2007).

After the early stages of aggregation, larger aggregates are built by non-specific, non-covalent (hydrophobic and salt-induced) interactions (**physical aggregation**). The disulfide-linked oligomers act as building blocks for physical aggregation, which increases with heating time (Schokker et al., 1999). Below 75 °C non-covalent interactions contribute little to the overall aggregation. At higher temperatures the dissociation and unfolding would be very fast, aggregation becomes rate limiting and only small disulfide-linked aggregates would be formed (Roefs & Dekruif, 1994).

The **free thiol group** of Cys^{121} plays a crucial role in the thermally induced aggregation. It initiates the thiol/disulfide exchange reactions and thus the intermolecular disulfide bonds. The exchange reactions stop, if all thiolates are oxidized to disulfides. Hoffmann et al. (1997) confirmed that the formation of disulfide-linked oligomers is completely inhibited when β-LG is heated in the presence of a thiol-blocking agent. At the early stage of heat treatment two monomeric forms were observed: native unfolded monomers with an exposed thiol group of Cys^{121} and non-native monomers with an exposed thiol group of Cys^{119}. The presence of the free Cys^{119} can be explained by intramolecular thiol/disulfide rearrangement during heating. The molten globule with the reactive Cys^{121} can become reversible after cooling (Croguennec et al., 2003; Croguennec et al., 2004).

Modification or substitution of the free thiol group enables an effective reduction of aggregation and can inhibit the irreversibility of the denaturation reaction to a certain extent. Burova et al. (1998) modified Cys^{121} with a thiol reagent, which resulted in the suppression of aggregation and allowed a refolding to the native-like structure. On the other hand, the thermal stability and thus the denaturation temperature were reduced by the covalent modification. Cho et al. (1994) introduced a

sixth thiol group by site-directed mutagenesis of β-LG. The additional disulfide bond substantially enhanced the thermal stability and inhibited chemical aggregation.

Concerning the overall reaction kinetics two rate limiting steps have been reported. According to Verheul et al. (1998) at low heating temperatures (67-78 °C), at pH values close to the isoelectric point and at a high ionic strength the structural unfolding is the rate limiting step. In contrary, at high heating temperatures (78-82 °C), a pH value far from the isoelectric point and at low ionic strength the aggregation reaction is rate limiting.

As mentioned above the reactivity and accessibility of the free thiol group strongly depends on the **pH value**. Thus, the thermal induced aggregation is very sensitive to this parameter. Bauer et al. (1998) reported that the formation of thiol/disulfide stabilized oligomers increased from pH 7.0 to 8.7 (Bauer et al., 1998). Hoffmann & van Mil (1997) and Hoffmann & van Mil (1999) investigated the denaturation at a pH range between 6.0 and 8.0. They observed that the aggregation rate increased and the size of the aggregates decreased at higher pH values. At a high pH value a large number of reactive intermediates with an exposed thiol group, were formed in early stages of heat treatment. The number of reactive thiol groups was quickly reduced and the aggregation process was terminated, because small disulfide-linked oligomers without a reactive thiol group were formed. In contrast, at a very acidic pH value (pH 2.0) the thiol groups were very unreactive and their contribution to the aggregation process would be negligible. At pH values close to the isoelectric point physical aggregation was promoted because of the low intermolecular charge repulsion (Zúñiga et al., 2010).

The **ionic strength** has two opposing effects (Renard et al., 1998; Schokker et al., 1999; Bauer et al., 1998). A high ionic strength increases the thermal stability due to decreased intramolecular repulsion and thus, higher conformational stability. On the other hand, a high ionic strength screens intermolecular repulsive forces and enhances the non-covalent aggregation rate. Since the unfolding reaction is the crucial initial step, a high ionic strength could finally lead to a reduced aggregation rate. The protein concentration mainly influences the size of aggregates. As Hoffmann & van Mil (1997) and Iametti et al. (1996) observed, the increase in concentration also increased the aggregate size.

Small **structural differences** can significantly influence the thermal stability of β-LG. Manderson et al. (1998) investigated the denaturation and aggregation behavior of the genetic variants A, B and C. They found out that the variant C has the highest and variant A the least stability, which was recently confirmed by Keppler et al. (2014c). Barbiroli et al. (2011) observed a stabilizing effect of non-covalent bound ligands. Bound palmitate induced a more compact structure and stabilized the

hydrophobic core and the dimer interface of the protein. It was also suggested that the bound fatty acid impeded the movement of the helix region, which retarded the exposure of the Cys^{121}. Mulsow et al. (2009) showed the stabilizing effect of the covalent modification of amino groups. Glycation enhanced the denaturation temperature of β-LG, probably because of the higher hydrophilicity of the protein which reduces hydrophobic aggregation.

Beside thermal denaturation, **pressure induced denaturation** of β-LG is practically relevant. Pressure denaturation is a stepwise process and starts with a partial collapse of the β-barrel and the exposure of the free thiol groups (up to 50 MPa), followed by revealing of hydrophobic regions (up to 123 MPa) and finally irreversible denaturation through thiol/disulfide exchange reactions at higher pressures (>200 MPa) (Stapelfeldt & Skibsted, 1999).

Uversky et al. (1997) reported that β-LG underwent conformational changes with increasing solvent hydrophobicity. **Organic solvents** caused the formation of a denatured intermediate state, as the molten globule state. The molten globule state was also induced by other mild denaturants and at acid and alkaline pH values. β-LG has very high pH stability and has even at pH 2.0 a native like structure (Molinari et al., 1996). **Chaotropic agents**, as urea, cause the unfolding of the protein which is irreversible due to intra- and intermolecular thiol/disulfide exchange reactions. Yagi et al. (2003) investigated the urea induced unfolding behavior of β-LG mutants in which Cys^{121} was replaced by alanine, serine or valine. The unfolding reaction was completely reversible for all mutants due to the prevented thiol/disulfide exchange reactions.

2.1.3 Binding properties

β-LG is similar in sequence and structure to the human serum retinol binding protein and belongs to the lipocalin family, a group of β-barrel containing proteins with hydrophobic binding abilities. Hence, some members of the lipocalins are also known for their specific transport function, it was suggested that the binding of retinol or fatty acids in the calyx is the biological function of β-LG (Sawyer & Kontopidis, 2000a). But the question of the physiological function is still in discussion and no clear answer can be made so far. The X-ray crystallography of β-LG in complexes with various ligands revealed that the central cavity is the main binding site for hydrophobic compounds but external binding sites have been suggested as well (Wu et al., 1999; Qin et al., 1998b; Wang et al., 1999). The ligand is bound by **non-covalent** forces like hydrophobic interactions, hydrogen bonding and van der Waales forces (Ozdal et al., 2013). The interaction of β-LG with a wide range of potential ligands has been studied. The most prevalent ones are retinol, fatty acids, vitamin D_2, cholesterol and phenols (Kontopidis et al., 2004; Wang et al., 1999). The protein provides a strong affinity for various

ligands and thus a low specificity of the binding (Konuma et al., 2007). The non-covalent binding reactions were mainly analyzed by fluorescence measurement and equilibrium dialysis (Kontopidis et al., 2004).

The interaction of the protein and a ligand influences the properties of both. The structure, stability, digestibility and functional properties of β-LG can be changed by ligand binding (Considine et al., 2007; Barbiroli et al., 2011; Stanic-Vucinic & Velickovic, 2013). β-LG-retinol complexes were found to be less susceptible to trypsin because of the more compact structure (Puyol et al., 1993). On the other hand the protein stabilized the ligand, but also influences its antioxidant activities and its bioavailability: EGCG bound to β-LG was protected from oxidative degradation (Shpigelman et al., 2012). The binding of aroma compounds decreased their release rate and their headspace concentration (Tromelin & Guichard, 2003).

Besides the binding of non-covalent ligands, β-LG can also be **modified covalently**. The functional groups of amino acid residues, like amino and thiol groups, can act as a binding site for covalent binding ligands. β-LG contains 15 free amino groups from lysine residues and a further available amino group from the N-terminal α-amino group of leucine (Morgan et al., 1999). As mentioned above only one free thiol group of Cys^{121} is available for potential ligands (chapter 2.1.1). Covalent modifications can occur naturally or were done for techno-functional reasons. The usage of the protein as a transporter for reactive, bioactive ligands is a new approach.

One of most relevant **natural occurring covalent modification** in foods is the glycation during Maillard reaction. This involves the condensation of reducing sugars with the ***ε***-amino group of lysyl residues under heating resulting in Amadori products. Depending on the kind of sugar and the extent of glycation thermal stability and functional properties like foaming and emulsifying properties of the protein can be improved (Chevalier et al., 2001). The denaturation temperature can be increased by about 5 °C if the degree of lysine modification was 22% (Mulsow et al., 2009). The reason is probably the enhanced hydrophilicity due to modification by sugar moieties which suppresses the aggregation reaction. On the other hand the Gibbs energy of unfolding of glycated β-LG was reduced by 20% (Van Teeffelen, Annemarie M.M. et al., 2005).

Beside the Maillard reaction, electrophilic compounds, like isothiocyanates, quinones, and aldehydes, can react with β-LG in food systems. Phenolic compounds are susceptible to oxidation and can build reactive *o*-quinones. By nucleophilic addition the *o*-quinones can react with functional groups of the protein. Mainly amino and thiol groups are the reaction partners, but methionine and tryptophan side chains have been reported as well (Rawel & Rohn, 2010). Such covalent reactions could affect conformational and functional properties, surface hydrophobicity, thermal stability,

digestibility and the nutritional quality of the protein (Ali et al., 2013; Rawel et al., 2003; Rawel et al., 2001). Unwanted covalent modifications of β-LG can be induced by reactive products of lipid oxidation. These reactions may lead to reduction of the nutritional value and functionality may be impaired, which could cause negative changes of sensory properties. The aldehyde malondialdehyde arose from oxidation of polyunsaturated fatty acids (PUFAs) and reacts with amino groups of the protein by formation of Schiff base and dihydropyridine-type adducts (Gürbüz & Heinonen, 2015). Since the fortification of foods with PUFAs is a continuing trend, the interaction between proteins and lipids should be considered and needs further investigation.

Various **chemical modifications** have been tested to improve the **techno-functional properties** and the digestibility of β-LG (Stanic-Vucinic & Velickovic, 2013). By succinylation the stability towards gastric conditions was even more improved, providing a suitable protection for gastro-sensitive compounds (Poulin et al., 2011). Whereas ethylation increased the peptic digestibility of β-LG, which lowered its allergenicity (Chobert et al., 1995). The covalent binding of stearic acid improved the emulsifying and foaming properties and decreased the allergenicity as well (Akita & Nakai, 1990).

Table 2-1 provides an overview of studies which focused on the modification of the thiol groups of β-LG. Nevertheless the examined ligands differed in size and properties, some common observations were made:

- modification is more efficient at pH values above 7.0 due to the higher reactivity of the thiol groups at a basic pH (chapter 2.1.1)
- modification induces structural changes resulting in:
    - partially enfolded tertiary structure
    - monomer-dimer equilibrium shifted towards monomeric form
    - faster thermally induced unfolding
    - inhibited thermally induced aggregation reaction
    - increased reversibility of unfolding reaction

From the techno-functional point of view the most promising advantage of the thiol specific modification is the inhibited disulfide-linked aggregation during heating which enables the reversibility of the unfolding reaction to a certain extent.

A new approach is the usage of β-LG as a **transporter** for bioactive, covalent bound compounds. Only a small number of studies already addressed this topic. Ferranti et al. (2011) considered β-LG as a transporter for covalently bound glutathione and fatty acids. Rade-Kukic et al. (2011) and Keppler et al. (2014a) investigated the usage β-LG as a carrier for covalently bound AITC. It was suggested that the binding to the protein the volatile compound loses its pungent and lachrymatory effect, improves techno-functional properties of the protein and provides its antimicrobial and health promoting effects concurrently. The idea was already patented (Rade-Kukic & Schmitt, 2010). AITC reacted with amino and thiol groups with a moderate affinity. About four to six molecules AITC were bound per molecule protein, if ligand was present in excess (40:1 M/M). Beside the above mentioned structural changes, AITC cleaved the disulfide bond of Cys^{66}-Cys^{160}, changed the emulsifying and foaming properties of the protein and blocked several tryptic cleavage sites (Keppler et al., 2014a). Furthermore the pungent odor and taste of AITC was significantly reduced through the binding to β-LG and the modified protein showed an antimicrobial activity (Rade-Kukic & Schmitt, 2010).

Table 2-1: Studies about covalent modification of thiol groups from β-lactoglobulin.

Ligand	Binding sites	Analytical methods	Results	References
Modification of thiol groups				
Mercapto propionic acid, mercaptoethanol	Cys^{121}	CD, gel-permeation-HPLC, high-sensitivity DSC	• Dimer-monomer equilibrium is shifted to monomeric form • Thermal stability reduced (unfolding reaction) • Thermal unfolding reversible	(Burova et al., 1998)
Mercapto propionic acid, mercaptoethanol, mercaptoethylamine, propanethiol	Cys^{121}	optical rotation	• Urea induced denaturation reversible • Reduced conformational stability, largest effect had aminoethyl derivate	(Cupo & Pace, 1983)
Thioredoxin/NADP	Cys^{66}, Cys^{106}, Cys^{119}, Cys^{121}, Cys^{160}	*in vitro* digestibility, SDS-PAGE, *in vivo* allergenicity test	• Reduction of disulfide bonds increased pepsin sensitivity and reduced allergenicity	(del Val et al., 1999)
Glutathione (oxidized)	Cys^{119}/ Cys^{121}	LC-MS	• Naturally occurring glutathionated β-LG in water buffalo milk • Structural features as transport protein were confirmed • Glutathionylation was reversible, can be split by mercaptoethanol	(Ferranti et al., 2011)
2-pyridine disulfide, DTNB	Cys^{121}	DSC, spectro-photometry	• PDS was more reactive than DTNB • Cys^{121} showed low reactivity (pH 7) • High NaCl concentration reduced SH/SS exchange reaction • Modification reduced protein stability and prevented dimerization during heating	(Owusu-Apenten et al., 2003)
DTNB	Cys^{121}	CD, fluorescence quenching, analytical ultracentrifugation, ^{1}H-NMR	• Modification induced confirmatory changes, molten globule-like structure at pH 7.5 and dimer dissociation	(Sakai et al., 2000)
IAEDANS, DTNB	Cys^{66}, Cys^{106}, Cys^{119}, Cys^{121}, Cys^{160}	spectro-photometry, MALDI MS/MS, HP-GPC, RP-HPLC	• At pH 7 SH group was inaccessible for IAEDANS, at pH 8 partially accessible for DTNB (0.18 M SH/M protein), in the presence of 8 M urea 0.99 M SH/M protein were accessible • Non-native SH groups were released at Cys^{66} and Cys^{160} after heating, indicating the SH/SS exchange of the free SH group from Cys^{119} or Cys^{121} with the disulfide bond Cys^{66}-Cys^{160}	(Kehoe et al., 2007)

MTSL (spin lable), MMTS	Cys^{121}	Fluorescence, DTNB-assay, ESR	• Modification did not affect retinol binding in the calyx but reduced the affinity for palmitic acid binding, indicating the fatty acids are bound in a hydrophobic pocket at the surface of the protein	(Narayan & Berliner, 1998)
p-Chloro-mercuribenzoate	Cys^{121}	spectro-photometry	• Reactivity of SH group was low at pH 2.0-6.7 and increased above 6.7 • Higher accessibility of Cys^{121} above pH 7.4	(Dunnill & Green, 1966)
Acrylamide	Cys^{66}, Cys^{106}, Cys^{119}, Cys^{121}, Cys^{160}	LC-MS, LC-MS/MS, protein hydrolysis	• One thiol group per intact molecule reacted • PH 9.5 allowed disengagement of Cys^{160} from disulfide bond and reaction with the ligand	(Curcuruto et al., 1998)
Modification of amino & thiol groups				
Allyl isothiocyanate	various amino and thiol groups	DTNB and OPA-assay, ANS fluorescence, CD, characterization of functional properties	• Ligand cleaved disulfide bonds at higher concentrations • Enhanced protein hydrophobicity • Changes in secondary and tertiary structure • Dissociation of dimers • Reduced heat induced aggregation at pH 7 and increased aggregation at pH 4 • Changed foaming and emulsifying properties	(Rade-Kukic et al., 2011)
Allyl isothiocyanate	various amino and thiol groups	Fluorescence quenching, equilibrium dialysis, headspace-water equilibrium, MS, LC-MS/MS	• Binding to one thiol group and 3 amino groups • AITC cleaved disulfide bond Cys^{66}-Cys^{160}	(Keppler et al., 2014a)
Allyl isothiocyanate	various amino and thiol groups	Protein hydrolysis, LC-MS/MS	• AITC binding blocked several cleavage sites of trypsin	(Keppler et al., 2014b)
5-Caffeoylquinic acid, dicaffeoylquinic acid (o-quinone)	ε-amino group of several lysine residues, Cys^{121}	CD, DSC, SDS-PAGE, RP-HPLC, MALDI-TOF-MS, protein hydrolysis, ANS fluorescence, DTNB-assay, TNBS-assay, fluorescence quenching, TEAC-assay, DPPH-assay, molecular modeling, characterization of functional properties	• Enhanced antioxidative properties of protein • Changed secondary structure, increase in random coil fraction • More hydrophilic surface property • Changed functional properties (solubility, emulsification) • Higher thermal stability, lower stability with respect to unfolding reaction	(Ali et al., 2013)

Quercetin, rutin (o-quinone, p-quinone methide derivatives)	free amino and thiol groups, tryptophan	SDS-PAGE, , TNBS-assay, fluorescence quenching, monobrombimane- assay, RP-HPLC, SELDI-TOF-MS, CD, ANS fluorescence, protein hydrolysis	• Quercetin was more reactive than rutin • One ligand molecule was bound by one protein molecule • Increased surface hydrophilicity • Perturbation of secondary and tertiary structure, especially by rutin • Solubility at pH 4 decreased	(Rawel et al., 2003)

Abbreviations: AITC – Allyl isothiocyanate, ANS – 1-Anilino-8-naphthalenesulfonate, CD – Circular dichroism, DPPH – 1,1-Diphenyl-2-picrylhydarzyl, DSC – Differential scanning calorimetry, DTNB – 5,5'-dithiobis(2-nitrobenzoic acid), EGCG – (-)-Epigallocatechin-3-gallate, HP-GPC – High performance gel permeation chromatography, IAEDANS – 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid, LC-MS – Liquid chromatography–mass spectrometry, MALDI – Matrix-assisted laser desorption ionization, MMTS – Methylmethanethiosulfonate, MS/MS – tandem mass spectrometer, MTSL – (1-oxy-2,2,5,5-tetramethylpyrrolinyl-3-methyl)-methanethiosulfona, NADP – Nicotinamide adenine dinudeotide phosphate, PDS – 2-pyridine disulfide, SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SELDI – surface-enhanced laser desorption ionization, TEAC – Trolox equivalent antioxidative capacity assay, TNBS – Trinitrobenzenesulfonic acid, TOF – Time-of-flight.

2.1.4 β-Lactoglobulin as nanotransporter

Research activities are increasingly focused on the use of β-LG as a functional ingredient, since it has the GRAS (generally recognized as safe) status, a high nutritional value, desired technological and versatile functional properties (de Wit, 1998). Beyond that, it is remarkably stable against gastric conditions and can protect bound ligands against the harsh milieu in the stomach as well as against degradation by other compounds of the complex food matrix (Bossios et al., 2011). All these favorable properties qualify β-LG as a suitable transporter for the delivery of bioactive compounds. Furthermore, new process developments facilitate enhanced protein quality and enable the fractionation of single whey proteins in a cost-efficient way (Etzel, 2004).

The transport function of β-LG was repeatedly discussed as its natural physiological function (Sawyer & Kontopidis, 2000a). Besides the investigation of naturally occurring interactions of β-LG with hydrophobic ligands, many studies were performed concerning the targeted, non-covalent binding of bioactive agents (Bello et al., 2012; Kanakis et al., 2011; Li et al., 2012; Loch et al., 2013; Shpigelman et al., 2012). β-LG as a transporter for covalently bound ligands is a more recent approach. As far as known, only allyl isothiocyanate (AITC) was investigated as covalent bioactive ligand for β-LG (Keppler et al., 2014a; Rade-Kukic et al., 2011). With respect to the continuing consumer trend for health and wellbeing, β-LG is a promising carrier to enable the enrichment of bioactive compounds in food.

2.2 Bioactive compounds from garlic

Garlic, *Allium sativum* L., is a traditional flavoring agent and known for its health benefits. Investigations about the medical effects of garlic revealed that its organosulfur compounds and their transformation products are primarily responsible for the pharmacological effect (Butt et al., 2009). Furthermore, the volatile organosulfur compounds, like thiosulfinates, contribute mainly to the typical smell and pungent taste of garlic and are a part of the defense mechanism of the plant (Amagase et al., 2001). Allicin (S-allyl 2-propene-1-sulfinothioate) is the major thiosulfinate in freshly crushed garlic (allicin content 0.2-0.6%) and contributes to 70-75% of total thiosulfinates (Lawson, 1996). For the first time, allicin was discovered in garlic by Cavallito & Bailey (1944). It is a fairly hydrophobic agent and its partition coefficients in octanol/water is log P = 1.1 (Miron et al., 2000).

2.2.1 Formation and stability

Allicin is not present in intact garlic cloves. Before processing the major sulfur-containing compounds in garlic are the non-proteinogenic amino acid S-allyl-L-cysteine sulfoxide (alliin) and γ-glutamyl-S-allyl-L-cysteine. If the plant tissue is damaged, like by chopping or chewing, allicin is formed by an **enzymatic reaction** within a few seconds (Figure 2-4) (Stoll & Seebeck, 1949; Cavallito & Bailey, 1944). The precursor **alliin** is hydrolyzed by the enzyme **alliinase** in presence of the cofactor pyridoxal-phosphate leading to the formation of allyl sulfenic acid, pyruvic acid and ammonia. Two molecules of allyl sulfenic acid condensate spontaneously to one molecule of allicin (Trio et al., 2014). The enzymatic activity of alliinase is inhibited by common protein denaturants. Hence, acidic conditions like in the stomach (pH < 3.5), cooking of garlic, as well as addition of ethanol inactivates the enzyme and thus the formation of allicin (Lawson, 1996). Alliin is only present in garlic (*Allium sativum*) and ramsons (*Allium ursinum*). Onions contain the isomer isoalliin (trans-(+)-S-(1-propenyl)-L-cysteine sulfoxide) (Lawson, 1996).

Beside the strong odor, a decisive shortcoming of allicin is its instability. Various authors investigated the chemical and biological **half-life** of allicin, whereby biological half-life was expressed as antibacterial activity. Different parameters influencing the stability have been discovered: temperature (Lee et al., 2014; Fujisawa et al., 2008b; Lawson & Gardner, 2005), pH value (Lee et al., 2014; Lawson & Hughes, 1992), solvent (Fujisawa et al., 2008a; Lawson, 1996; Freeman & Kodera, 1995), and concentration (Lee et al., 2014; Lawson, 1996). The **degradation products** comprise mainly of ajoenes, allyl sulfides and vinyldithiins (Figure 2-4), their prevalence is determined by external conditions (Lawson, 1996).

Under acidic condition thiosulfinates are relatively stable, but at **pH values** above 6 their degradation is remarkable accelerated (Lee et al., 2014). Furthermore, with increasing temperatures the stability of thiosulfinates decreases. Fujisawa et al. (2008b) determined a half-life of 347 days at 4 °C of allicin, whereas at room temperature it was 9 days and at 37 °C only one day. A high thiosulfinate concentration has also a negative effect on the stability of the same (Lee et al., 2014).

Although, allicin is readily soluble in non-polar **solvents**, the stability in n-hexane and ethyl acetate is poor. A higher stability was shown in more polar solvents, like methanol, ethanol and water (Freeman & Kodera, 1995; Fujisawa et al., 2008a). The highest stability was reported to be in 20% aqueous ethanolic solution, i.e. a chemical half-live of 12 d at room temperature (Fujisawa et al., 2008a). The increased stability in polar solvents is probably caused by the formation of hydrogen bonds with water or the hydroxyl groups of ethanol. The major degradation products in aqueous solutions are diallyl-, allyl methyl-, di- and tri-sulfides, while in non-polar solvents allicin is mainly transformed into 2-vinyl-4H-1,3-dithiin, 3-vinyl-4H-2,3-dithiin and (E/Z)-ajoene (Lee et al., 2014).

Freeman & Kodera (1995) investigated the stability of allicin in the blood cell and plasma fraction and observed that allicin immediately reacts with compounds in the blood. Since it was not detectable after 3 minutes in the blood cell fraction, but in the plasma fraction, it had a half live of 50 min.

With respect to **food** products, the stability of allicin is not influenced by major components such as carbohydrates and proteins. But oils, especially those with a high concentration of polyunsaturated fatty acids, negatively influence the stability, resulting in a chemical half-life of 3.1 h in oil (Lee et al., 2014).

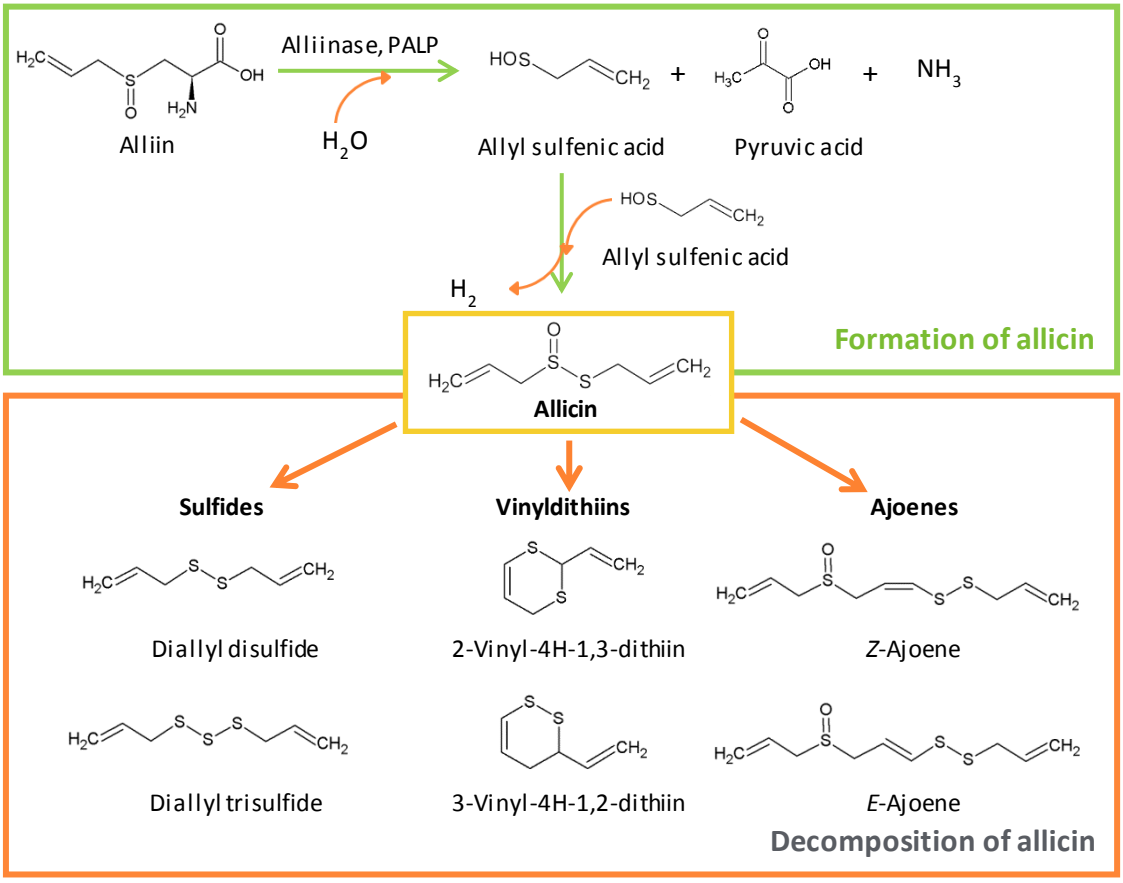

Figure 2-4: Basic formation and decomposition processes of allicin. PALP – pyridoxal-phosphate.

2.2.2 Garlic products

Table 2-2 summarizes the main organosulfur compounds and properties of different garlic preparations. The quantitatively most important product is **dehydrated garlic**, which is used as flavoring agent and also as nutraceutical in capsulated form. Garlic cloves are sliced or chopped and subsequently dried. Finally the dehydrated product is ground, powdered or granulated (Santhosha et al., 2013). Garlic powder is in its composition most similar to fresh garlic, although the content of

many bioactive agents are reduced due to processing conditions. The highest quality is obtained by freeze drying of the whole garlic cloves without damaging the cell structure, which could completely preserve the alliin content and alliinase activity.

Garlic powder supplements do not contain any allicin until disintegration in the gastrointestinal tract, where alliin can be converted to allicin by alliinase. The potential amount of allicin generated is called allicin yield or potential and is an important quality parameter. However, **allicin formation** from supplements can be a challenge that should not to be underestimated. The allicin-producing enzyme alliinase is irreversibly inactivated below pH 3.5, the usual range of gastric fluid. Therefore, most garlic powder supplements are enteric-coated to prevent the inactivation of alliinase in the stomach. But even at a natural pH value the enzyme activity decreases fast at body temperature (Lawson & Gardner, 2005). Lawson & Wang (2001) investigated the **allicin release** of 24 brands of enteric-coated garlic powder supplements under simulated gastrointestinal conditions. With one exception, all brands released less than 15% (on average 13%) of their claimed allicin potential, although the enteric coating effectively protected alliinase under gastric conditions. The authors suggested that an impaired alliinase activity and slow tablet disintegration were the main reasons for the low release. The alliinase activity can be impaired by harsh processing conditions and by the presence of tablet excipients. Significant variations in allicin release were also observed between different batches of the same brand (Lawson & Gardner, 2005). These variations clearly demonstrate the importance of controlling the allicin release under simulated gastrointestinal conditions of the used product, particularly in a clinical trial. Otherwise the results of the study are questionable and cannot be extrapolated to fresh garlic. It is likely that this problem is responsible for contradictory reports in the previous studies.

Garlic extracts are obtained by an aqueous ethanol extraction of whole or sliced garlic. After certain incubation the extract is filtrated, concentrated or dried. A variation of the fresh garlic extract is the **aged garlic extract** (AGE), which is stored at room temperature for up to 20 month (Butt et al., 2009). Due to the initial destruction of plant tissue, thiosulfinates, including allicin, are formed. During the aging process volatile, unstable organosulfur compounds are transformed into stable, less volatile compounds. γ-Glutamyl-cysteine is completely hydrolyzed to **S-allyl cysteine** (SAC). Due to protein hydrolysis cysteine is released and reacts with allicin to **S-allylmercaptocysteine**. After a storage time of three month thiosulfinates are no longer present due to degradation and evaporation (Colín-González et al., 2012).

The final product of fresh or aged garlic extraction can be lyophilized and contains mainly water-soluble organosulfur compounds such as SAC (content is about 0.25%), S-allylmercaptocysteine (SAMC) and only small amounts of oil-soluble compounds (Ried et al., 2010). AGE is only used as

nutraceutical, mainly in capsulated form, and is claimed to be "odorless", which means free of garlic taste and typical garlic "after breath". Furthermore it was suggested that AGE is safer and cause less adverse effects due to the absence of irritating compounds (Amagase, 2006).

Garlic essential oil comprises 0.2-0.5% of garlic clove and is obtained by steam distillation. The cloves are crushed in water and heat-distilled resulting in a separating oily liquid. Distillation is often combined with organic solvent extraction to increase the yield (Lawson, 1996). The major organosulfur compounds are di- and polysulfides, like DADS and diallyl trisulfide (DATS), water soluble compounds and allicin are completely eliminated. The essential oil is diluted with vegetable oil because of the strong smell and commercially available as garlic oil capsules (Amagase et al., 2001).

Oil macerates are produced by grinding garlic cloves and mixing them with vegetable oil. The present organosulfur compounds are degradation products of allicin, such as dithiins, ajoene and sulfides (Amagase et al., 2001). Oil macerates were used as condiments.

Table 2-2: Commercial garlic products.

Product	Main bioactive compound	Properties and usage
Freshly crushed garlic	Allicin	• Mash, used as food and flavoring agent • Highest allicin content • Strong taste and smell • Not stable, not storable
Dehydrated garlic	Alliin, alliinase	• Powder, granulates, used as supplement and spices • No allicin present, but allicin can be built when hydrated (allicin potential/allicin yield) • Alliin content and alliinase activity determine allicin potential
Aged garlic extract	SAC, SAMC, other water-soluble compounds	• Liquid or powder, used as supplement
Garlic essential oil	Diallyl disulfide, diallyl trisulfide, other di- and polysulfides	• Liquid, used as supplement and spice mixtures • Strong odor • No allicin or other water soluble compounds
Oil macerate	Dithiins, ajoene, sulfides	• Garlic flavor, used as condiment

Abbreviations: SAC – S-allyl cysteine; SAMC – S-allyl mercaptocysteine.

2.2.3 Delivery systems for allicin

Different methods have been tested to generate a stable, bioactive form of allicin. Two main approaches can be distinguished: 1) the transformation of the thioallyl group to another molecule via covalent reaction with allicin, 2) the stabilization of allicin itself by carrier systems. The first approach was patented two times. Ott (2005) and Parkin & Zhang (2011) described among other claims the transformation of the thioallyl-group to a **thiol containing protein** or peptide for the usage in nutraceuticals or food items. For *in vitro* and animal studies stable thioally-derivates have been synthesized with allicin and different thiol containing compounds, such as cysteine, GSH, captopril (D-3-mercapto-2-methylpropanoyl-L-proline, inhibitor of angiotensin converting enzyme) and 6-mercaptopurine (immunosuppressive drug) (Miron et al., 2012; Miron et al., 2010; Oron-Herman et al., 2005; Miron et al., 2004; Rabinkov et al., 2000). Especially the latter are not suitable for food products, but they could be used for multi-functional drugs. Miron et al. (2001) patented the formation of S-allylmercaptoglutathione (SAMG) for pharmaceutical use.

Lu et al. (2014) used the second approach (i.e. stabilization by carrier systems) by encapsulation of allicin in **liposomes** and reported an improved stability of allicin and a sustained-releasing potential of liposomes. Nikolic et al. (2004) examined the complexation of allicin with **β-cyclodextrin**, Wang et al. (2012) also encapsulated allicin with β-cyclodextrin and porous starch resulting in enhanced temperature and pH stability and solubility of allicin while retaining its antimicrobial activity. The three mentioned studies indicated all an enhanced stability of allicin by encapsulation or complexation, but did not show how much the half-life of allicin could be prolonged actually. Thus it cannot conclude if these techniques are suitable to overcome allicin's drawbacks and enable the enrichment in foods.

2.2.4 Metabolism and bioavailability

The metabolic pathway of organosulfur compounds of garlic is not completely known and only a few studies about their metabolism in the human body are published (Table 2-3). Especially the revelation of allicin's metabolism and bioavailability is difficult, because it is not detectable in blood, urine or stool, even after consumption of high amounts of freshly crushed garlic or pure allicin (Lawson & Wang, 2005). After *in vitro* addition to blood allicin disappears within a few minutes (Lawson & Wang, 2005; Freeman & Kodera, 1995). It has been shown that allicin easily permeates through cell membranes and enters erythrocytes, followed by a rapid reaction with **intracellular thiol groups**, causing its fast disappearance in blood (Miron et al., 2000). Glutathione (GSH) and cysteinyl residues of proteins have been reported as probable targets of allicin, resulting in the formation of the **S-allylmercapto-conjugates** and allyl sulfenic acid (Figure 2-5) (Pinto et al., 2006; Rabinkov et al.,

1998). The released ally sulfenic acid can also react with a thiol group and form a further derivate, or form again allicin through self-condensation (Borlinghaus et al., 2014). **SAMG** can undergo a SH/SS exchange with another GSH by releasing allyl mercaptan and formation of oxidized glutathione (glutathione disulfide, GSSG). **Allyl mercaptan** is an intermediary product and can be methylated by S-adenosylmethionine to **allyl methyl sulfide** (AMS) (Lawson & Wang, 2005).

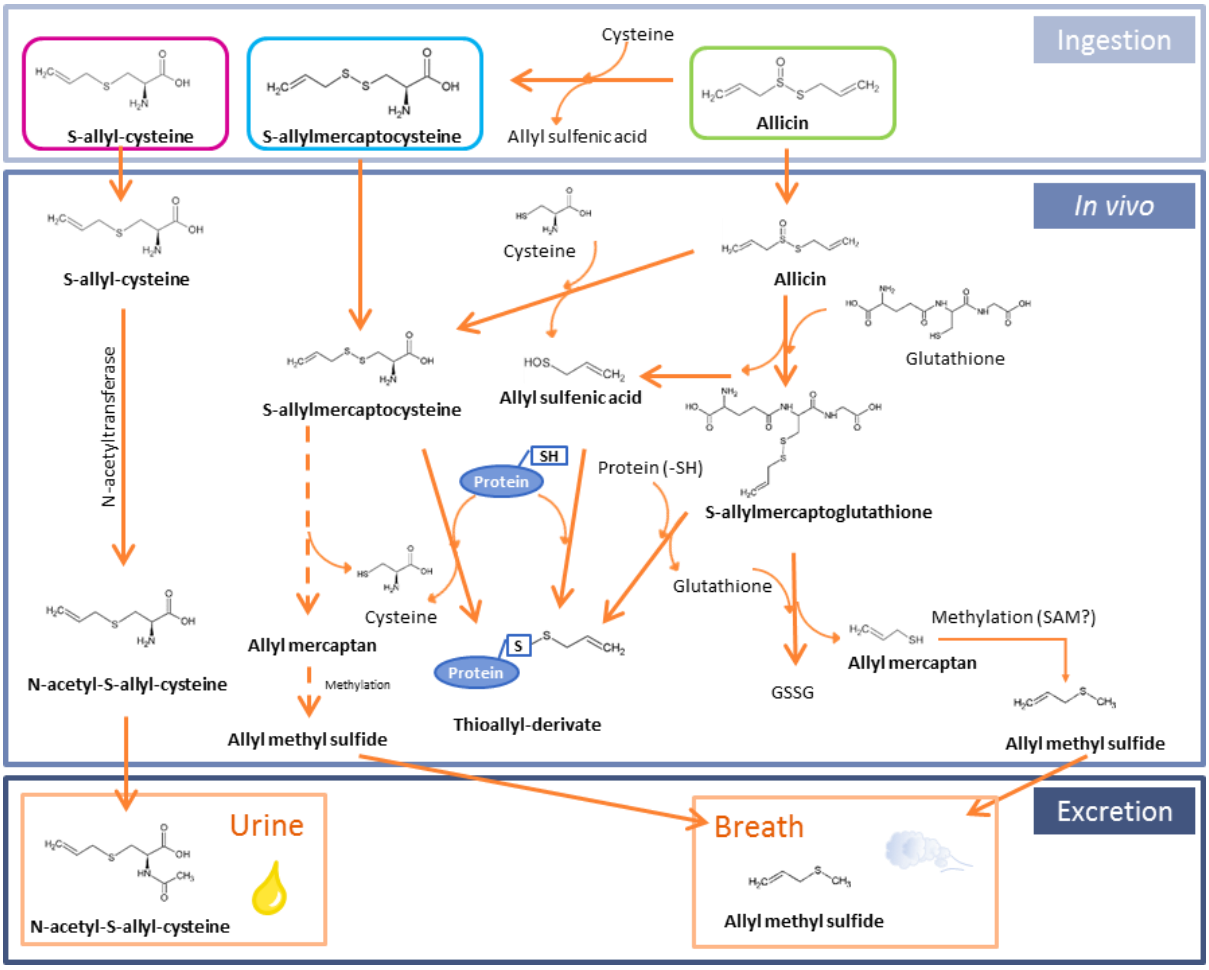

Figure 2-5: Main metabolic pathways of allicin, S-allyl cysteine and S-allylmercaptocysteine. Dotted arrow indicates a supposed pathway that has not been proved so far. GSSG – glutathione disulfide; SAM – S-adenosylmethionine.

Lawson & Wang (2005) analyzed the metabolism of allicin to **AMS** and found that allicin is almost completely (90%) converted into AMS (molar ratio 1:1), which makes AMS a suitable marker for the bioavailability of allicin. AMS is absent in breath gas without prior garlic consumption and increases in a dose-dependent manner after ingestion. The maximum level is reached after 3-4 hours and it is detectable up to 30 hours after consumption, indicating that AMS is a product of systemic metabolism (Taucher et al., 1996).

Besides AMS, other sulfur containing metabolites in the breath gas have been associated with garlic consumption. DADS, diallyl sulfide (DAS), AMS and allyl mercaptan have been detected as well, but at lower quantities (Taucher et al., 1996; Hansanugrum & Barringer, 2010). Since garlic products used in human studies contain not only allicin, other organosulfur compounds are present simultaneously and contribute to the sulfur containing metabolites as well. Therefore the association of a specific metabolite to allicin is limited.

Next to allicin the water soluble derivates SAC and SAMC are of particular interest in this work. **SAC** is the main bioactive component of AGE. Since it is far more stable than allicin, it was found in human blood after ingesting AGE. Nagae et al. (1994) descripted the pharmacokinetics of SAC in animal models (rats, mice, dogs). SAC was highly absorbed and distributed in plasma, liver and kidney. The bioavailability was 103% and 87% in mice and dogs, respectively. Furthermore, they revealed the main elimination pathway of SAC: acetylation of SAC by acetyltransferase in liver and/or kidney to N-acetyl-S-allyl cysteine (NASAC), which is excreted with urine. This elimination pathway is the only one known for SAC, because it is not metabolized to AMS in breath gas (Lawson & Wang, 2005). Cope et al. (2009) reported a high variability of the conversion rate of SAC to NASAC in humans. The half-life of NASAC ranged from "not detectable" to 5 h with high intraindividual variations. Kodera et al. (2002) confirmed the high bioavailability, resorption, and stability of SAC in a human study. Quantification of SAC in plasma resulted in a half-life of 10 h and a clearance time of more than 30 h.

SAMC is present in AGE in low quantities and it can also be formed by allicin reacting with free cysteine before or after absorption. In the metabolism of allicin, SAMC acts as a stable mediator that can transfer the thioallyl group via SH/SS exchange. SAMC is metabolized to AMS in breath gas in a molar ratio of 1:1, like allicin (Lawson & Wang, 2005). The detection in plasma was not tested yet.

The **bioavailability** of allicin and its water-soluble derivates seems to be high. According to an in vivo study with ^{35}S-labeled allicin in rats the absorption rate is at least 65%, thus the cumulative excretion in urine and feces was 86% (Lachmann et al., 1994). A human study showed that the molar amount of ingested allicin caused a similar extent of excreted AMS in breath gas (Lawson & Wang, 2005). The authors concluded an estimated absorption of at least 95%. However, from a chemical point of view one molecule allicin could theoretically produce two molecules AMS.

Table 2-3: Bioavailability analysis of allicin or its derivates in human studies.

Garlic product	Dose[1]	Analyzed metabolites		Comments	References
		Matrix	Metabolite		
Human studies					
Fresh garlic Garlic powder, enteric-coated capsule AGE	4 g (12.6 mg allicin) 1.4 g (12.8 mg allicin yield) 1.8 g (1.8 mg SAC)	Breath gas	AMS	Average AMS C_{max}=174 ng/l (after intake of fresh garlic) Average AMS C_{max}=207 ng/l (after intake of garlic powder)	(Lawson & Gardner, 2005)
Fresh garlic Allicin (synthesized), capsulated Garlic powder, enteric-coated capsule Garlic powder, dissolved, capsulated	7 g, 20 g (38 mg/108 mg allicin) 30 mg, 59 mg (allicin) 10.5 g (44 mg allicin) 3 g (38 mg allicin)	Breath gas	AMS	Average AMS C_{max}=1200 ng/l (after intake of 108 mg allicin from fresh garlic) Allicin, SAMC, DADS were about completely metabolized to AMS, SAC and DAS were not metabolized to AMS	(Lawson & Wang, 2005)
Fresh garlic AGE	10 g n.s.	Breath gas Blood	AMS, DAS, DADS SAC	AMS was main metabolite in breath gas SAC C_{max}= 500 nM (800 ppb)	(Rosen et al., 2001)
Fresh garlic	6 g (21 mg allicin)	Breath gas	AMS	AMS C_{max}=11 µg/l	(Suarez et al., 1999)
Fresh garlic	38 g	Breath gas	AMS	AMS C_{max}=90 µg/l	(Taucher et al., 1996)
Garlic powder, enteric-coated capsule (different brands)	12-21 capsules (48 mg allicin yield)	Breath gas	AMS	total AMS C_{AUC}=9.1 µg-h/l	(Lawson & Wang, 2001)
AGE	2.56 g/d	Blood	SAC	Average serum level of SAC in treated group was 220 ng/ml, in control group 100 ng/ml after 45 d	(Nantz et al., 2012)
AGE, liquid	4 ml (1.22 g dry matter)	Blood	SAC		(Budoff et al., 2004)
AGE	500 mg (0.67-0.8 mg SAC)	Blood	SAC	SAC C_{max}=12-25 ng/ml	(Kodera et al., 2002)
AGE	2.4-7.2 g (6-18 mg SAC)	Blood	SAC	SAC base line level was 30-60 ppb, after AGE intake > 100 ppb	(Steiner & Li, 2001)

[1]Dose of garlic product, dose of bioactive compound in parentheses if known. Abbreviations: AGE – Aged garlic extract; AMS – Allyl methyl sulfide; DADS – Diallyl disulfide; DAS – Diallyl sulfide; n.s. – not specified; SAC – S-allyl cysteine; SAMC – S-allylmercaptocysteine.

2.2.5 Bioactivity

Since ancient times garlic has been known for its therapeutic and health promoting properties, including anticancerogenic, antioxidative, antiatherosclerotic, antibacterial, antiinflammatory and antidiabetic activities (Borlinghaus et al., 2014; Trio et al., 2014; Miron et al., 2008). Cholesterol- and triglyceride-lowering properties have been mentioned as well, but further studies indicated that these features are questionable (Zeng et al., 2013; Gardner et al., 2007). A regular intake of a usual serving size, like one clove, is sufficient to exert health benefits (Witte et al., 1996; Steinmetz et al., 1994). Table 2-4 provides an overview of selected *in vitro* and *in vivo* studies about biological activities of allicin and its derivates SAC and SAMC.

According to many researchers **allicin** is the major biologically active compound in garlic and responsible for most of its health benefits (Borlinghaus et al., 2014; Butt et al., 2009; Miron et al., 2000). Since allicin is not detectable in blood after oral intake or *in vitro* addition, some authors suggest that allicin is not the bioactive compound of garlic (Lawson & Wang, 2005; Freeman & Kodera, 1995; Amagase, 2006). However, it has been suggested that allicin readily passes through cellular membranes and exerts many of its bioactivities by redox-reaction with intracellular thiol containing molecules and by its antioxidative activity (Miron et al., 2010; Rabinkov et al., 1998). The reaction with the SH-group of proteins, like enzymes, can change their structure and function and thereby allicin is able to regulate the activity of these. GSH and cysteinyl residues are the most prevalent targets, resulting in the formation of the corresponding **S-allylmercapto-conjugates**. The transformation of the thioallyl-moiety via SH/SS exchange reactions seems to play a crucial role for the mediation of allicin's effects and it is not clear if the thioallyl or the thiosulfinate group is the key pharmacophore (Miron et al., 2010; Hunter et al., 2005; Rabinkov et al., 1998).

Rabinkov et al. (2000) demonstrated the SH/SS exchange reaction by **SAMC** and **SAMG** with thiol containing enzymes resulted in inactivated S-allylmercapto-derivates in equal measure as found for allicin. The inactivation of the enzymes was reversible and could be restored by SH/SS exchange with another thiol containing compound. However, allicin showed a higher efficiency in enzyme inactivation than SAMC and SAMG, probably because one molecule allicin can generate two thioallyl-moieties, in contrast to SAMC and SAMG. The advantage of SAMC over allicin is its stability and it may serve as reservoir to prolong the activity of allicin (Miron et al., 2010). The reaction of allicin with cysteine or GSH is strongly dependent on pH value. With increasing pH in a range of 4.0 to 7.0 the reaction rate increased significantly, the maximum yield was observed at pH 8.4 (Miron et al., 2010; Rabinkov et al., 2000).

Horev-Azaria et al. (2009) clarified further the mechanism underlying the biological activities of allicin. Allicin influenced the **gene expression** and the **GSH level** of treated cells. The increased GSH

level was probably induced by up-regulation of glutamate cysteine lygaze modifier subunit, the rate limiting enzyme in GSH biosynthesis. The increased GSH synthesis enhanced the antioxidant potential of the cells, because only the concentration of the reduced form (GSH) was raised. The gene expression of phase II detoxifying enzymes thioredoxin reductase 1 and 2 and heme oxygenase-1 was up-regulated. This up-regulation could be induced by the mild oxidative conditions caused by the oxidant allicin, which leads to the activation of the redox-sensitive transcription factors. The induction of the Nrf2/Keap 1 system by allicin was reported and confirmed this hypothesis. The Nrf2/Keap 1 system is responsible for the expression of different antioxidative enzymes (Borlinghaus et al., 2014). The enhanced antioxidative cellular protective mechanisms induced by allicin can protect the cells from oxidative stress, which is associated with many pathological diseases. Other authors confirmed the lowering effect of intracellular reactive oxygen species (ROS) by allicin (Rabinkov et al., 1998). SAMC exhibits also a cell protective mechanism against ROS by scavenging peroxides and increasing the intracellular glutathione level to an even higher extent than allicin (Horev-Azaria et al., 2009; Imai et al., 1994).

The **redox-mediated mechanisms** induced by allicin or its thioallyl-derivates seem to be responsible for many of its health promoting effects, such as anticarcinogenic and antiatherosclerotic properties. Allicin showed diverse mechanisms of cardiovascular disease prevention: suppression of cholesterol biosynthesis (coenzyme A modulated), reduction of platelet-aggregation, reduction of hypertension (mediated by H_2S) and inhibition of endothelia cell damage caused by low density lipoprotein oxidation (Gonen et al., 2005; Shouk et al., 2014; Supakul et al., 2014; Zeng et al., 2013).

Epidemiological studies revealed that a high consumption of garlic is inversely associated with the incidence of different types of cancer (Fleischauer et al., 2000). Animal and *in vitro* studies confirmed the **anticarcinogenic** effect of allicin. The induction of apoptosis, suppression of metastasis and inhibition of cell proliferation are the mainly suggested underlying mechanisms (Trio et al., 2014; Arditti et al., 2005; Oommen et al., 2004). Similar anticarcinogenic effects were also shown for SAMC (Liang et al., 2011; Howard et al., 2007; Xiao et al., 2003; Shirin et al., 2001; Sigounas et al., 1997a).

Allicin is known for its **antibacterial** activity that was already described by Cavallito & Bailey (1944) and several times confirmed (Fujisawa et al., 2009; Cutler & Wilson, 2004). Its antibacterial power corresponds only to 0.2-8% of clinically used antibiotics (streptomycin, vancomycin, colistin) on weight basis, but allicin was able to inhibit 30 different strains of methicillin-resistant Staphylococcus aureus, which is increasingly prevalent in hospitals (Fujisawa et al., 2009; Cutler & Wilson, 2004). The supposed underlying mechanism is again the modification of SH-containing enzymes. If allicin reacted prior with thiol-containing compounds or it was degraded to DADS, its antibacterial activity was lost (Fujisawa et al., 2009).

Beside the numerous incidences for the biological activity of allicin and its derivates, several **contradictory results** were generated, especially from *in vivo* studies (Zeng et al., 2013; Khoo & Aziz, 2009; Banerjee et al., 2003). The diversity of the garlic products used is one of the major reasons for the differences between studies. As mentioned above, the kind and concentration of bioactive organosulfur compounds in garlic products varies a lot, because of the enzyme-related and degradation processes depending on the production conditions. Large quality differences between garlic supplements have been revealed, because most garlic powder products release far less allicin than it ought to be (Lawson & Wang, 2001).

Table 2-4: Bioactivity of allicin and its derivates.

Bioactive compound	Effect	Assay	Results	References
In vitro studies				
Allicin	Anticarcinogenic	Lymphocytic leukemia cells	• In situ generation of allicin by site-directed reaction • Allicin induced apoptosis of cancer cells	(Arditti et al., 2005)
Allicin	Anticarcinogenic, antioxidative	Human promyelocytic leukemia-derived cells, human myelomonocytic cells	• Allicin inhibited growth of cancer cells and induced apoptotic events • Mechanism: allicin activated mitochondrial apoptotic pathway by GSH depletion and by changes in the intracellular redox status	(Miron et al., 2008)
Allicin, SAMC	Anticarcinogenic	Human and murine tumor cell lines (colon carcinoma, colorectal carcinoma, cervical carcinoma)	• Allicin induced apoptosis of cancer cells and antiproliferative mechanisms	(Oommen et al., 2004)
Allicin	Antioxidative	ESR (spin trapping), enzymatic assay	• Antioxidative properties • Inactivation of thiol-containing enzymes by reaction with allicin	(Rabinkov et al., 1998)
Allicin	Antibacterial	S. aureus (Gram-positive), E. coli (Gram-negative)	• Allicin was only 0.2-8% as potent as clinically used antibiotics • Antibacterial activity of allicin was completely abolished by addition of thiol containing compounds	(Fujisawa et al., 2009)
SAC, SAMC	Anticarcinogenic	Human colon cancer cells	• SAMC, but not SAC, inhibited the growth of cancer cell • SAMC induced apoptosis, associated with an increase in caspase3-like activity • SAMC enhanced jun kinase activity and increased endogenous levels of reduced glutathione	(Shirin et al., 2001)
SAMC	Anticarcinogenic	Human colon adenocarcinoma cells	• SAMC suppressed significantly the growth and metastasis of cancer cells	(Liang et al., 2011)
SAMC, other disulfide conjugates with cysteine and GSH	Anticarcinogenic, anti-inflammatory	Murine hematoma cells	• SAMC and SAMG showed highest activity in induction of phase II detoxification enzymes and inhibition of NO production • Cancer-preventive and anti-inflammatory activities	(Zhang et al., 2010)

SAC, SAMC	Anticarcinogenic	Human prostate carcinoma cells	• SAMC, but not SAC, showed antiproliferative effect • SAMC and SAC induced higher level of reduced GSH	(Pinto et al., 1997)
SAC, SAMC, AGE	Antioxidative	Chemiluminescence assay, TBARS assay, DPPH assay	• SAC and SAMC showed radical scavenging activity and are mainly responsible for the antioxidative effect of AGE	(Imai et al., 1994)
In vivo studies				
Allicin	Antiatherosclerotic	Apolipoprotein E-deficient and LDL-receptor knockout mice	• Reduced the atherosclerotic plaque area • Suggested mechanisms: lipoprotein modification, inhibition of LDL uptake and degradation by macrophages	(Gonen et al., 2005)
Garlic	Anticarcinogenic	Cohort of 41,837 women (212 positive cases)	• Epidemiologic study • Regular, high intake of garlic (≥ 1 serving/week) was associated with 50% lower risk for colon cancer	(Steinmetz et al., 1994)
Garlic	Anticarcinogenic	Humans (325 men, 163 women, 488 controls)	• Epidemiologic study • Inverse association between high garlic consumption (≥ 3 servings/week) and adenomatous polyps incidence (can progress to colorectal carcinomas)	(Witte et al., 1996)
SAMC	Anticarcinogenic	Prostate cancer mouse model	• SAMC significantly inhibited tumor growth and reduced number of metastases	(Howard et al., 2007)
SAMC	Anticarcinogenic	Mice, inoculated with prostate cancer cells	• SAMC reduced proliferation and metastasis of cancer cells	(Liang et al., 2011)
AGE	Antihypertensive	Humans, with uncontrolled systolic hypertension (n = 79)	• Mean systolic blood pressure was significantly reduced	(Ried et al., 2013)
AGE	Cardiovascular disease preventive	Humans, healthy (n = 34)	• Inhibited platelet aggregation	(Steiner & Li, 2001)
AGE	Immunmodulatory	Humans, healthy (n = 120)	• Indications for enhanced immune cell function and reduction of cold and flu severity	(Nantz et al., 2012)
AGE	Cardiovascular disease preventive	Humans, with coronary artery disease (n = 19)	• Rate of progression of coronary calcification was inhibited	(Budoff et al., 2004)

Abbreviations: AGE – Aged garlic extract; AMS – Allyl methyl sulfide; ESR – Electron spin resonance; DADS – Diallyl disulfide; DAS – Diallyl sulfide; DPPH – 2,2-diphenyl-1-picrylhydrazyl; GSH – Reduced glutathione; LDL – Low density lipoprotein; SAC – S-allyl cysteine; SAMC – S-allylmercaptocysteine; SAMG – S-allylmercaptoglutathione; TBARS – Thiobarbituric acid reactive substance.

3. Analysis of the binding reaction: Manuscript 1

β-Lactoglobulin as nanotransporter – part I: Binding of organosulfur compounds

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3.1 Abstract

The binding reaction of allicin and diallyl disulfide with β-lactoglobulin and the influence of pH value and protein denaturation on this reaction have been examined in the present study. Regardless of the structural similarity of the both organosulfur compounds, their binding behavior was significantly different. Both ligands were covalently bound by the free thiol group of the protein whereas the affinity for allicin was significantly higher. In addition, diallyl disulfide was non-covalently bound. The binding reaction of both ligands was very sensitive to the pH value during incubation. The optimal pH range was between pH 8.0 and 9.0. Protein denaturation increased the reaction rate and reduced the number of binding sites for allicin whereas the number of non-covalent binding sites increased for diallyl disulfide. Based on these findings, it can be proposed that the covalent modification of β-lactoglobulin functions as a specific transporter stabilizing allicin or diallyl disulfide.

3.2 Introduction

Garlic, *Allium sativum* L., is a traditional flavoring agent and known for its health benefits. Epidemiological studies revealed that a high consumption of garlic is inversely associated with the incidence of different types of cancer (Fleischauer et al., 2000). Furthermore garlic may reduce the risk of cardiovascular diseases (Butt et al., 2009). Allicin (diallyl thiosulfinate) is the major biologically active compound derived from garlic. Numerous health promoting effects of garlic can be attributed to allicin, which include antimicrobial, antioxidant, antiatherosclerotic and anticancerogenic properties (Borlinghaus et al., 2014; Miron et al., 2008). Allicin is enzymatically formed from alliin (S-allyl-L-cysteine sulfoxide) by alliinase upon tissue damage like crushing (Rahman, 2007). Due to its reactive character allicin is relatively unstable and contributes largely to the typical garlic smell and pungency which limits the usage as a bioactive ingredient in functional foods (Amagase et al., 2001). Diallyl disulfide (DADS) is a volatile degradation product of allicin showing bioactive properties as well (Butt et al., 2009). Allicin exerts many of its biological effects by redox-reaction with intracellular free thiol groups (Miron et al., 2010). Whereby S-mercapto conjugates, such as S-allylmercaptocysteine (SAMC) the reaction product of allicin and cysteine, are produced. These conjugates can react with further thiol groups by transferring the S-allyl moiety via thiol-disulfide exchange reaction (Borlinghaus et al., 2014; Pinto et al., 2006; Rabinkov et al., 1998).

The present study investigates the binding of allicin and DADS towards β-lactoglobulin (β-LG), the major protein in whey of ruminant milk. The binding to β-LG could form a stable, non-volatile derivate which is less sensory perceptible and has a higher solubility in water. The application of β-LG as transporter for non-covalently bound ligands had been reported frequently (Bello et al., 2012;

Loch et al., 2013; Shpigelman et al., 2012). To use β-LG as a transporter for covalently bound ligands is more recent approach. To our knowledge only allyl isothiocyanate (AITC) was investigated as covalent bioactive ligand at β-LG (Keppler et al., 2014a; Rade-Kukic et al., 2011). Covalent modifications of β-LG occur naturally as well as during food processing, e.g. glycation of amino groups through Maillard reaction, reaction with electrophilic compounds like aldehydes and quinones (Ali et al., 2013; Curcuruto et al., 1998).

We hypothesize that allicin or DADS can be bound by the whey protein β-LG under specific conditions. Therefore the overall binding reaction, the reaction with different functional groups of β-LG and the reaction conditions have been investigated in the present study.

3.3 Materials and Methods

3.3.1 Materials

β-LG was isolated according to Keppler et al. (2014a). Briefly, whey protein isolate (DSE 6668, Fonterra, New Zealand) was dissolved and α-lactalbumin, bovine serum albumin and immunoglobulins were precipitated at pH 3.8. Subsequently, the supernatant was freeze dried and resulted in a final product containing 98% β-LG in the protein fraction; 90.3% protein (Kjeldahl analysis, N x 6.38); 0.18% lactose; < 0.3% fat; 7.16% ash and 2.3% moisture.

For the synthesis of allicin a modified procedure from Small et al. (1947) was used. A solution of *m*-chloroperbenzoicacid (7.52 g, 43.71 mM) in dichloromethane was added to an ice cooled solution of diallyl disulfide (6.4 mL, 43.71 mM) in 125 ml dichloromethane. The reaction mixture was stirred for 90 minutes followed by addition of anhydrous Na_2CO_3 (40 g) with vigorous stirring for another hour. Then the reaction mixture was filtered and the excess solvent was removed by rotary evaporation. The crude substance was purified by flash column chromatography using ethyl acetate and pentane. The product was isolated as a pale yellow oil (5.02 g, 86%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.83-5.96 (m, 2H), 5.16-5.46 (m, 4H), 3.71-3.86 (m, 4H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 132.88, 125.81, 124.02, 119.05, 59.84, 35.02.

DADS, 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), o-phthalaldehyde (OPA) and L-leucine were purchased from Sigma-Aldrich (St. Louis, USA). N-Acetyl-L-cysteine (NAC) was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany).

3.3.2 Sample preparation

The experiments were conducted with native and heat-denatured β-LG. For heat denaturation a 100 µM β-LG solution was heated at 90 °C for 30 min and freeze dried (48 h, Gamma 1-16 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) afterwards. The preparation of the β-LG/ligand mixtures was similar to Keppler et al. (2014a). β-LG (native or denatured) was dissolved in water and was stirred for 1 h and stored overnight at 4 °C to complete hydration. Afterwards usually pH was adjusted to 8.5 using 0.1 M NaOH. To examine the influence of the pH value on the binding reaction pH values of 4.0, 7.0 (adjusted with 0.1 M HCl) and 8.0, 9.0 (adjusted with 0.1 M NaOH) were tested as well. The final concentration of β-LG was 500 µM. The ligand solution was prepared freshly before each trial. The stock solutions of DADS and allicin were prepared with ethanol and diluted to obtain different concentrations (5 to 750 mM). β-LG was mixed with each ligand separately. To one volume of β-LG solution 2% ligand solution at different concentrations was added under stirring to obtain different ligand/β-LG molar ratios ranging from 0.2 to 20 and 0.25 to 30 for allicin and DADS, respectively. As a control 2% ethanol was added to the β-LG solution. The samples with allicin and DADS were incubated for 24 h protected from light at 4 °C and room temperature. Preliminary experiments (analyzed by RP-HPLC) showed that an incubation time of 24 h is necessary to complete the binding reaction. All analyses were done after incubation, followed by equilibration at room temperature for 2 h. All samples were prepared in triplicate.

3.3.3 Characterization of the binding between β-LG and allicin or DADS

Determination of free thiol groups (RSH)

The amount of free thiol groups was determined using Ellman's assay according to Kehoe et al. (2007) and Keppler et al. (2014a). The samples with different β-LG/ligand ratios were diluted 20-fold in 50 mM Tris-glycine buffer (pH 8) with and without 8 M urea. 40 µl of DTNB (10 mM in Tris-glycine buffer) was added to 2 ml of the diluted solution and incubated for 10 min. If DTNB reacts with thiol groups the yellow dianion of 5-thio-2-nitrobenzoate is formed. The absorbance of the samples was measured at 412 nm using a spectrophotometer (Helios Gamma, UV-Vis, Thermo Spectronic, Cambridge, UK) and was corrected for the absorbance of the corresponding ligand concentration and DTNB. By means of the molar absorption coefficients of 13600 M^{-1} cm^{-1} the concentration of 5-thio-2-nitrobenzoate and thus the amount of free thiol groups per molecule of β-LG was calculated (Ellman, 1959).

Determination of free amino groups (OPA)

The amount of free amino groups was determined according to the method of Rade-Kukic et al. (2011) and Keppler et al. (2014a). The method is based on the reaction of OPA in conjunction with reduced sulfhydryl groups with primary amines of the protein with formation of fluorescent derivatives. 0.1 ml of the sample was mixed with 0.5 ml water and 9.1 ml NAC solution (0.3% NAC (w/v) in 0.1 M borate buffer, pH 9.3) and incubated at 50 °C for 10 min. 0.3 ml OPA solution (3.4% OPA (w/v) in MeOH) was added and the mixture was incubated for further 30 min at 50 °C. After cooling down to room temperature for 30 min the absorbance of the mixture was measured at 340 nm using the same spectrophotometer as was mentioned above. The absorbance value was corrected for the absorbance of the corresponding ligand concentration and OPA. The concentration of free amino groups was calculated using a calibration function of L-leucine in a concentration range of 10-150 µM.

Fluorescence quenching

Fluorescence measurements were carried out according to Keppler et al. (2014a). The quenching of tryptophan fluorescence of β-LG was used to analyze the binding of allicin and DADS. Therefore the samples with different ligand/protein molar ratios (0-30 M/M) were diluted to 15 µM β-LG. The fluorescence was measured at room temperature using a Varian Cary Eclipse right angle fluorescence spectrophotometer (Varian Australia PTY Ltd.) and a 1 x 1 cm quartz cell. The excitation and emission wavelength were 294 nm and 340 nm, respectively, employing 5 nm bandwidths. The fluorescence values were corrected for the inner-filter effect by measuring the absorbance at the applied excitation and emission wavelength for each sample using the spectrophotometer as was mentioned above. According to van de Weert (2010) the observed fluorescence F_{obs} was corrected to F_{corr} in the following way:

$$F_{\text{corr}} = F_{\text{obs}} * 10^{\frac{A_{\text{ex}}+A_{\text{em}}}{2}}$$

Where A_{ex} and A_{em} are the absorption values at the excitation (294 nm) and at the emission wavelength (340 nm), respectively. To verify that the observed fluorescence quenching is related to true binding reactions two further experiments were conducted. Binding decreases at higher temperatures and quenching should be less as well (van de Weert & Stella, 2011). Therefore the fluorescence was determined at 40 °C additionally to the measurements at room temperature. The UV absorption spectrum of β-LG was compared with the spectrum of β-LG with each ligand because true binding changes the absorbance (Keppler et al., 2014b).

RP-HPLC analysis

The samples were diluted to a β-LG concentration of 15 µM and were filtered through 0.2 µm syringe filters (regenerated cellulose membrane, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). RP-HPLC was performed using the Agilent 1100 Series HPLC with a diode-array detector and PLRP-S column (300 Å, 8 µm, 150 x 4.6 mm, Agilent Technologies, Santa Clara, USA). For the analytical separation the injection volume was 20 µl at a flow rate of 1 ml/min and a column temperature of 40 °C using eluents A (0.1 % (v/v) TFA in water) and B (0.1 % TFA (v/v) in ACN). The elution used gradient steps of 35-38% B (1-8 min), 38-42% B (8-16), 42-46% B (16-22 min), 46-100% B (22-22.5 min) and 100-35% B (23-23.5 min). The detection wavelength was 205 nm.

Calculation of binding parameters

The binding constants of allicin and DADS to β-LG were calculated by nonlinear regression of the saturation-binding curves using the software Graphpad Prism version 6 (Graphpad Software, La Jolla, USA). The binding curves were obtained by plotting the molar binding ratio of ligand to β-LG *(B, M/Ml)* against the ligand concentration *(mM)*.

From the analysis of free thiol and amino groups, *B* was calculated based on the assumption that each free functional group can bind one molecule of ligand. Only the RSH values that were determined with urea were used for the calculation of binding parameters. The corrected fluorescence values were transformed to a saturation-binding curve using the fractional quench method of Levine, 1977 according to Keppler et al. (2014a). The area of the unmodified and modified β-LG A and B (genetic variants) in the RP-HPLC chromatogram was used to determine the binding parameters. The relative amount of the modified β-LG of each sample with ligand in relation to the area of β-LG without ligand (control) was calculated and corresponded to the molar binding ratio *B*. Because the calculation model is limited to a maximum of one binding site, only the affinity of the binding reaction was calculated. The number of binding sites was assessed by the number of peaks of the modified β-LG A and B, based on the assumption that the protein becomes more hydrophobic through each bound ligand. All binding parameters were calculated as means ± standard error of the mean (SEM) of the samples prepared in triplicate.

3.4 Results and discussion

The interaction of allicin and DADS with β-LG was characterized on different levels: total binding was evaluated using RP-HPLC and FQ and specific binding sites were analyzed with colorimetric methods (detection of free thiol and amino groups).

3.4.1 Total Binding

The overall binding was measured by RP-HPLC and FQ. Figure 3-1 presents the RP-HPLC chromatograms of β-LG A and B (genetic variants) with the ligands in different molar ratios. With increasing concentration of allicin the area of the native β-LG decreased. Concurrently a new peak appeared for each genetic variant at a higher retention time. Based on these results it is very likely that only one binding site of β-LG was modified by allicin. At a molar ratio of 0.6 M/M the maximum transformation was reached. The affinity constant K_a (i.e. 151.1 mM^{-1}, Table 3-1) was calculated by using the relative amount of the modified β-LG of each sample with ligand in relation to the area of β-LG without ligand. The binding parameters for allicin based on FQ data were similar to the results of the RP-HPLC analysis and gave n=1.07 [M/M] binding site on β-LG and an apparent affinity of 138.9 mM^{-1}.

The chromatograms of β-LG and DADS showed as well a decrease of the native β-LG peak areas with increasing ligand concentration but to a much lesser extent than for allicin (Figure 3-1, B). Even at a high molar ratio (40 M/M) about 60% of the protein remained unmodified. The first peaks of the modified β-LG A and B exhibited the same retention time as β-LG which was modified by allicin. Interestingly, further small peaks of the modified β-LG A and B were formed at an even higher retention time. The additional peak for each genetic variant indicated a more hydrophobic modification of β-LG A and B and thus a further binding site. Based on the RP-HPLC findings it can be concluded that the additional binding sites resulted in slightly more hydrophobic physico-chemical properties. The maximum number of binding sites determined by FQ was n=3.56 ± 0.17 M/M.

Figure 3-2 A and B show the saturation-binding curves of allicin and DADS obtained by different methods. The curves and K_a for allicin indicate the high binding affinity. Depending on the method used the saturation level was reached at a molar ratio of 0.6-1.0 M/M. For DADS a five times higher ligand concentration was necessary to reach the saturation level.

The higher binding affinity of allicin in comparison to DADS in the present study was expected. Due to the thiosulfinate group allicin is more electrophilic and reactive than DADS which causes the higher affinity (Hunter et al., 2005).

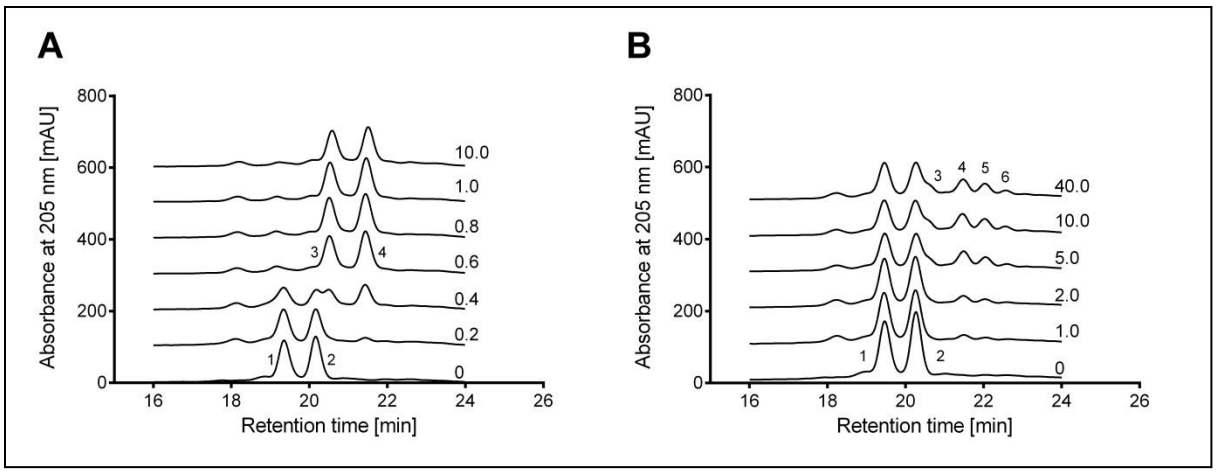

Figure 3-1: Detail of the RP-HPLC chromatograms of β-lactoglobulin (β-LG) with allicin (A) and diallyl disulfide (B). The number at the end of each profile indicates the corresponding molar ligand-protein ratio (M/M). Numbers at the profile peaks: 1 – native β-LG B; 2 – native β-LG A; 3 – modified β-LG B (one ligand/molecule bound); 4 – modified β-LG A (one ligand/molecule bound); 5, 6 – modified β-LG (probably >1 ligand/molecule bound or protein aggregates).

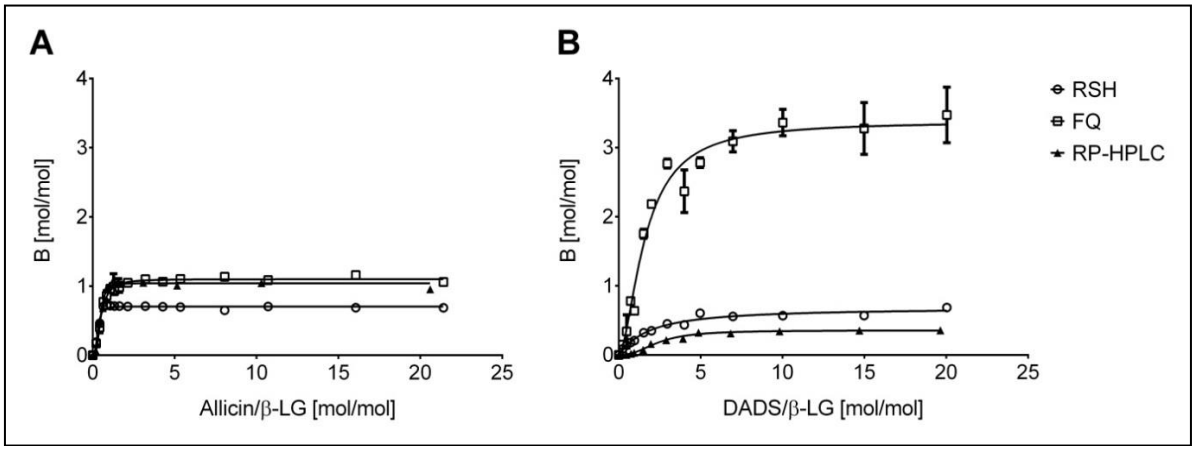

Figure 3-2: Molar binding ratio of the ligands allicin (A) and diallyl disulfide (DADS) (B) to 500 µM native β-LG as a function of the molar ratio between ligand and protein. Binding was determined by measurement of free thiol groups (RSH), fluorescence quenching (FQ) and RP-HPLC. Incubation pH was 8.5. B – bound ligand per M β-LG; DADS – diallyl disulfide; β-LG – β-lactoglobulin.

Both FQ and RP-HPLC are suitable methods to gather the overall binding reaction of the protein and were used in similar studies (Keppler et al., 2014a; Rade-Kukic et al., 2011). Rade-Kukic et al. (2011) observed as well a peak shift to higher retention times in RP-HPLC chromatogram of β-LG induced by covalent binding of AITC.

Table 3-1: Binding parameters of allicin and diallyl disulfide to native β-LG measured at pH 8.5 by different methods.

Method	Allicin		Diallyl disulfide	
	Maximum number of binding sites *n* [M/M]	Apparent affinity constant K_a [mM^{-1}]	Maximum number of binding sites *n* [M/M]	Apparent affinity constant K_a [mM^{-1}]
RSH	0.71 ± 0.01	139.8 ± 5.74	0.67 ± 0.03	24.3 ± 2.87
OPA	0	-	0	-
FQ	1.07 ± 0.02	138.9 ± 7.74	3.56 ± 0.17	38.0 ± 4.27
RP-HPLC	≈1*	151.1 ± 6.41	≈2*	15,5 ± 4.42

FQ – fluorescence quenching; K_a – apparent affinity; *n* – number of binding sites; OPA – measurement of free amino groups; RP-HPLC – reversed phase high pressure liquid chromatography; RSH – measurement of free thiol groups. * Values are based on the number of new formed peaks for each genetic variant and not on calculations. All results were measured in triplicate and listed as mean ± standard error mean.

A higher number of binding sites was determined by FQ for DADS compared to RP-HPLC method. With respect to non-covalent interactions FQ is a frequently utilized and might detect non-covalent interactions more sensitive than RP-HPLC. Nevertheless, an overestimation of the binding reaction by FQ cannot be ruled out. The high ligand excess of DADS may have induced protein aggregation due to competition with water molecules. Oligomer formation of the protein was shown to cause binding overestimation by FQ (Muresan et al., 2001). To verify that the change in fluorescence was only related to static binding, possible interferences (i.e. inner filter effect, collisional quenching) had to be either excluded or corrected (van de Weert & Stella, 2011; Keppler et al., 2014b). The inner filter effect was corrected by using absorption calculations. To investigate collisional quenching the fluorescence was measured at different temperatures (20 °C, 40 °C) and the absorption spectrum (250-300 nm) of β-LG with and without ligand was determined (data not shown). The results of both methods indicated that collisional quenching can be ruled out.

In summary, FQ and RP-HPLC provide complementary and supportive information on the binding characteristics. The analysis by RP-HPLC is less influenced by confounding factors than FQ and the data interpretation is easier (van de Weert & Stella, 2011). In addition, information about physico-chemical characteristics of the modified protein are obtained, hence the higher retention time indicates a higher hydrophobicity of the molecule. Thus, binding parameters based on RP-HPLC results are able to determine the proportion of the modified β-LG, the detection of different species (number of peaks), but not the degree of modification.

3.4.2 Binding of functional groups

Allicin binding. The concentration of the free amino groups did not significantly change with increasing allicin concentration (data not shown). In contrast the measurement of the free thiol groups (RSH) revealed that allicin bound to one thiol group. The binding parameters determined by RSH corresponded to the results of RP-HPLC and FQ (Table 3-1). The RSH data of allicin further showed that after the complete binding of free thiol groups the concentration of SH-groups per molecule β-LG was slightly increased with an increasing molar ratio (Figure 3-3). At a molar ratio of 0.6 M allicin/1 M β-LG 94% of thiol groups were blocked.

It is highly probable that the free thiol group of Cys^{121} is the main binding site because it is the only free thiol group of β-LG. Cys^{121} is located in the inner area of the protein and its accessibility is limited (Burova et al., 1998). Due to this steric hindrance only about 20% of the free thiol groups can react with the DTNB and are detectable by the RSH method (Figure 3-3), which is in accordance with other studies (Kehoe et al., 2007). Nevertheless the results of RSH method, RP-HPLC and FQ taken together indicated that allicin reacted immediately and almost quantitatively with the free thiol group. Presumably the reaction was not retarded by the steric hindrance because of the small size of allicin (molecular weight 162.27 g/M) and its amphiphilic character that facilitates the diffusion into the inner part of the protein.

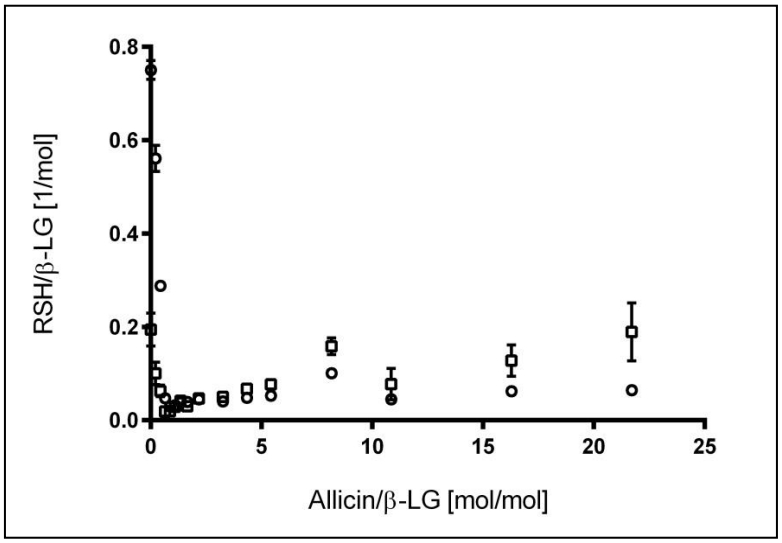

Figure 3-3: Reactive thiol groups (RSH) per molecule β-lactoglobulin (β-LG) as function of molar ratio between allicin and β-LG. The determination of RSH was done without (circles) and with urea (squares).

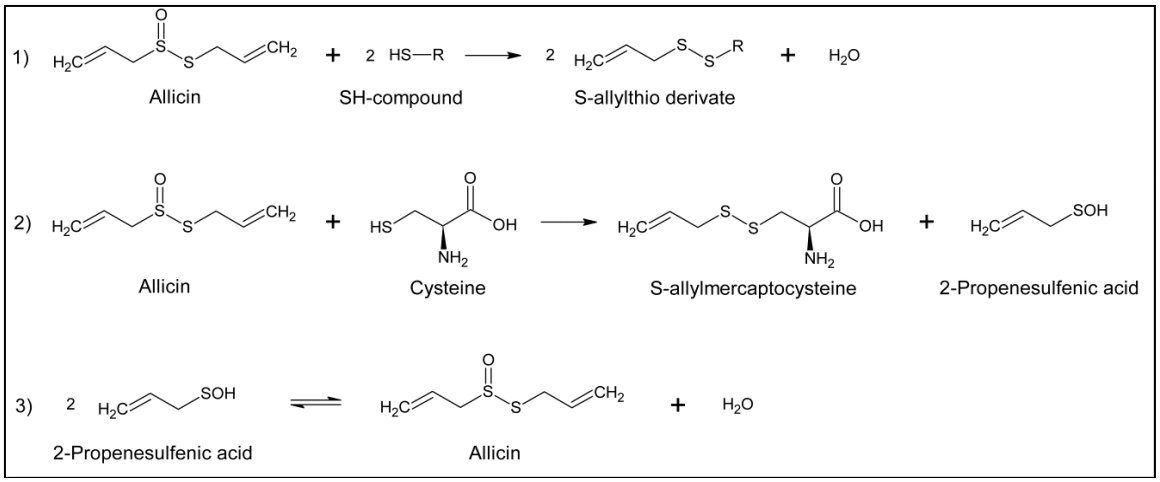

Figure 3-4: Reaction between allicin and thiol groups in general (1) and of cysteine (2). The released 2-propenesulfenic acid can form allicin again by self-condensation (3).

The binding of allicin with 94% of the free thiol groups of β-LG at a ratio close to 2:1 between β-LG and allicin as found in the present study was also reported by Miron et al. (2000) and Hunter et al. (2005). If allicin reacts with a thiol group, 2-propenesulfenic acid is formed, which can react with a further thiol group or can form allicin by self-condensation (Trio et al., 2014). In consequence, the theoretical stoichiometric ratio is 2:1, as shown in Figure 3-4, and the reaction product of allicin and cysteine is S-allylmercaptocysteine (Rabinkov et al., 2000). The results of RSH and RP-HPLC are in accordance with the proposed mechanisms, i.e. the thiol-disulfide exchange reaction between allicin and thiol containing proteins (Rabinkov et al., 1998; Miron et al., 2010).

The increase of thiol group concentration found in the present study may be caused by the cleavage of disulfide bonds of β-LG through the high concentration of free ligand, as it was shown for AITC (Keppler et al., 2014a; Rade-Kukic et al., 2011). Since the results of FQ and RP-HPLC indicate that it is unlikely that allicin binds a further thiol group by cleaving a disulfide bridge, another explanation seems to be more likely: the release of 2-propensulfenic acid through the degradation of allicin favored by the basic conditions reduced the pH value and hence the reactivity of thiol groups (Figure 3-4). With increasing allicin excess the reduction of the pH value during incubation was caused. The lowest pH value measured after incubation was 7.4. At this pH the affinity for the reaction is significantly reduced, as shown in Figure 3-5, and only a part of the thiol groups react which explains the slight increase of free thiol groups at high allicin excess (Figure 3-3).

DADS binding. DADS also reacted only with the free thiol group of β-LG. The reaction was not detectable without previous denaturation of the protein. If β-LG was unfolded about 75% (0.75 ± 0.03 M/M β-LG) of the free thiol groups were detectable. 70% of these reacted with DADS at the saturation level. According to the analysis of thiol and amino groups there were only 0.7 M/M

binding sites for DADS, contrary to the results of RP-HPLC and FQ indicating a molar ratio of circa 2 M/M and 3.6 M/M (Table 3-1), respectively. Thus, about one binding site can be assigned to the covalent reaction with the free thiol group, further binding sites seem to be attributed to non-covalent interactions. The stable and hydrophobic character of DADS could enable the binding in the calyx or in the hydrophobic pockets at the surface of β-LG (Kontopidis et al., 2002).

The measurement of the free thiol (RSH) and amino groups (OPA) revealed that both ligands react only with the thiol groups and the binding to amino groups can be ruled out. At the incubation pH of 8.5 the majority of the thiol groups of cysteine (pK 8.5) were deprotonated and thus more reactive than the protonated amino groups (pK 10.4) (Thurlkill et al., 2006). The reaction of allyl sulfides with thiol groups has been described in several studies but not the reaction with amino groups which is in accordance with our findings (Miron et al., 2010; Rabinkov et al., 1998; Hunter et al., 2005). In contrast other covalent ligands, like AITC and caffeoylquinic acids, bind to the amino groups of the protein although with a low affinity (Rade-Kukic et al., 2011; Ali et al., 2013). In case of AITC, the reason is the higher electrophilicity compared to allicin and DADS and therefore the higher attraction to the less nucleophilic amino groups. AITC binds to the free thiol group at Cys^{121} as well but the affinity constant K_a was considerably lower (K_a=4.35 mM^{-1}) than for allicin and DADS (Keppler et al., 2014a; Rade-Kukic et al., 2011). The attack of the deprotonated thiolate of cysteine is much stronger for the disulfide exchange reaction with the sulfur containing compounds of garlic than for the electrophilic AITC (Nagy, 2013).

In summary, RSH and OPA methods revealed the involved functional groups in the reaction and the data enable the calculation of binding parameters. However, for very reactive ligands like allicin the methods are not applicable to test the influence of the pH value during incubation. Because of the required alkaline buffer systems (pH 8.0, 9.3) the thiol groups are in a reactive state. Furthermore if urea is used the free thiol group is exposed and more accessible due to the unfolding of the protein. Even the incubation conditions were not appropriate for the binding reaction, as any non-bound, reactive ligand can immediately bind to the protein when the sample was mixed with the buffer and urea. This observation has been made for allicin and β-LG at an incubation pH of 7.0 and 4.0. The results of RSH in the presence of urea were the same for the different incubation pH values contrary to all other methods. Thus the sample preparation of the RSH method provides favorable binding conditions, the original state after incubation can be changed until DTNB is added which causes false positive results.

3.4.3 Influence of the pH value during incubation

The influence of different pH values (4.0-9.0) on the binding reaction of allicin or DADS with β-LG was tested by RP-HPLC and FQ. According to RP-HPLC method the maximum number of binding sites *n* and the highest affinity (K_a) for the binding reaction of allicin was achieved at the pH value of 8.5 (n=1.0 ± 0.02 M/M; K_a=151.1 ± 5.6 mM^{-1}).Whereas the results of FQ indicated that the maximum binding was at pH 8.0 (n=1.1 ± 0.01 M/M; K_a=166.9 ± 18.8 mM^{-1}) (Figure 3-5). Compared to pH 8.5 (n=1.1 ± 0.01 M/M; K_a=138.9 ± 7.7 mM^{-1}) and at pH 9.0 (n=1.1 ± 0.01 M/M; K_a=124.1 ± 6.5 mM^{-1}) *n* was the same, but K_a decreased slightly. At a pH value below 8.0 the binding reaction was significantly reduced. By RP-HPLC a minor binding reaction was measured at pH 7.0 (n=0.1 ± 0.04 M/M; K_a=23.1 ± 4.2 mM^{-1}). With FQ no binding reaction was detectable under these conditions. For DADS the results were similar. The maximum binding was at pH 8.5, no binding reaction was measured at 7.0 and 4.0 by RP-HPLC and FQ.

The strong influence of the pH value during incubation of ligand and protein on the binding reaction is caused by the pH dependent reactivity of the thiol groups (Fernandes & Ramos, 2004). Only at a basic pH the majority of the thiol groups of Cys^{121} (pK 8.5) were deprotonated and reactive, which is crucial for the binding reaction. Rabinkov et al. (2000) analyzed the pH dependency of the binding of allicin to a thiol containing peptide. They also observed an increasing reaction rate with increasing pH value (pH 4.5-7.0). In contrast to our results the reaction took also place at acidic conditions even though at a low rate. The crucial difference to a thiol containing peptide is the limited accessibility of the thiol group of β-LG and the influence of the vicinal amino acids. Beside the reactivity of the functional group the pH influences the conformation of the protein (Hoffmann & van Mil, 1999; Dunnill & Green, 1966). At pH above 7.4 the accessibility of the thiol group is increased through the Tanford transition (Tanford et al., 1959). Below this pH the thiol group is buried in the inner part of the protein and less available for the reaction. In combination with the low reactivity of the thiol group at a neutral and acidic pH the binding reaction is inhibited. Therefore covalent modifications of β-LG have been reported mainly at alkaline conditions (Curcuruto et al., 1998; Ali et al., 2013; Rade-Kukic et al., 2011; Keppler et al., 2014a).

3.4.4 Influence of the protein heat denaturation on binding

The influence of protein heat denaturation (i.e. at a given protein concentration for a defined time period as described in materials and methods part) on the overall binding reaction of allicin and DADS to β-LG was measured by FQ.

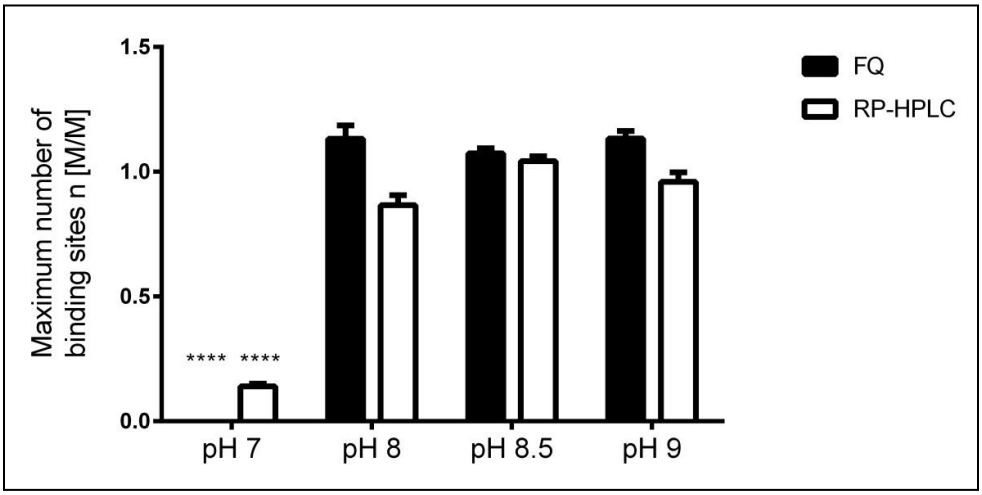

Figure 3-5: Maximum number of binding sites (n [M/M]) of allicin to 500 µM native β-LG as a function of the pH value during incubation. Binding was determined by fluorescence quenching (FQ) and RP-HPLC. (****) for $P < 0.001$.

According to FQ the maximum number of binding sites after protein denaturation was n=0.86 ± 0.08 M/M and the affinity K_a was 527.7 ± 359.0 mM^{-1} for allicin. The measurement of the different functional groups revealed that there was no binding at the amino groups like under all other conditions. According to RSH method n of allicin was lowered to 0.22 ± 0.01 M/M but the affinity of the reaction was tenfold higher (K_a=1264.1 ± 2718.0 mM^{-1}) compared to the native state of the protein (K_a=139.8 ± 5.74 mM^{-1}). With regard to DADS, FQ showed an increase in the maximum number of binding sites (n=6.1 ± 0.05 M/M) and a lower affinity (K_a=17.8 ± 7.9 mM^{-1}) compared to the native β-LG, whereas RSH resulted in a lower number of binding sites (n=0.16 ± 0.02 M/M) and a lowered affinity as well (K_a=12.8± 5.2 mM^{-1}).

The apparently lower number of binding sites of the denatured β-LG according to RSH is caused by the reduced number of available thiol groups per molecule (SH/β-LG=0.24 ± 0.01 M/M). Probably heat induced covalent and non-covalent aggregation was responsible for lower availability, since the free thiol groups becomes exposed during heating and can form intermolecular disulfide bonds (Croguennec et al., 2003; Creamer et al., 2004). Allicin and DADS were bound to a similar extent as by the native protein concerning the reduced number of available thiol groups. According to FQ and RSH the main difference compared to the native protein state is the affinity of reaction. Due to the unfolding of the protein during heating the free thiol group of Cys^{121} becomes exposed and its reactivity is enhanced which finally causes the high affinity (Creamer et al., 2004). To prevent the lower number of available thiol groups suitable physicochemical conditions (like temperature < 90 °C) during heat treatment can be chosen to obtain a non-native monomer with an exposed free thiol group (Croguennec et al., 2003).

The increased affinity of the denatured protein was not detected for DADS. Instead, the number of binding sites increased according to FQ. The thermally induced unfolding of β-LG exposes adhesive hydrophobic regions which enables more interactions with small hydrophobic ligands such as DADS (Busti et al., 2005). Therefore, the number of non-covalent binding sites for DADS was presumably increased by denaturation of β-LG which is consistent with the findings for other hydrophobic non-covalently binding ligands (Li et al., 2012; Shpigelman et al., 2010). In conclusion, the prevalence of non-covalent interactions (shown by FQ) with DADS was increased by denaturation, whereas covalent interactions (shown by RSH) were reduced.

3.5 Conclusions

It has been demonstrated that the closely structural related organosulfur compounds allicin and DADS were bound by β-LG in a different manner. The affinity for the covalent reaction with the free thiol group of the protein was significantly higher for allicin compared to DADS. Additionally, non-covalent interactions were detected for DADS. The methods used for binding analysis enabled the differentiation between covalent and non-covalent interactions. The pH value influenced the interaction with both ligands in the same way, protein denaturation gave rise to partly contrary effects. The binding can be formed specially, as the strong pH dependency of the reaction, it can be assumed that the reaction would not take place in a significant extent under neutral or acidic conditions. The binding allows stabilization of the bioactive garlic constituents allicin and DADS. Further, binding may reduce the flavor-activity. With respect to a functional food ingredient allicin is probably the more appropriate ligand because it was completely bound by the protein in a stable manner. While DADS remained partly free, even if the protein was in excess, thus it can still contribute to the garlic like sensory perception.

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4. Analysis of the protein modification: Manuscript 2

β-Lactoglobulin as nanotransporter – part II: Characterization of the covalent protein modification by allicin and diallyl disulfide

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4.1 Abstract

The whey protein β-lactoglobulin has been proposed as a transporter for covalent bound bioactive compounds in order to enhance their stability and reduce their sensory perception. The garlic derived compounds allicin and diallyl disulfide were bound covalently to the native and heat denatured protein. The binding site and the influence of the modification on the digestibility were determined by mass spectrometric analysis of the modified β-lactoglobulin. Further, the conformation of the modified protein was assessed by circular dichroism and dynamic light scattering. The free thiol group of Cys^{121} turned out to be the major binding site. After proteolysis with trypsin at pH 7 but not with pepsin at pH 2, a limited transfer to other cysteinyl residues was observed. The covalently bound ligands did not mask any proteolytic cleavage site of pepsin, trypsin or chymotrypsin. The modified β-lactoglobulin showed a native like conformation, besides a moderate loosening of protein folding. The covalent binding of organosulfur compounds to β-lactoglobulin provides a bioactive ingredient without impairing the digestibility and functional properties of the protein.

4.2 Introduction

β-Lactoglobulin (β-LG) is the major protein in whey of ruminant milk. The globular molecule consists of 162 amino acids and has a molecular weight of approximately 18.3 kDa. β-LG possesses two intramolecular disulfide bonds (Cys^{66}-Cys^{160}, Cys^{106}-Cys^{119}) and one free thiol group (Cys^{121}) which is buried between the β-barrel and the major α-helix (Burova et al., 1998). Furthermore, β-LG contains 16 free amino groups that can act as binding site for potential covalent ligands as well (Morgan et al., 1999). The tertiary structure is dominated by the β-barrel and consists of nine anti-parallel β-sheets and a major α-helix at the C-terminal end of the polypeptide chain (Brownlow et al., 1997). The β-barrel forms a hydrophobic cavity inside the protein, where hydrophobic compounds can be bound (Kuwata et al., 1999). In its quaternary structure β-LG is mostly present in monomeric or dimeric form. At physiological conditions the dimer is predominant, but the equilibrium is influenced by different parameters (Aymard et al., 1996). Due to the solubility of β-LG over a wide pH range (pH 2-9), its GRAS (generally recognized as safe) status, various techno-functional properties and high nutritional value it is frequently used as an additive in food products. It contributes largely to the functional properties of whey protein concentrate and isolate, like emulsification, gelation, water binding, viscosity development and foaming (de Wit, 1998; Renard et al., 1998; Morr & Ha, 1993). In addition, it has been shown that peptides derived by enzymatic proteolysis of β-LG exert biological activities, e.g. antihypertensive, antioxidant and antimicrobial effects (Hernandez-Ledesma et al., 2008). All these properties together qualify β-LG as a suitable transporter of bioactive compounds.

Allicin (S-allyl 2-propene-1-sulfinothioate) is the major thiosulfinate derived from freshly crushed garlic and mainly responsible for the typical garlic flavor and many health-promoting effects (Borlinghaus et al., 2014). Dially disulfide (DADS) is a stable degradation product of allicin that exerts these health benefits to a lesser extent (Reuter et al., 1996). Due to the reactive character of allicin it is relatively unstable, i.e. the chemical half-life is ranging from several hours to some days, depending on the conditions (Fujisawa et al., 2008; Hunter et al., 2005; Lawson & Gardner, 2005). The binding of organosulfur compounds (allicin, DADS) to β-LG could form a stable bioactive derivate, but the properties of the protein could be changed by the modification as well. Changes of the conformation, physico-chemical properties and digestibility have been observed after covalent modifications of β-LG by different ligands (Rade-Kukic et al., 2011; Chevalier et al., 2001; Sakai et al., 2000; Rawel et al., 1998). Recently we have shown that β-LG is able to bind allicin and DADS covalently (Wilde et al., 2016). The free thiol group was hypothesized to provide the binding site for the organosulfur compounds, but it was not proved so far. Through hydrolysis of the modified protein followed by mass spectrometry the modified amino acid can be identified and the digestibility can be assessed (Keppler et al., 2014b; Ali et al., 2013).

The aim of this study was to investigate the structure of β-LG modified with allicin or DADS. By proteolysis and subsequent mass spectrometry (MS) the binding site was determined. In addition, the influence of the modification on the digestibility and conformation of β-LG were analyzed.

4.3 Materials and Methods

4.3.1 Materials

β-LG was isolated according to Keppler et al. (2014a) and contained 98% β-LG in the protein fraction; 90.3% protein (Kjeldahl analysis, N x 6.38); 0.18% lactose; < 0.3% fat; 7.16% ash and 2.3% moisture. Allicin was synthesized as described by Wilde et al. (2016) and the purity was 86%. All other chemical compounds were analytical grade and were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

4.3.2 Sample preparation

Sample preparation was done as previously described in Wilde et al. (2016). Shortly, native and heat-denatured β-LG were solved in water to give a final concentration of 500 µM. The pH value was adjusted to 8.5. The ligands were separately solved in ethanol and diluted to different concentrations (5 to 750 mM). One volume of β-LG was mixed with 2% of ligand solution at different concentrations

resulting in ligand/β-LG molar ratios of 0.2 to 20 and 0.25 to 30 for allicin and DADS, respectively. Ethanol was used for control sample. The samples were incubated for 24 h, under protected from light, at 4 °C. All samples were prepared in triplicate.

4.3.3 LC-MS analysis of intact β-LG

To analyze the modification of the free sulfhydryl-group after incubation with allicin or DADS respectively, high accuracy mass spectra of the intact proteins were acquired with a Fourier transform mass analyzer. LC-ESI MS analysis was performed on an Ultimate 3000 nano-HPLC system, equipped with an Acclaim PepMap100 nano-column (75 µm x 15 cm, 3 µm, 100 Å, Dionex), coupled online to an LTQ Orbitrap Velos mass spectrometer (Thermo, Bremen, Germany) via a nanospray ion source with a 30 µm PicoTip emitter (New Objective, Woburn, MA). β-LG samples were diluted to a concentration of 5 pmol/µl with 0.1% formic acid. After injection of 1 µl the analyte was washed for 5 min on a PepMap C18 guard column (300 µm x 10 mm, Dionex) with 0.1% aqueous TFA at a flow rate of 30 µl/min. Ion paring reversed-phase (IP-RP) HPLC separation was performed using a 27 min linear gradient from 95% eluent A (water with 0.1% FA) to 90% eluent B (80% ACN, 0.1% FA) at a flow rate of 300 nl/min, followed by isocratic elution for 13 min; afterwards, the column was equilibrated with 5% B for 15 min. UV detection was performed at 214 nm.

The mass spectrometer was operated with Xcalibur software (v2.1.0.1140). The spray voltage was set to 1.37 kV at capillary temperature of 197 °C. MS conditions were: AGC target 1 x 10^6; maximum inject time 500 ms. Full scans of 300 – 2000 *m/z* range were acquired in positive ion mode and recorded in profile, with a resolution of 100000 and calibrated by lock mass of the polysiloxane contaminant peak at *m/z* 445.120024.

4.3.4 Enzymatic hydrolysis of modified β-lactoglobulin

Pepsin digestion was performed with the native and heat-denatured β-LG samples without modification and after modification by equal molar amounts of allicin and twice the amount of DADS. β-LG reaction mixtures were adjusted to pH 2.5 with 0.1 M HCl. Pepsin was added in a protease/protein ratio of 1:5 (w/w) and incubated for 16 h at 37 °C. 2 pmol of the digested protein were analyzed by LC-MS/MS.

Prior digestion with trypsin or chymotrypsin a washing step was performed by using molecular weight cut-off filters (MWCO 3 kDa, Millipore). Briefly, 200 µg of β-LG which had been incubated at 1:0 or 1:1 with allicin (M/M) or at 1:0, 1:1, 1:2, 1:5 or 1:15 with DADS (M/M) were made up to 500 µL

using 100 mM HEPES buffer (pH 7). Samples were spun down for 20 min at room temperature at 14000 *x g* and washed once with HEPES buffer. Samples were spun down (20 min, 14 000 *x g*) and 10 µg were digested overnight at 37 °C with either trypsin or chymotrypsin at a ratio of 1:100 (w/w). Samples were diluted to 500 fmol/µL using 0.1% trifluroacetic acid and 1 µL was injected for LC-MS/MS analysis.

4.3.5 LC-MS/MS analysis of β-LG digests

LC-MS/MS analysis of digested β-LG was performed on the same instruments described for intact protein LC-MS analysis. HPLC separation was performed using a 60 min linear gradient from 95% eluent A (water with 0.1% FA) to 55% eluent B (80% ACN, 0.1% FA) at a flow rate of 300 nl/min, followed by a sharp increase to 95% eluent B in 1 min and isocratic elution for 10 min; afterwards, the column was equilibrated with 5% B for 10 min. For MS analysis scans of 300 – 2000 *m/z* range were recorded in profile mode with a resolution of 30000. The five most intense precursors (minimum signal intensity 500 and rejecting charge state 1) were selected for both CID fragmentation and HCD fragmentation with a repeat count of 2, a repeat duration of 20 s and subsequent dynamic exclusion of the selected *m/z* values (±5 ppm) for 60 s. The CID isolation window was set to 3 Da, the AGC target was 1×10^4 with a maximum inject time of 400 ms, activation time of 10 ms, and an Activation Q of 0.25. Normalized collision energy (NCE) for CID fragmentation was set to 36%. The isolation window for HCD precursors was set to 3 Da, AGC target was 1×10^5 with a maximum inject time of 500 ms and activation time was 0.1 ms. NCE of 45% was used for fragmentation and HCD spectra were acquired with a resolution of 15000. All MS^2 spectra were recorded in centroid mode.

4.3.6 Data processing

MSConvert was used for peak picking and conversion of Thermo raw files to "mgf" file format (Chambers et al., 2012). The MassMatrix MS/MS search engine was used to search MS data against a sequence database containing 112 common laboratory contaminants (http://www.thegpm.org/crap) in addition to the β-LG sequence variants A and B (Xu et al., 2008). Peptides between 6 and 40 amino acids were considered. Cleavage sites were restricted to the peptidase used for digestion, allowing 3, 6 or 9 (maximum) missed cleavages for trypsin, chymotrypsin or pepsin digests, respectively. The peptide mass tolerance was set to 10 ppm with a fragment mass tolerance of 0.04 Da. The 5 most confident protein identifications were searched for disulfide cross linkages, considering a maximum of 2 cross links per peptide.

4.3.7 Circular dichroism

The influence of allicin binding on secondary and tertiary structure of β-LG was investigated. Before CD analysis, samples were diluted in distilled water to the final β-LG concentrations of 5.4 µM, and 54 µM, for the far-UV analysis and near-UV analysis, respectively.

CD spectra were acquired at room temperature using a Jasco J-700 spectropolarimeter (Jasco, Germany). The instrument is regularly calibrated with camphorsulfonic acid in water (1 mg/ml in 1 mm cell), whose CD spectrum provides a two-point calibration: a negative CD band at 192.5 nm (ellipticity = ca. - 69 mdeg, molar ellipticity = ca. - 15600 deg. $cm^2.dmole^{-1}$) and a positive band at 290.5 nm (ellipticity = ca. 33 mdeg; molar ellipticity = ca. 7800 deg. $cm^2.dmole^{-1}$). Far and near-UV CD spectra covered 178-260 and 250-320 nm, respectively, and readings were recorded every 0.5 nm. Spectra were obtained using quartz cells (Jasco, Germany). The far-UV CD spectroscopic measurements were carried out with a 0.1 cm light path-length cell, whereas for near-UV CD spectroscopic measurements a 0.5 cm light path-length cell was used. Scan speed was 50 nm/min and each spectrum was the average of 4 scans integrated with the data processor. All CD spectra were baseline corrected using appropriate blanks and converted to molar ellipticity using the available software option. The measurements were also monitored by the corresponding pattern of the high tension voltage traces (HTV). Measurements of the protein solutions (178-260 nm) gave values of 300-800 V and for tertiary structure 280-350 V. Thus, the measured values were in reasonable range. For the calculation of the secondary structure allocation the data was truncated to 190 nm.

The secondary structural content of the protein was calculated by analyzing CD spectra between 190 and 240 nm, with the curve-fitting software CDPro using CONTIN method that compared spectrum with the spectra of 43-protein reference set containing proteins of different conformations (Sreerama & Woody, 2000; Sreerama et al., 2000). Prior to analysis, CD spectra were corrected for the concentration of solutions, the path length of the scanning cell (0.1 cm) and the mean residue weight (113 Da, according to β-lg-bovine-p02754 – http://www.uniprot.org/uniprot/P02754 ; MW= 18355 Da; 162 amino acid residues).

4.3.8 Particle size measurement

The particle size of native β-LG solutions with and without allicin were measured by dynamic light scattering (DLS) on a Zetasizer Nano System (Malvern Instruments Inc., Worcester, UK). The measurements were performed at 25 °C in a quartz cell with square aperture and at a scattering

angle of 173 ° (viscosity of the sample: 0.88 cP; refractive index of protein: 1.45; refractive index of water: 1.33). The samples were prepared in triplicate and each sample was measured three times.

4.4 Results and discussion

4.4.1 Mass spectrometry of the intact protein

The mass spectrum of the unmodified β-LG showed the presence of the two β-LG variants A and B (molecular weights: 18238.07 Da and 18277.15 Da). Table 4-1 (Supplementary) summarizes the masses of the native and reduced form of both variants. The mass spectrum of native β-LG shows a signal cluster of intact protein ions between *m/z* 900 and *m/z* 2000 with charge states ranging from +10 to +20 (Supplementary, Figure 4-1). The most abundant charge states observed for native β-LG signals in ESI-MS spectra were +13 and +14. The calculated average *m/z* for charge state +14 of β-LG B is 1306.5183 (monoisotopic *m/z*: 1305.6778) with the most abundant isotope peak expected at *m/z* 1306.4653. The *m/z* signal of β-LG A at charge state +14 is expected at *m/z* 1312.6676 (monoisotopic *m/z*: 1311.8233) with the base peak at *m/z* 1312.6108. The calculated masses and isotopic distribution are in agreement with the acquired spectra (Figure 4-1). Denatured β-LG shows a second charge state series of *m/z* signals (ranging from +21 to +25) corresponding to masses of the three possible dimers of β-LG variants A and B, i.e. A-A, B-B and A-B. (Supplementary, Figure 4-2). The signals observed at *m/z* 1314.2443 and 1308.1012 correspond to the sodium adducts of variants A and B respectively (ΔM experimental: +22.973; ΔM calculated: +22.989) and were absent or of much lower intensity in samples cleaned by liquid chromatography prior to MS analysis.

The *m/z* values and isotopic distribution acquired for the modified protein correspond to a single modification of cysteine to S-allylmercaptocysteine (+C_3H_4S; ΔM experimental: +72.016; ΔM calculated: +72.028). The calculated average *m/z* for charge state +14 of modified β-LG variant B is 1311.6704 (monoisotopic *m/z*: 1310.8209) with the most abundant isotope peak expected at *m/z* 1311.6084. The *m/z* signal of modified β-LG variant A at charge state +14 is expected at *m/z* 1317.8197 (monoisotopic *m/z*: 1316.9664) with the base peak at *m/z* 1317.7539 (Figure 4-1). The formation of the S-allylmercapto-derivate of cysteine through the reaction with allicin is in accordance with previous studies (Pinto et al., 2006; Rabinkov et al., 1998).

MS analysis of heat-denatured or native β-LG after modification did not show signals indicating doubly or triply modified β-LG. With respect to allicin, these findings are in accordance with the results of the binding analysis used in the previous study (RP-HPLC, fluorescence quenching (FQ), detection of free thiol groups/RSH assay) (Wilde et al., 2016). Regarding DADS, the results are in

accordance with the findings of the RSH assay, i.e. DADS was bound at only one binding site like allicin. In contrast, results of RP-HPLC ($n \approx 2$ M/M) and FQ ($n = 3.56$ M/M) indicated more than one binding site for DADS (Wilde et al., 2016). It can be suggested that DADS is bound in a covalent and non-covalent way by β-LG which explains the higher number of overall binding sites detected by RP-HPLC and FQ. Further, the high ligand excess can induce the formation of larger protein aggregates, as shown by Table 4-3, which can cause binding overestimation by FQ.

At a molar ratio of 0.5 M allicin/1 M β-LG the signals of unmodified β-LG are barely detectable and almost all β-LG molecules were modified, in agreement with the supposed stoichiometric ratio of 2:1 (Figure 4-1) (Hunter et al., 2005). In presence of DADS β-LG was mostly unmodified even at a molar ratio of 15 M DADS/1 M β-LG (data not shown). The observed extent of the reaction with allicin and DADS is in accordance with the results of RP-HPLC and RSH assay in the previous study (Wilde et al., 2016). After reaction with allicin at high excess (molar ratios of 7.5 or 10) the MS spectra showed signals corresponding to the unmodified protein. This phenomenon was caused by the reduction of the pH value during incubation, which reduced the reactivity of thiol groups. By degradation of allicin, favored by the basic conditions, 2-propensulfenic acid can be released and lower the pH value. Incomplete binding was also indicated by the results of RSH method in the previous study (Wilde et al., 2016). In contrast, high excess of the stable DADS did not affect the pH, which supports the mentioned hypothesis.

The modification of β-LG both by allicin and DADS resulted in the formation of S-allylmercaptocysteine. Subsequent measurements were only conducted with allicin, exemplarily for both ligands.

4.4.2 Mass spectrometry of the hydrolyzed protein

To locate the site of modification, modified and native β-LG were digested with the proteases trypsin, chymotrypsin or pepsin prior to LC-MS analysis of the resulting peptide mixtures. The modified protein was formed at a ligand-protein ratio of 1:1 resulting in the complete binding of the ligand. Thus, inhibition of the enzyme activity by the free ligand can be excluded.

LC-MS analysis of chymotryptic and tryptic digested β-LG samples showed a sequence coverage of 98% and 100% respectively; pepsin digestion resulted in a sequence coverage of 70%. In all three digestions sequence coverage did not differ between modified and unmodified β-LG. In contrast, pepsin digestion of heat denatured β-LG consistently showed a sequence coverage of 90%.

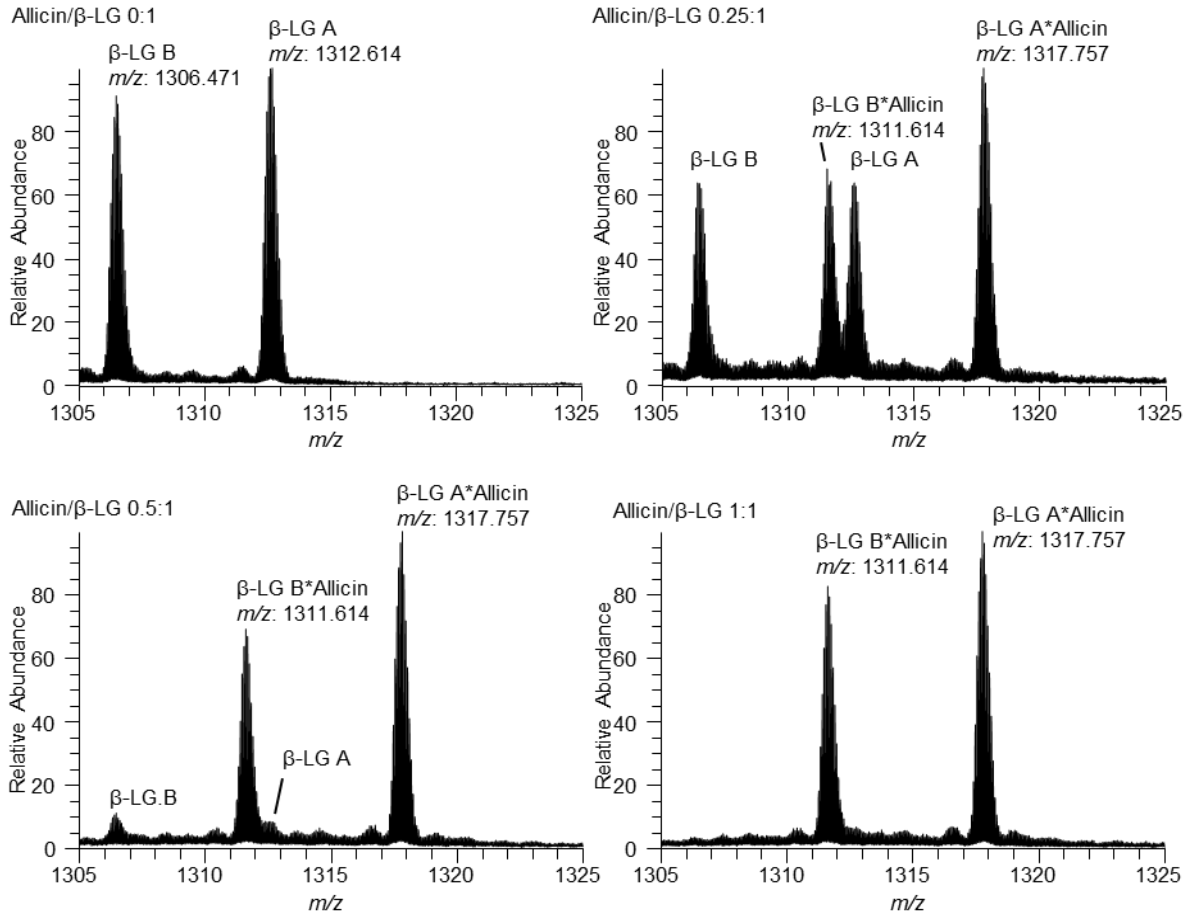

Figure 4-1: LC-MS spectra of the intact β-lactoglobulin (β-LG) ion z=14 after modification with increasing molar ratios of allicin (0:1 M/M – 1:1 M/M). *M/z* – mass/charge.

Table 4-1: S-allylmercaptocysteine modification sites detected by LC-MS/MS analysis of allicin modified β-lactoglobulin (β-LG) digests. The number of peptide spectrum matches for modifications on the five cysteine residues of β-LG digested by the proteases trypsin, chymotrypsin and pepsin are shown in addition to the sum identification scores of the peptide spectrum matches (PSM).

	Trypsin		Chymotrypsin		Pepsin		Pepsin, β-LG denatured	
Cysteine	Modified PSM	∑ pp-score	Modified PSM	∑ pp-score	Modified PSM	∑ pp-score	Modified PSM	∑ pp-score
C66	3	86.5	0	0	2	102.7	1	26.1
C106	27	298.6	0	0	1	11.1	0	0
C119	16	220.8	4	69.8	128	3131	105	2018.6
C121	32	477.6	11	189.3	357	6616.5	116	2435.4
C160	4	114.6	2	62.8	0	0	0	0

These results indicate that S-allylmercaptocysteine modification of the substrate did not noticeably affect the efficiency of digestion by e.g. changing the accessibility of β-LG to the enzymes as was the case with the denatured protein. Furthermore, the derived peptides contained fragments of bioactive peptides (e.g. VLDTDYK, YLLF, CMENSA) that can be released by further digestion (Hernandez-Ledesma et al., 2008) (Supplementary, Table 4-2). The main specificity of trypsin is peptide bonds that contain arginine and lysine; chymotrypsin cleaves preferentially at aromatic amino acids like tryptophan, tyrosine and phenylalanine; while pepsin is a non-specific protease but prefers aromatic and hydrophobic amino acid residues (Rawlings et al., 2012). As the digestion of the modified β-LG was not affected, it can be concluded that modification was restricted to cysteinyl residues. Under physiological conditions β-LG is resistant to pepsin, but trypsin and chymotrypsin contribute largely to its digestion (Mandalari et al., 2009; Reddy et al., 1988). Hence, it can be assumed that the covalent modification of β-LG by allicin does not affect the protein digestion *in vivo*, at least with respect to gastric and small intestine enzymes.

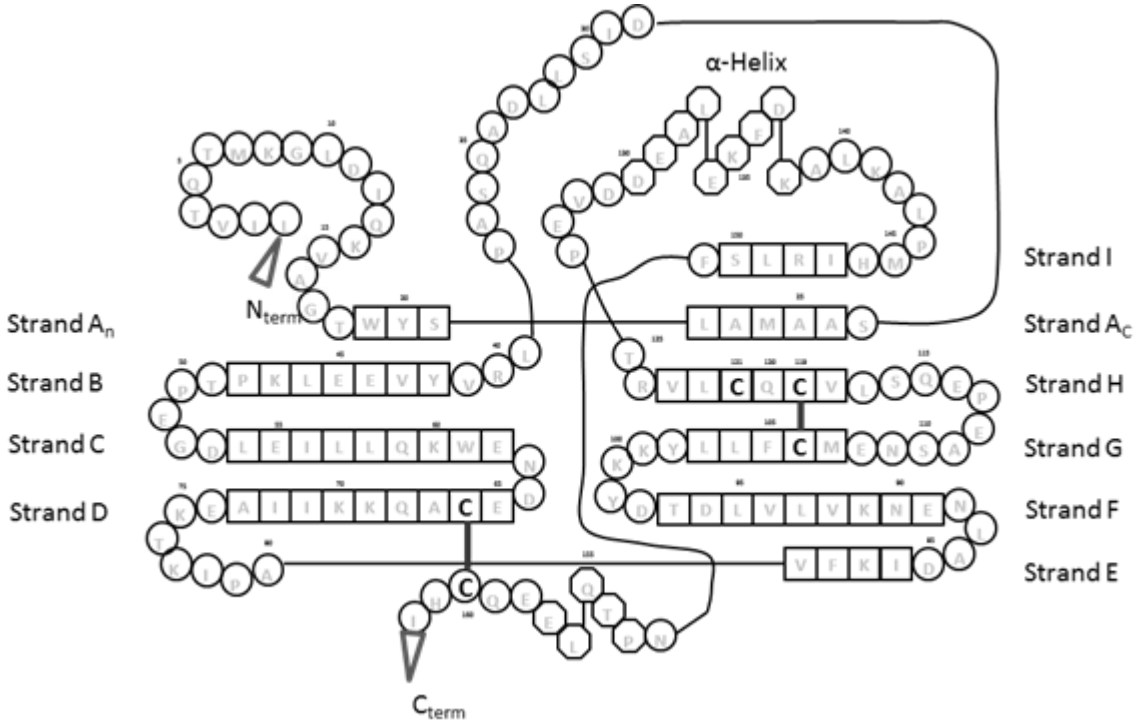

Figure 4-2: Schematic depiction of the primary and secondary structure of β-lactoglobulin. Cysteine residues are bold. Schema modified according to Keppler et al. (2014a). Amino acid sequence according to Vincenzo Fogliano et al. (1998).

The protein samples were not treated with a reducing agent, leaving disulfide bonds and cysteine modifications intact. Identified modification sites are summarized in Table 4-1 by the number of

peptide spectrum matches (PSM) corresponding to the modification at the five cysteine residues and the sum of the identification scores of these PSMs. All identified peptides with a p-value < 0.05 (pp-score > 2.5 and pp_{tag}-score > 1.3) were counted. In addition, Table 4-2 and Supplementary Table 4-2 show the sequence of identified peptides after tryptic and chymotryptic digestion of modified β-LG. Figure 4-2 illustrates the position of cysteinyl residues and disulfide bonds within the sequence of the intact protein.

Table 4-2: Identified peptides after chymotryptic and tryptic digestion of β-lactoglobulin (β-LG) incubated with allicin at a molar ratio of 1:1. Cysteine residues are bold. The listed peptides are exemplary, not all identified peptides are shown.

Peptide	Cysteine residue	β-LG variant	Charge [*z*]	*m/z*	RT [min]	TIC
Chymotryptic digestion						
ENDE**C**^{66}AQKKIIAEKTKIPAVF-SFNPTQLEEQ**C**^{160}HI	Disulfide	A	4	980.24	30.12	2.88E+06
C^{106}MENSAEPEQSLV**C**^{119}Q**C**^{121}L	Disulfide, Allicin*	A	2	986.39	33.33	1.67E+07
C^{106}MENSAEPEQSLA**C**^{119}Q**C**^{121}L	Disulfide, Allicin*	B	2	972.37	32.70	9.32E+05
C^{106}MENSAEPEQSLV**C**^{119}Q**C**^{121}	Disulfide, Allicin*	A	2	920.84	39.22	1.96E+06
C^{106}MENSAEPEQSLA**C**^{119}Q**C**^{121}	Disulfide, Allicin*	B	2	906.83	36.96	1.35E+07
SFNPTQLEEQ**C**^{160}HI	Allicin	A,B	2	809.36	39.00	7.87E+05
SFNPTQLEEQ**C**^{160}HI	SH	A,B	2	773.35	33.70	1.90E+06
Tryptic digestion						
WENDE**C**^{66}AQK	SH	A	2	561.73	27.39	3.67E+06
WENDE**C**^{66}AQK	Allicin	A	2	597.73	27.27	1.62E+05
WENDE**C**^{66}AQKK-LSFNPTQLEEQ**C**^{160}HI	Disulfide	A	5	582.07	30.15	2.19E+06
YLLF**C**^{106}MENSAEPEQSLV**C**^{119}Q**C**^{121}LVR	Disulfide, SH*	A	3	891.74	42.92	2.79E+04
YLLF**C**^{106}MENSAEPEQSLV**C**^{119}Q**C**^{121}LVR	Disulfide, Allicin*	A	3	915.74	46.75	1.36E+06
LSFNPTQLEEQ**C**^{160}HI	SH	A,B	2	829.90	37.94	3.32E+06
LSFNPTQLEEQ**C**^{160}HI	Allicin	A,B	2	865.90	42.68	1.15E+06

* A site specific attribution to individual cysteine residues is not possible. Abbreviations: *m*/z – mass/charge; RT – retention time; SH – thiol group; TIC – total ion current.

Peptides containing an S-allylmercapto-derivate exhibited belated retention times compared to peptides with the same sequence and a free thiol group, indicating a higher hydrophobicity (Supplementary, Table 4-2). Digestion with chymotrypsin and in particular with pepsin demonstrated that Cys^{121} and Cys^{119} were the prevalent sites of modification. Due to the close proximity of both cysteine residues in the protein secondary structure detected peptides usually contain both cysteine residues (Calleri et al., 2005; Creamer et al., 2004). An unequivocal differentiation between the modification of Cys^{121} and Cys^{119} by allicin is not possible as the two co-eluting peptide species are isobaric and are co-isolated for fragmentation. Interestingly, the residue at position 121 was nonetheless identified by more than twice the number of spectra in the peptic digest of modified native β-LG, while the modified heat-denatured β-LG showed almost equal numbers of spectra for both residues. This observation is in line with the results of various methods by which Cys^{121} is assigned as the free thiol of the native β-LG (i.e. NMR spectroscopy, crystallography, MS/MS) (Kuwata et al., 1999; Brownlow et al., 1997; Yen et al., 2000). However, denaturation was shown to favor the alternating topology with the free thiol on Cys^{119} (detected by crystallography and MS/MS) which is in line with the present findings (Yen et al., 2000; McKenzie & Shaw, 1972). With respect to the native β-LG, it can be concluded that the free thiol group at position 121 is the binding site for allicin and DADS.

After chymotrypsin and pepsin digestion the modification by the thiol allyl moiety was mainly restricted to peptides containing Cys^{121} and Cys^{119}. In contrast, S-allylmercaptocysteine modifications were more distributed in trypsin digested samples, which was probably due to disulfide exchange reactions (Miron et al., 2010). The disulfide bond might not be stable under the digestion conditions of 37 °C and pH 7. Thus, the native disulfide topology is more stable upon digestion at pH 2, preventing disulfide interchange reactions as well as the diffusion of the modification to other cysteine residues.

4.4.3 Influence of the ligand binding on protein folding of β-LG

The secondary protein structure of β-LG was further analyzed by the far-UV CD spectrum. The native protein contained 13.8% α-helix, 35.6% β-sheet, 22.0% β-turn and 28.7% unordered structure elements. Only minor differences in secondary structure between the native and the modified β-LG were detected. The content of α-helix (modified: 8.5%) decreased absolutely by about 5% and concurrently the content of β-sheet (modified: 39.7%) increased. The near-UV spectrum showed a decrease of the maximum at 270 nm, 280 nm and 288 nm (Figure 4-3). Binding of allicin reduced the ellipticity at 267 nm and 258 nm, the bands are attributed to cysteine and disulfide bonds,

respectively. Furthermore the minimum at 285 nm which is ascribed to Trp[61] was also reduced. An increase and blue shift was observed at the maximum 255 nm.

The observed structural elements of the native state of the protein is in accordance with other studies (Ali et al., 2013; Rade-Kukic et al., 2011). The bound allicin affected the tertiary structure of β-LG which was clearly indicated by the changes in the near-UV CD spectrum. In our previous study the increased hydrophobicity of β-LG modified by allicin was shown by RP-HPLC (Wilde et al., 2016). Thus, it can be suggested that the loosening of the globular structure and the attached thiol allyl moiety contribute to a higher hydrophobicity of the protein. This finding is consistent with the observations of other thiol binding ligands to β-LG (Ali et al., 2013; Rade-Kukic et al., 2011). The conformational changes induced by AITC led also to a higher hydrophobicity and consequently to a better absorption to interfaces, as air/water or oil/water, resulting in enhanced emulsification and foaming properties of the modified protein (Rade-Kukic et al., 2011). A similar effect on the techno-functional properties of β-LG can be assumed for the modification by allicin. Burova et al. (1998) modified the free thiol group of β-LG with other thiol reactants (i.e. mercaptopropionic acid, mercaptoethanol) and observed no significant effect on far-UV CD spectrum, but changes of the aromatic bands in the near-UV CD spectrum, which is in agreement with our results.

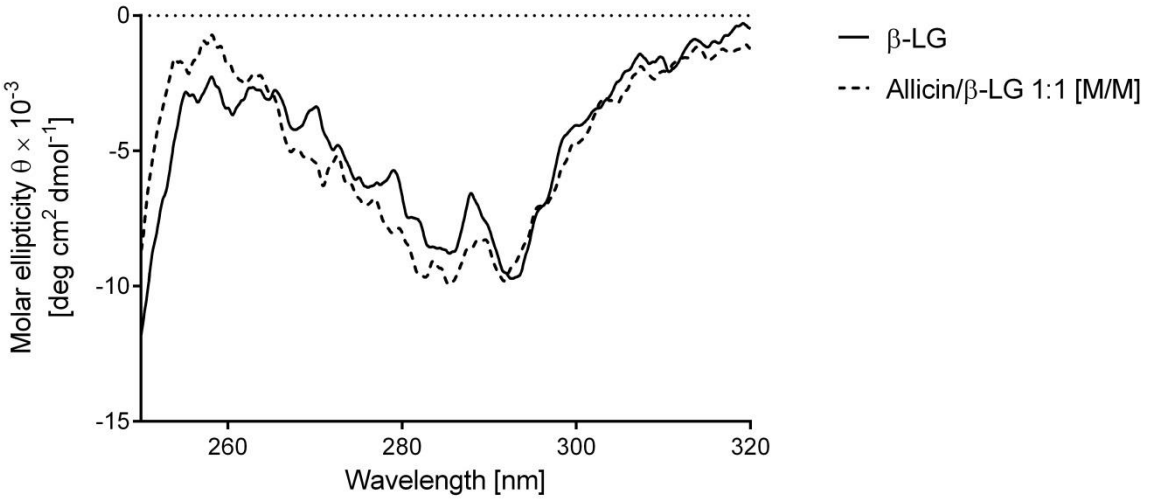

Figure 4-3: Near-UV CD spectrum of native β-lactoglobulin (β-LG) and β-LG incubated with allicin at a molar ratio of 1:1.

4.4.4 Influence of the ligand binding on protein aggregation of β-LG

Size measurements revealed that the binding of allicin has no influence on the quaternary structure of β-LG. Table 4-3 summarizes the results. The unmodified protein had an average hydrodynamic

diameter of 5.6 nm which corresponds to the dimeric structure. With increasing ligand concentration up to a molar ratio of 2 M allicin/1 M β-LG the hydrodynamic diameter increased slightly (6.1 nm). If allicin was present in excess (i.e. all free thiol groups are blocked, remaining free allicin in solution, ligand-protein ratio of 0.75 M/M) a small fraction of larger aggregates (> 30 nm) was observed. At a ligand excess of 5 M/M the predominant particle size sharply increased to about 140 nm. The remaining free allicin in solution or its degradation products seemed to induce the non-covalent aggregation of β-LG. It can be hypothesized that this phenomena is based on a competition of allicin with water molecules in the solvation shell of the protein molecule.

The prevalence of the dimeric form at the present condition (neutral pH, 25 °C) is in accordance with literature (Bauer et al., 1998; Aymard et al., 1996). However, after ligand addition other studies with thiol-modifying ligands found a shift towards the monomeric form (diameter of about 4 nm) of β-LG (Owusu-Apenten et al., 2003; Sakai et al., 2000; Burova et al., 1998), which was not detectable in the present study. It was suggested that the bound ligand enhances the dissociation into monomers by destabilizing the rigid hydrophobic core and the nearby dimer interface (Sakai et al., 2000; Burova et al., 1998). The extent of the destabilizing effect was shown to be dependent on the properties of the thiol reagent (Sakai et al., 2000; Cupo & Pace, 1983). None of the above named studies used allicin for thiol group modification. Most likely the small size and uncharged nature of allicin is responsible for the different behavior, i.e. not causing dimer dissociation.

Table 4-3: Particle size of β-LG with increasing allicin concentration measured by dynamic light scattering. All measurements were conducted in triplicate and listed as mean ± standard deviation (SD).

Allicin/β-LG [M/M]	Peak	Hydrodynamic diameter	
		Size (± SD) [nm]	Volume (± SD) [%]
0	I	5.6 (± 0.21)	99.8 (± 0.11)
0.25	I	5.6 (± 0.08)	99.9 (± 0.10)
0.5	I	5.8 (± 0.20)	99.7 (± 0.16)
0.75	I	5.8 (± 0.25)	99.7 (± 0.02)
1.0	I	6.0 (± 0.46)	91.0 (± 14.82)
	II	44.29 (± 19.48)	6.36 (± 10.49)
2.0	I	6.2 (± 0.36)	99.1 (± 0.13)
	II	75.65 (± 4.05)	0.9 (± 0.13)
5.0	I	141.1 (± 2.79)	97.3 (±4.66)
	II	29.03 (± 50.29)	2.69 (±4.66)

4.5 Conclusions

The covalent binding of allicin and DADS to β-LG and their influence on the protein structure have been examined in the present study. Both ligands led to a single covalent modification of a cysteine residue to S-allylmercaptocysteine. This relatively small structural modification did not induce considerable conformational changes beside a more loosely protein folding and higher hydrophobicity. Hence, similar techno-functional properties as the native protein and improved interfacial properties can be assumed. Further, the digestibility by physiological relevant proteases and the release of bioactive peptides was not impaired. The major modification site in peptides of β-LG was Cys^{121}. Following this, β-LG seems to be a suitable transporter for the bioactive thiol allyl moiety of allicin and DADS. A bioavailability study of β-LG modified by allicin is presently underway.

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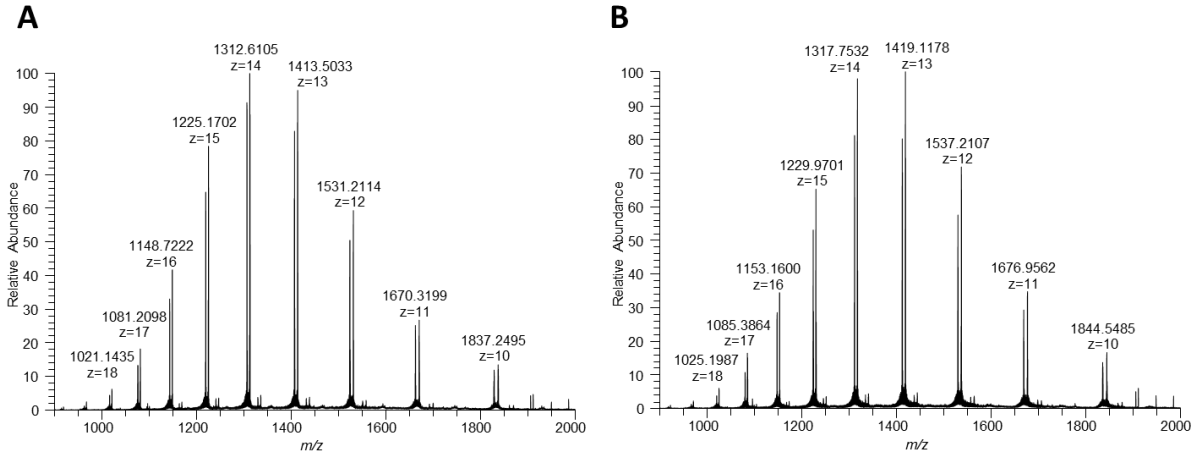
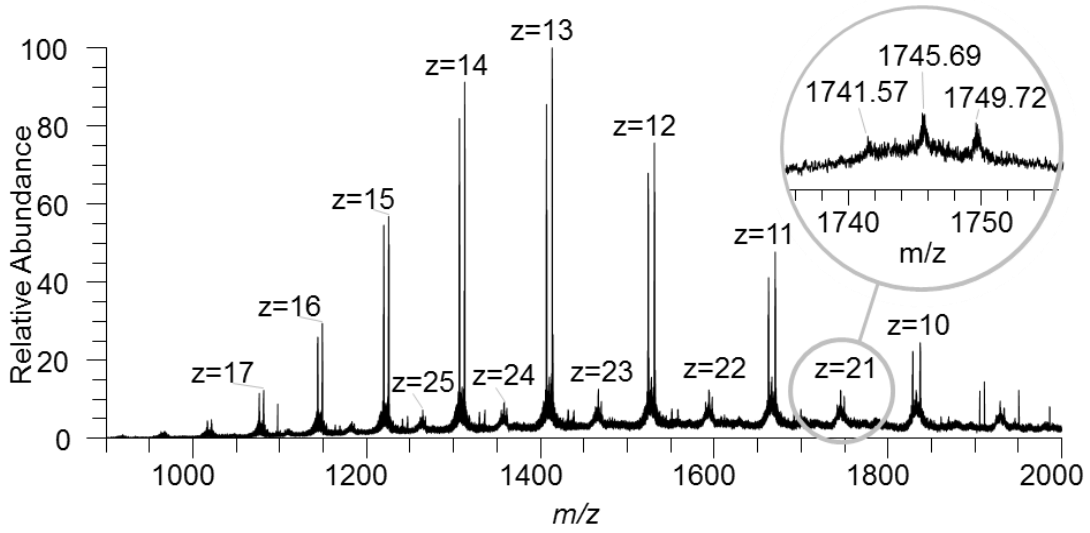
4.6 Supplementary

Supplementary Table 4-1: Mass spectrometry results of native β-lactoglobulin (β-LG).

β-LG variant	Monoisotopic mass [Da]	Molecular weight [Da]	Formula
β-LG A, native	18226.3393	18238.0736	$C_{821}H_{1318}N_{206}O_{250}S_9$
β-LG A, reduced cysteine residues	18230.3706	18367.276	$C_{821}H_{1322}N_{206}O_{250}S_9$
β-LG B, native	18265.3871	18277.1541	$C_{817}H_{1312}N_{206}O_{248}S_9$
β-LG B, reduced cysteine residues	18269.4184	18281.1859	$C_{817}H_{1316}N_{206}O_{248}S_9$



Supplementary Figure 4-1: Full high resolution MS spectra of the modified (A) and unmodified (B) β-LG ion cluster.



Supplementary Figure 4-2: Full high resolution MS spectra of denatured β-LG ion cluster. The insert (grey circle) shows signals of the dimers with a charge *z*=21.

Supplementary Table 4-2: Identified peptides after chymotryptic and tryptic digestion of β-lactoglobulin (β-LG) incubated with allicin at a molar ratio of 1:1. Cysteine residues are bold. The listed peptides are exemplary, not all identified peptides are shown.

Peptide	Cysteine modification	β-LG variant	Charge [*z*]	*m/z*	RT [min]	TIC
Chymotryptic digestion						
SFNPTQLEEQC^{160}HI	SH	A,B	2	773,35	33,70	1,90E+06
SFNPTQLEEQC^{160}HI	Allicin	A,B	2	809,36	39,00	7,87E+05
NPTQLEEQC^{160}HI	SH	A,B	2	656,30	27,10	1,14E+06
C^{106}MENSAEPEQSLAC^{119}QC^{121}L	Disulfide, Allicin	B	2	972,37	32,70	9,32E+05
C^{106}MENSAEPEQSLAC^{119}QC^{121}L	Disulfide, Allicin	B	3	648,58	32,62	1,01E+05
C^{106}MENSAEPEQSLVC^{119}QC^{121}L	Disulfide, Allicin	A	3	657,93	33,34	2,06E+05
C^{106}MENSAEPEQSLVC^{119}QC^{121}L	Disulfide, Allicin	A	2	986,39	33,33	1,67E+07
EEQC^{160}HI	SH	B	2	379,66	19,26	1,43E+04
C^{106}MENSAEPEQSLVC^{119}QC^{121}L-SFNPTQLEEQC^{160}H	2 x Disulfide	A	3	1142,15	36,30	6,65E+05
ENDEC^{66}AQKKIIAEKTKIPAVF-SFNPTQLEEQC^{160}HI	Disulfide	A	6	653,83	30,12	7,19E+05
ENGEC^{66}AQKKIIAEKTKIPAVF-SFNPTQLEEQC^{160}H	Disulfide	B	6	644,16	29,60	3,50E+05
C^{106}MENSAEPEQSLAC^{119}QC^{121}L-SFNPTQLEEQC^{160}HI	2x Disulfide	B	3	1132,80	34,90	4,02E+05
C^{106}MENSAEPEQSLAC^{119}QC^{121}L	Disulfide, Allicin	B	3	648,58	32,62	1,01E+05
C^{106}MENSAEPEQSLAC^{109}QC^{121}	Disulfide, Allicin	B	2	906,83	36,96	1,35E+07
C^{106}MENSAEPEQSLVC^{109}QC^{121}	Disulfide, Allicin	A	2	920,84	39,22	1,96E+06
C^{121}LVRTPEVDDEALEKF-SFNPTQLEEQC^{160}HI	Disulfide	A,B	2	852,40	33,21	5,60E+04
ENDEC^{66}AQKKIIAEKTKIPAVF-SFNPTQLEEQC^{160}HI	Disulfide	A	4	980,24	30,12	2,88E+06
ENGEC^{66}AQKKIIAEKTKIPAVF-SFNPTQLEEQC^{160}HI	Disulfide	B	4	965,74	29,60	2,23E+06
ENGEC^{66}AQKKIIAEKTKIPAVF-	2x Disulfide	B	4	1042,74	30,67	1,81E+04

C^{106}MENSAEPEQSLAC^{119}QC^{121}L						
ENDEC^{66}AQKKIIAEKTKIPAVF-C^{106}MENSAEPEQSLVC^{119}QC^{121}L	2x Disulfide	A	4	1064,26	32,20	2,85E+04
Tryptic digestion						
WENDEC^{66}AQK	SH	A	2	561,73	27,39	3,67E+06
WENGEC^{66}AQK	Allicin	B	2	568,73	26,28	4,20E+05
WENDEC^{66}AQK	Allicin	A	2	597,73	27,27	1,62E+05
WENDEC^{66}AQKK	SH	A	2	625,78	27,39	1,24E+04
WENGEC^{66}AQK	SH	B	2	532,73	27,74	4,14E+04
WENGEC^{66}AQKK-LSFNPTQLEEQC^{160}HI	Disulfide	B	5	570,47	29,92	2,06E+06
WENDEC^{66}AQKK-LSFNPTQLEEQC^{16}0HI	Disulfide	A	5	582,07	30,15	2,19E+06
WENGEC^{66}AQK-LSFNPTQLEEQC^{160}HI	Disulfide	B	4	680,81	31,42	5,23E+06
WENDEC^{66}AQ-LSFNPTQLEEQC^{160}HI	Disulfide	A	4	695,31	31,68	4,66E+06
WENGEC^{66}AQKK-LSFNPTQLEEQC^{160}HI	Disulfide	B	4	712,83	29,92	6,45E+06
WENDEC^{66}AQKK-LSFNPTQLEEQC^{160}HI	Disulfide	A	4	727,33	30,15	8,06E+06
WENGEC^{66}AQK-LSFNPTQLEEQC^{160}HI	Disulfide	B	3	907,41	31,42	2,34E+07
WENDEC^{66}AQK-LSFNPTQLEEQC^{160}HI	Disulfide	A	3	926,74	31,68	1,99E+07
WENGEC^{66}AQKK-LSFNPTQLEEQC^{160}HI	Disulfide	B	3	950,11	29,92	1,69E+07
WENDEC^{66}AQKK-LSFNPTQLEEQC^{160}HI	Disulfide	A	3	969,44	30,15	1,97E+07
WENDEC^{66}AQK-LSFNPTQLEEQC^{160}HI	Disulfide	A	2	1389,61	31,68	8,76E+05
WENGEC^{66}AQK-LSFNPTQLEEQC^{160}HI	Disulfide	B	2	1360,61	31,42	1,24E+06
WENGEC^{66}AQK-YLLFC^{106}MENSAEPEQSLAC^{119}QC^{121}LVR	2x Disulfide	B	3	1236,21	35,75	8,65E+04
YLLFC^{106}MENSAEPEQSLVC^{119}QC^{121}LVR	Disulfide, Allicin	A	3	915,74	46,75	1,36E+06
YLLFC^{106}MENSAEPEQSLAC^{119}QC^{121}LVR	Disulfide, Allicin	B	2	1359,10	45,12	3,04E+05
YLLFC^{106}MENSAEPEQSLVC^{119}QC^{121}LVR	Disulfide, Allicin	A	2	1373,11	46,75	1,41E+05
YLLFC^{106}MENSAEPEQSLAC^{119}QC^{121}LVR	Disulfide, SH	B	3	882,40	41,78	6,31E+04
YLLFC^{106}MENSAEPEQSLVC^{119}QC^{121}LVR	Disulfide, SH	A	3	891,74	42,92	2,79E+04
YLLFC^{106}MENSAEPEQSLAC^{119}QC^{121}LVR	Disulfide, Allicin	B	3	906,40	45,12	4,46E+06
LSFNPTQLEEQC^{160}HI	Allicin	A,B	3	577,60	42,68	2,67E+04

LSFNPTQLEEQC^{160}HI	SH	A,B	2	829,90	37,94	3,32E+06
LSFNPTQLEEQC^{160}HI	Allicin	A,B	2	865,90	42,68	1,15E+06
VLVLDTDYKKYLLFC^{106}MENSAEPEQSLV C^{119}QC^{121}LVR	Disulfide, Allicin	A	4	980,73	44,4	2,16E+04

Abbreviations: *m*/z – mass/charge; RT – retention time; SH – thiol group; TIC – total ion current.

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5. Sensory properties and application in food: Manuscript 3

β-Lactoglobulin as nanotransporter for allicin:

Sensory properties and applicability in food

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5.1 Abstract

The thiosulfinate allicin is a labile, bioactive compound of garlic. In order to enrich allicin in a functional food, a delivery system which stabilizes the compound and masks its intensive flavor is necessary. In the present study allicin was covalently bound to the whey protein β-lactoglobulin and the incorporation of this transporter in a food matrix was tested. The sensory properties of the pure functional ingredient as well as of an enriched beverage were characterized by quantitative descriptive analysis. The concentration of volatile compounds was analyzed by head space gas chromatography-mass spectrometry. The garlic related organoleptic properties of garlic powder were significantly improved by the binding of allicin in combination with spray drying. After purification of the modified β-lactoglobulin the garlic taste and smell were barely perceptible. β-Lactoglobulin modified with allicin provided a stable functional ingredient that can be used to enrich a broad range of food products.

5.2 Introduction

The continuing demand of consumers for health and well-being promoting products has led to an enormous increase in the number of functional foods that contain specific bioactive compounds (Benshitrit et al., 2012). The enrichment and fortification of these compounds in food is a major scientific and technologic challenge because many bioactive ingredients are relatively labile, resulting in a fast inactivation or degradation during food processing, storage and digestion. To ensure their high bioaccessibility and bioavailability, delivery systems have been developed to protect the compounds from degradation and enable their release at the desired absorption site (Vos et al., 2010). In addition, bioactive compounds, such as phytochemicals, can cause bitter or astringent tastes or unpleasant off-flavors. Since consumers are not willing to compromise on taste for health benefits, potential adverse effects on sensory properties need to be overcome (Verbeke, 2006).

The whey protein β-lactoglobulin (β-LG) provides structural and physico-chemical properties that facilitate the transport of small, hydrophobic ingredients. The globular protein is folded into a hydrophobic calyx which functions as the major non-covalent binding site beside hydrophobic pockets on the surface of the protein (Qin et al., 1998). Furthermore, β-LG has diverse techno-functional properties, GRAS (generally recognized as safe) status, a high nutritional value, and is soluble over a wide pH range (de Wit, 1998). Additionally, the protein can reduce the sensory perception of hydrophobic or volatile compounds due to its binding properties (Shpigelman et al., 2012; Seuvre et al., 2002). However, due to its compact globular structure and its resistance to gastric conditions, it is the main allergen in bovine milk (del Val et al., 1999). The use of β-LG as a

transporter for non-covalently bound ligands was frequently reported, but the targeted covalent binding of bioactive compounds is a more recent approach (Keppler et al., 2014; Teng et al., 2013; Shpigelman et al., 2012; Rade-Kukic et al., 2011).

Allicin, the major thiosulfinate in fresh crushed garlic, is mainly responsible for the characteristic taste and smell of garlic (Bautista et al., 2005; Salazar et al., 2008). The organosulfur compound exerts various health promoting effects, such as the reduction of the risk of certain cancers and cardiovascular diseases (Borlinghaus et al., 2014; Fleischauer et al., 2000). With respect to functional foods, these effects are two of the most important health related properties for consumers, therefore allicin is an interesting functional ingredient (Kraus, 2015). However, allicin is fairly unstable (e.g. at pH values above 6, at higher temperatures, in the presence of oil) and rapidly degrades during food processing and storage which limits its bioaccessibility (Lee et al., 2014). For example, spray drying has been reported to cause 25-70% degradation of allicin (Rodriguez-Jimenes et al., 2014). Beside the instability of allicin, a decisive shortcoming is its smell. Frequent garlic consumers associate garlic with its health promoting effects, whereas the aversion of seldom and non-users is mainly caused by the malodorous odor, especially in breath (Rosin et al., 1992).

Through the covalent binding of allicin to β-LG a stable, non-volatile S-allylmercapto-derivate of the free cysteinyl residue is formed. The digestibility of β-LG modified by allicin is not affected and S-allylmercaptocysteine could be released and absorbed like other amino acids (Wilde et al., 2016a). S-allylmercaptocysteine is a metabolite of allicin and acts as a stable reservoir of the S-allyl moiety to mediate and prolong its activity. Therefore the health related effects of S-allylmercaptocysteine were suggested to be similar to those of allicin (Miron et al., 2010; Rabinkov et al., 1998). So far, the transfer to a food-grade level and the sensory properties of the bound allicin have not been tested. Therefore, the objective of the present study was to produce β-LG modified with allicin at a food-grade level by taking the influence of various process parameters into account. Further, a suitable food matrix for the enrichment of the functional ingredient was developed. Finally, the sensory properties of the modified protein and the functional food were assessed.

5.3 Materials and Methods

5.3.1 Materials

For the production of the modified β-LG and the study drink (beverage model for experiments) only food and pharmaceutical grade ingredients were used. Whey protein isolate (WPI) (BiPRO, Davisco Foods International, Inc., Eden Prairie, US) with 97.7% protein and 75% β-LG in dry matter. Fresh

garlic bulbs, instant coffee, sugar, lactose, cocoa powder, coffee whitener (main ingredients: glucose syrup, vegetable fat) and cream were purchased from a local grocery store. Carrageen Satiagum ADC 25 (Cargill Deutschland GmbH, Krefeld, Germany) and vanilla flavor (Symrise AG, Holzminden, Germany) were generous gifts. Sodium hydroxide (Panreac Applichem, Darmstadt, Germany) and hydrochloric acid (Merck, Darmstadt, Germany) were food and pharmaceutical grade, respectively.

All chemicals used for chemical analysis were analytical grade. Allicin was synthesized according to a modified procedure from (Small et al., 1947) as described by Wilde et al. (2016b).

5.3.2 Preparation of garlic powder

Garlic cloves of five different garlic cultivars (white and purple-type, from China, Spain and France) were separately processed to garlic powder. At first garlic cloves were manually peeled and cut into 3-4 mm thick slices. In a perforated plastic bag the slices were frozen in liquid nitrogen and freeze dried afterwards (laboratory freeze dryer, Gamma 1-16 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). The dried slices were ground by an analysis mill. The allicin content of the garlic powder was measured by RP-HPLC with an Agilent 1200 Series system (Agilent Technologies, Santa Clara, US). Therefore, the powder was dissolved in water (5 mg/ml) and filtrated by a syringe filter (0.2 µm pore size). RP-HPLC analysis was conducted with a C-18 column Nucleodur Gravity (100 mm x 2 mm i.d., 1.8 µm particle size, Macherey-Nagel GmbH & Co. KG, Düren, Germany). The mobile phase consisted of 5 mM ammonium acetate dissolved in water, pH 6.6 (eluent A) and acetonitrile with 0.1% formic acid (eluent B) at a flow rate of 0.2 ml/min with a gradient program as follows: 40% B (0-10 min), 100% B (15-19 min), 5% B (20-22 min), 40% B (25-35 min). The injection volume was 5 µl, the UV detector operated at 205 nm and the column temperature was 25 °C. Quantification of allicin was done by calibration with allicin standard.

5.3.3 Preparation of β-LG modified with allicin

For the binding reaction of allicin from garlic powder to β-LG from WPI, the WPI was dissolved and stirred for one hour. The powder of the garlic cultivar with the highest allicin yield, rose garlic (Ail rose de Lautrec), was used for the binding experiments with β-LG. The garlic powder was dissolved separately and filtered before it was added to the protein solution. The pH of the mixture was adjusted to 8.5 by 0.1 M NaOH and the final concentration of WPI was 26 g/L (corresponds to 1000 µM β-LG). To test different ligand-protein ratios the final concentration of garlic powder was varied between 2.9-5.8 g/L (corresponds to 250-500 µM allicin). For the control sample no garlic powder was added. The solution was stirred for one hour and incubated at 4 °C for 24 h. Two different drying

techniques were tested. For the freeze drying the solution was filled in dishes after incubation, frozen at -25 °C and freeze dried using the same freeze dryer (see 5.3.2). For spray drying two different pH values of the solution during the drying process were tested. A part of the samples was left unchanged after incubation and had a pH value of about 8.0. The other part was adjusted to pH 6.0 by 0.1 M HCl. Finally, the solutions were spray dried on a pilot plant spray dryer (Mobile Minor 2000, Niro A/S, Copenhagen, Denmark) using a rotating atomizer disc at a flow rate of 47 ml/min, at 180 °C/70 °C inlet/outlet temperature and an outlet pressure of 4 bar.

For the analysis of the thermal stability of unmodified and modified β-LG pure β-LG and allicin were used. The samples were prepared as described by Wilde et al. (2016b) by using different molar ratios (β-LG/allicin: 1:0 mol/mol; 2:1 mol/mol; 1:1 mol/mol).

5.3.4 Characterization of modified β-lactoglobulin

Degree of denaturation

The influence of the covalent modification by allicin on the thermal stability of β-LG was analyzed. Therefore, pure β-LG modified by pure allicin was diluted to 100 µM β-LG and heated in a water bath for 30 min at different temperatures (70, 75, 80, 85, 90 °C). Afterwards the samples were cooled down in an ice bath and the degree of denaturation was determined. In addition, the degree of denaturation was measured of the samples prepared with β-LG from WPI and with allicin from garlic powder, before and after drying processes.

The determination was done by the content of acid soluble β-LG in the samples according to methodical provision of the German Industrial Standard (DIN 10473) (German Industrial Standard, 1997). The method is based on the isoelectric precipitation of denatured β-LG at pH 4.6. The β-LG concentration was analyzed by RP-HPLC using the Agilent 1100 Series HPLC with a diode-array detector and PLRP-S column (300 Å, 8 µm, 150 x 4.6 mm, Agilent Technologies, Santa Clara, USA). The injection volume was 20 µl at a flow rate of 1.0 ml/min and a column temperature of 40 °C using eluents A (0.1% (v/v) TFA in water) and B (0.1% TFA (v/v) in ACN). The elution used gradient steps of 35-38% B (1-8 min), 38-42% B (8-16), 42-46% B (16-22 min), 46-100% B (22-22.5 min) and 100-35% B (23-23.5 min). The detection wavelength was 205 nm. The relative difference of the β-LG concentration in a sample before and after precipitation corresponded to the degree of denaturation.

Degree of modification

The area of the unmodified and modified β-LG A and B (genetic variants) in the RP-HPLC chromatogram was used to determine the degree of modification. The WPI powder containing unmodified (control) or modified β-LG was dissolved (0.9 mg/ml) and analyzed by RP-HPLC with the same method as mentioned above for acid soluble β-LG. The unmodified product showed two peaks for β-LG genetic variants A and B. These peaks had a higher retention time when β-LG reacted with allicin. The relative difference of the unmodified β-LG concentration in the sample, to the control, corresponded to the degree of modification (Wilde et al., 2016b).

5.3.5 Study drink development

The study drink was proposed to be comparable to a chilled ready to drink coffee drink. Commercially available coffee drinks consist of about three quarters of milk. For the study drink milk was replaced by WPI, containing unmodified or modified β-LG, to assure that β-LG was the most prevalent protein in the drink. A commercial coffee drink was used for comparison during the development process. Table 5-1 (Supplementary) shows the ingredients of the final product. The production process is shown in Figure 5-1 (Supplementary) schematically. The beverage was chilled at 4 °C until sensory analysis.

5.3.6 Sensory analysis

Sensory evaluation was done by a trained panel with 10 panelists. Through quantitative descriptive analysis the intensity of defined attributes was measured on a six-point-scale (0 = not perceptible, 5 = strongly perceptible) (Stone et al., 1974). The panel selected 5 attributes for a garlic powder solution, concerning odor and taste. White bread and filtered tap water were used as palate cleansers.

Samples were prepared as follows. Dried WPI containing β-LG modified with allicin was tested in water and in the study drink (see 5.3.5). In a pretrial appropriate concentrations for both media were alternated. Four aqueous samples were prepared which were composed as follows: 1) garlic powder (0.25 mg/ml); 2) garlic powder (0.25 mg/ml) and WPI (1.7 mg/ml), added separately without prior binding reaction; 3) WPI containing modified β-LG (1.95 mg/ml), containing WPI and garlic powder, after binding reaction and drying; 4) ultrafiltrated WPI containing modified β-LG (1.95 mg/ml). The ultrafiltration was done to remove the remaining free compounds of the garlic powder that were still present in the product after binding reaction and drying. Therefore, the WPI containing modified β-LG was dissolved in water (mg/ml) and filtrated by using ultrafiltration units (Vivaspin, MWCO: 10 kDa, Sartorius AG, Göttingen, Germany). After centrifugation (8000 x *g*, 15 min) permeate was

removed. 10 ml water were added to the retentate and centrifugation was repeated to wash the protein fraction. Afterwards further 10 ml water were added to the retentate in the upper part of the filtration unit and the protein adhering to the membrane was dissolved by vigorous shaking. The final protein concentration was determined by RP-HPLC and adjusted to the same level as the non-filtrated WPI sample (1.95 mg/ml).

Four study drinks (see 5.3.5) were prepared which contained the following additives: 1) garlic powder (3.1 mg/ml) and WPI (29.0 mg/ml), added separately without prior binding reaction; 2) WPI containing modified β-LG (32.1 mg/ml), containing WPI and garlic powder, after binding reaction and drying; 3) WPI containing unmodified (native) β-LG (29.0 mg/ml). For the sensory analysis 30 ml of each sample was filled in white cups.

5.3.7 Headspace gas chromatography-mass spectrometry (HS GC-MS)

The volatile sulfur-containing compounds from garlic powder with and without WPI were analyzed by HS GC-MS using a 6890 gas chromatograph equipped with a MS detector 5975 and a DB-5MS column, 60 m x 0.32 mm i.d., 0.25 µm film thickness (all from Agilent Technologies, Santa Clara, US). 1 ml of sample solution was transferred to a 20 ml headspace vial which was incubated at 50 °C for 30 min. After incubation the syringe, tempered to 75 °C, transferred 500 µl gas sample into the injector. The operating conditions were as follows: injector temperature 230 °C, split ratio 1:1, carrier gas flow 1 ml/min, temperature program: 40 °C - 250 °C, heating rate 10 °C/min, initial and final temperatures were held for 3 min and 2 min, respectively. The MS detector conditions were as follows: electron ionization mode at 70 eV over the range of 27-600 amu. The source temperature was maintained at 230 °C and the GC-MS interface at 250 °C. Standard reagents of allicin and diallyl disulfide were used for calibration.

5.3.8 Statistical analysis

The results were subjected to a statistical analysis of variance (ANOVA) and Tukey's multiple comparisons test. The calculations were performed by using GraphPad Prism (version 6.00, GraphPad Software, San Diego, USA).

5.4 Results and discussion

5.4.1 Food grade production of modified β-lactoglobulin (β-LG)

WPI rich in β-LG (i.e. β-LG >70%) and garlic powder with a high allicin potential were used to modify β-LG with allicin. After the binding reaction of allicin to β-LG the mixture of WPI and garlic powder was dried by freeze- or spray drying.

Garlic powder

To obtain a garlic powder with a high allicin yield different garlic cultivars were examined. The allicin concentration and the diallyl disulfide (DADS) signal intensity of the headspace of five different garlic powders were analyzed (Supplementary Figure 5-2). The cultivar with the highest allicin yield was a rose garlic from Lautrec in France (Ail rose de Lautrec). The allicin and DADS abundance correlated strongly (r=0.99) which indicated the prevalence of allicin and its degradation to DADS during GC measurement, which was also reported elsewhere (Block, 2011). Further, a high pungency, which indicates a high allicin yield, has been previously described for purple-type cultivars (Pardo et al., 2007), confirming the present results. Following this, the powder of the rose garlic was used for the binding experiments with β-LG from WPI to minimize the necessary amount of garlic powder added.

Modification of β-LG

The modification of β-LG through the binding reaction with compounds from garlic powder was analyzed using RP-HPLC (Figure 5-1). The chromatogram of the WPI containing unmodified β-LG shows the different whey proteins: α-lactalbumin, bovine serum albumin and the genetic variants A and B of β-LG. After the incubation with allicin the area of the native protein peaks decreased and two new peaks at higher retention times were formed revealing a higher hydrophobicity for the modified protein. Through the binding reaction the free thiol group of β-LG at Cys^{121} is modified to S-allylmercaptocysteine (SAMC), as shown by Wilde et al., 2016a. Different ratios of allicin to β-LG were tested (1:2; 1:3; 1:4). The degree of modification was 64.3 ± 0.05% at a molar ratio of 1:3. The stoichiometric ratio of the reaction between allicin and β-LG is 1:2 (Wilde et al., 2016b). I.e. allicin reacted completely with the protein. Corresponding to the molar ratio the degree of modification changed: 1:2 mol/mol – 86.9 ± 0.83% modified; 1:4 mol/mol – 56.4 ± 0.95% modified.

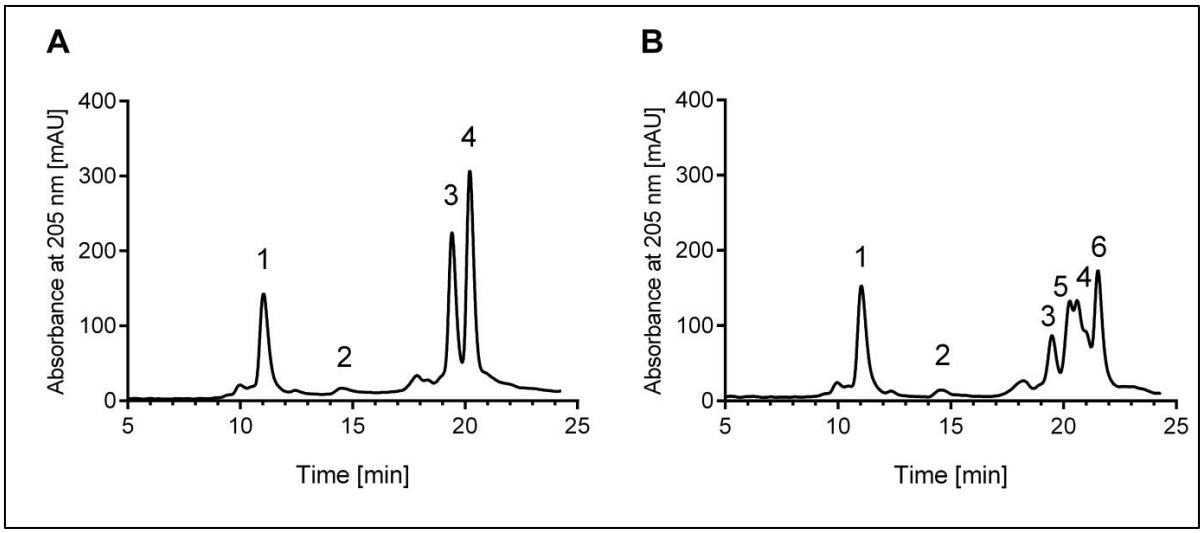

Figure 5-1: Detail of the RP-HPLC chromatograms of unmodified and modified WPI. The molar ratio of allicin to β-lactoglobulin (β-LG) was 1:3 mol/mol in the sample with the modified WPI. Identified peaks in A and B: 1) α-lactalbumin; 2) bovine serum albumin; 3) β-LG B; 4) β-LG A. Additionally identified peaks in B: 5) modified β-LG B; 6) modified β-LG A.

Drying of modified β-LG

After protein modification with garlic powder the influence of the drying process (freeze or spray drying) and the ligand-protein ratio on the DADS concentration in the headspace was analyzed.

Since allicin was completely bound the detected amount of DADS in the headspace of the mixture decreased significantly (Figure 5-2). As shown before allicin contributes largely to the detected DADS intensity of garlic powder (Supplementary Figure 5-2). After the binding of allicin the remaining amount of DADS is probably caused by DADS originally present in garlic powder. The concentration of the volatile DADS was 94% lower after spray drying compared to freeze drying ($P \leq 0.001$) (Figure 5-2). The higher loss of volatile sulfur compounds during the drying process at elevated temperatures compared to freeze drying has been shown before (Leino, 1992). Rodriguez-Jimenes et al. (2014) reported a decrease of the allicin concentration of garlic extract of 23-73% through spray drying under different conditions. They demonstrated that the kind of carrier material, its concentration as well as the inlet and outlet air temperatures were crucial for the retention of allicin. It has been shown that a high concentration of carrier material (i.e. 60%) enables an extensive encapsulation of the organosulfur compounds and a high retention during drying (Balasubramani et al., 2015). In the present study it was intended to achieve the opposite, namely an extensive loss of free volatile organosulfur compounds to reduce the garlic smell and taste of the dried WPI containing modified β-LG. Therefore the low dry matter of the WPI feed solution (about 3%) was used for spray drying.

With respect to the ligand-protein ratio, an influence on the headspace DADS concentration was only seen after freeze drying (Figure 5-2). Presumably because the total amount of volatile DADS was already very low after spray drying, a higher protein excess did not induce a further significant reduction. In contrast after freeze drying a higher ligand to protein ratio (1:8) significantly reduced the DADS concentration in the headspace ($P \leq 0.01$). This result can be explained by the ability of β-LG to increase the retention of volatile aroma compounds by hydrophobic interactions (Seuvre et al., 2002). With increasing ligand-protein ratio this effect was enhanced as well.

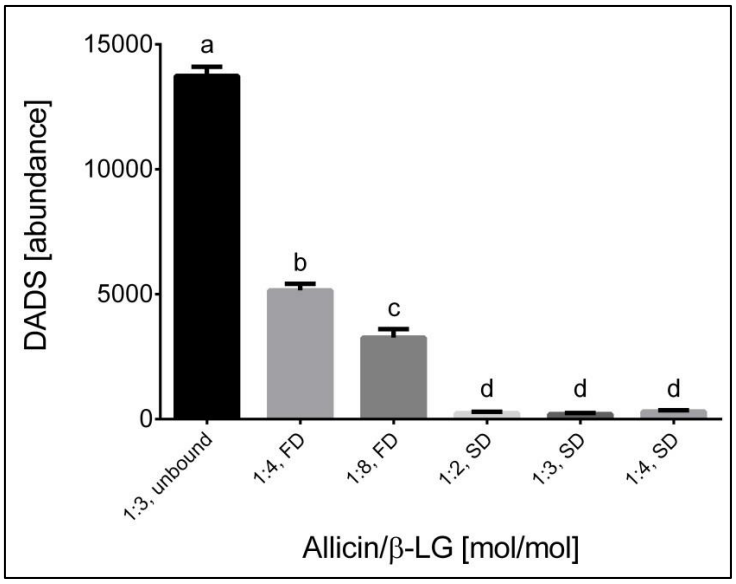

Figure 5-2: Diallyl disulfide (DADS) abundance ± SD analyzed by headspace GC-MS of dissolved WPI and garlic powder with different ligand-protein ratios. Samples were freeze dried (FD) or by spray dried (SD) at pH 8.0. Molar ratios of allicin from garlic powder to β-lactoglobulin from WPI varied from 1:2 to 1:8 mol/mol. The binding reaction was carried out prior the drying process. 8 mg sample was dissolved in 2 ml water for headspace analysis. For comparison one sample contained WPI and garlic powder without prior binding reaction (unbound) and drying. Different letters denote significantly different values ($P < 0.01$).

Denaturation of β-Lactoglobulin

The influence of the modification by garlic powder, on the thermal stability of β-LG was analyzed (Figure 5-3). After heat treatment for 30 min at different temperatures (70, 75, 80, 85, 90 °C) the degree of denaturation was determined. The modification by garlic powder significantly reduced the degree of denaturation after heating at temperatures in the range of 70-90 °C. If β-LG was modified at a molar ratio of 1:2 mol/mol allicin to β-LG, this effect was only observed at temperatures <90 °C. Samples modified at the ligand-protein ratio of 1:1 mol/mol showed a low degree of denaturation (≤ 11%) even after heating at 90 °C. Further, the degree of denaturation of the sample at this protein-

ligand ratio did not correlated with the heating temperature as shown for the other samples, i.e. unmodified β-LG and modified β-LG (allicin/ β-LG 1:2 mol/mol).

The results clearly demonstrated that the modification of the thiol group of β-LG reduced the irreversible denaturation of the protein. This effect was dependent on the degree of modification, since only the modification of all thiol groups prevented denaturation efficiently. Croguennec et al. (2003) also observed a decreased denaturation of β-LG during heat treatment if the free thiol group was blocked by the reagent N-ethylmaleimide. The free thiol group plays an important role in the thermal denaturation process of β-LG, hence it induces the formation of disulfide linked aggregates which leads to the irreversibility of the process (Busti et al., 2005; Hoffmann & van Mil 1999). If the free thiol group is blocked the formation of covalent stabilized aggregates is limited (Croguennec et al., 2003). Therefore the derivatization of Cys^{121} by allicin influenced the denaturation behavior of the protein notably.

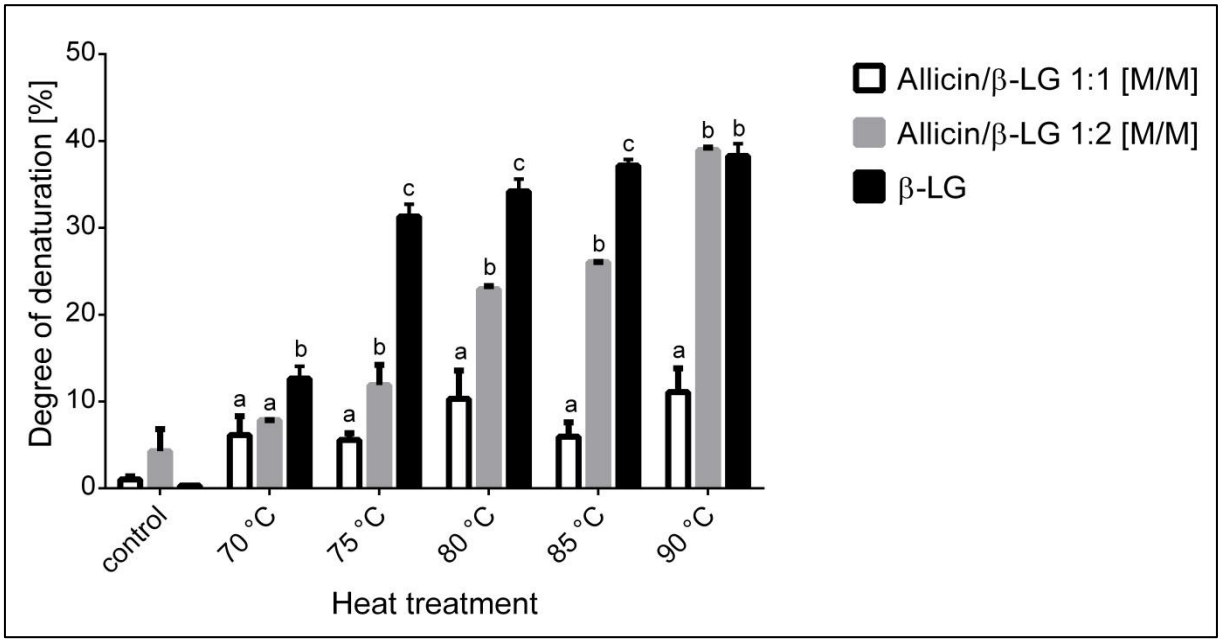

Figure 5-3: Degree of denaturation ± SD of unmodified and modified β-lactoglobulin (β-LG) after heat treatment at different temperatures. Heating time was 30 min. β-LG concentration was 100 µM, pH value was 7.0. Control samples were not heat-treated. Different letters denote significantly different values between samples heated at the same temperature ($P < 0.05$).

Further, the degree of denaturation was determined for samples prepared by β-LG from WPI and allicin from garlic powder. Figure 5-4 shows the influence of the pH value (i.e. pH 6.0 or pH 8.0) and the ligand-protein ratio (1:2, 1:3 or 1:4) on the degree of β-LG denaturation during spray drying. The pH value of the solution significantly influenced the denaturation and had the strongest effect among

the tested conditions: If the solution was dried at a pH of 6.0 the denatured β-LG proportion was remarkably lower compared to drying at pH 8.0 with the same ligand-protein ratio ($P \leq 0.0001$). An alkaline pH value (pH 8.0) during drying favored the denaturation which has been reported by other authors before (Hoffmann & van Mil, 1999; Verheul et al., 1998). Due to rising intramolecular electrostatic repulsion the conformational stability of β-LG decreases with increasing pH value from the isoelectric point to an alkaline pH value. Additionally, the reactivity of the free thiol group of Cys^{121} is much higher at pH 8.0 compared to 6.0 which leads to a higher reaction rate of the aggregation process (Hoffmann & van Mil, 1999; Verheul et al., 1998). Further, the accessibility of the thiol group is increased due to the Tanford transition at pH ≈ 7.4 and the presence of the thiolate anion is increased because it is closer to the pK value (pH 8.5) (Thurlkill et al., 2006; Tanford et al., 1959).

The ligand-protein ratio affected the degree of denaturation because of the different thermal stability of the unmodified and modified β-LG, as shown above for the samples prepared with pure β-LG and allicin (Figure 5-3): When the majority of the free thiol groups was modified by allicin, the proportion of denatured β-LG after spray drying was significantly reduced ($P \leq 0.0001$). This effect was only obvious for the ligand-protein ratios of 1:2 mol/mol to 1:3 mol/mol (Figure 5-4). Between 1:3 mol/mol and 1:4 mol/mol no further difference regarding the degree of denaturation was observed (1:3 = 64.3%; 1:4 = 56.4%).

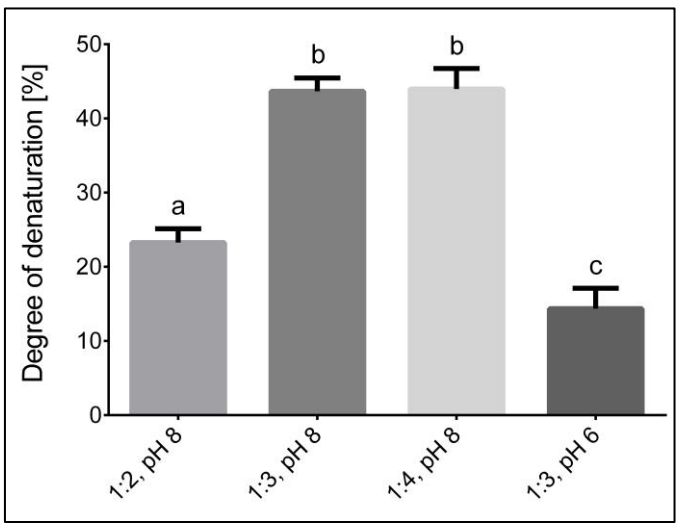

Figure 5-4: Degree of denaturation ± SD of modified β-lactoglobulin (β-LG) from WPI with garlic powder after spray drying at pH 8.0 and 6.0. Molar ratios of allicin from garlic powder to β-LG varied from 1:2 to 1:4 mol/mol. Different letters denote significantly different values ($P < 0.01$).

5.4.2 Sensory evaluation of aqueous solutions with garlic constituents combined with whey protein

For the description of the taste, following attributes were determined by the panel: "fresh garlic", "garlic powder" and "pungency", for the description of the odor: "fresh garlic", "garlic powder" and "musty". The evaluation of garlic powder in aqueous solution showed that the binding of allicin to β-LG had an enormous influence on the perceived intensity for all attributes (Figure 5-5A). The effect on the taste was even more distinct compared to the odor. With respect to the odor the intensity for "fresh garlic" was significantly lower if allicin was bound to the protein ($P \leq 0.01$). The odor intensity of "garlic powder" was also reduced but a remarkable reduction of the intensity was only reached by ultrafiltration ($P \leq 0.01$). The same effects were shown for the attribute "mustiness" ($P \leq 0.05$). This means that the perception of "garlic powder" and "mustiness" was not caused by bound allicin but by other non-bound organosulfur compounds of garlic powder which were removed by ultrafiltration of the WPI containing modified β-LG.

With respect to the taste the intensity of "fresh garlic" and the "pungency" were significantly reduced by the binding of allicin ($P \leq 0.0001$). The strong correlation of the intensity of these attributes is probably caused by allicin because it is a strong flavor compound and the major thiosulfinate (Bautista et al., 2005; Salazar et al., 2008). The control sample containing WPI and unbound allicin from garlic powder underlined the importance of the binding reaction for the taste perception, because the presence of the whey proteins in the control sample had a minor effect on the taste intensity. In contrast to the odor perception, the taste of "garlic powder" was mainly reduced by the binding of allicin but no further reduction through the ultrafiltration was noticed. Probably because of the low intensity of "garlic powder" no further differentiation was possible.

Through the binding reaction of allicin the free cysteinyl residue of β-LG is modified to SAMC. There is nothing known about the excitation of sensory neurons by SAMC. Allicin mediates its pungency by activating members of the transient receptor potential (TRP) family of cation channels that also respond to a variety of pungent compounds, like capsaicin and allyl isothiocyanate (Bautista et al., 2005). The covalent modification of cysteine residues is the supposed mechanism of the channel activation of TRPV1 (Salazar et al., 2008). Since SAMC is less reactive than allicin and is buried in the inner of the globular protein it is very unlikely that it is able to mediate pungency. Furthermore, SAMC is covalently bound within the polypeptide chain of β-LG and is not volatile like the odor-causing organosulfur compounds of garlic (Amagase, 2006).

Besides allicin, garlic powder contains various other dialkyl sulfinates (e.g. allyl methanethiosulfinate, methyl 2-propenethiosulfinate, allyl *trans*-1-propenethiosulfinate) and degradation products (e.g. diallyl disulfide, diallyl trisulfide) that contribute to the remaining garlic like sensory impression (Lawson & Hughes, 1992). In particular the formation of degradation products is very likely during 24

h incubation at an alkaline pH value and heating during spray drying (Lawson, 1996). The results of GC-MS analysis confirmed the presence of DADS in the headspace of the WPI containing modified β-LG, even at a very low level (Figure 5-2).

5.4.3 Study drink

Development of the study drink matrix

The aim was to obtain a palatable beverage containing an amount of about 10 g WPI with β-LG modified with allicin from garlic powder. This amount delivered 18 mg of bound allicin corresponding to the uptake of approximately one garlic clove. Further, a matrix was required which can mask the garlic flavor of residual organosulfur compounds from the WPI-garlic-powder-mixture.

The final product exhibited a similar sensory profile as commercially chilled coffee drinks. The crucial difference was that the developed drink contained no fresh milk as WPI was the main protein source in the drink of the present study. Referring to commercial coffee drinks milk is usually the main ingredient (75-80%). For the development of the study drink no milk was used, to exclude the effects of other proteins than β-LG. An interaction of allicin with α-lactalbumin and bovine serum albumin from WPI was ruled out by RP-HPLC analysis (Figure 5-1). Beside the WPI other ingredients, like lactose, coffee whitener and cream, were added to create a similar sensory profile to a milk containing drink. The addition of coffee whitener and cream resulted in an increased fat content in order to contribute to the masking effect of garlic flavor and pungency. The notable bitterness of the drink was probably caused by WPI, instant coffee and cocoa powder (Ye et al., 2012; Beecher et al., 2008; Frank et al., 2007). Carrageen was added to improve the mouthfeel, but also to reduce the perception of garlic flavor. This effect was described before (Cook et al., 2003). Finally, at the drink-serving temperature of 4 °C of the beverage contributed to a low volatility of the organosulfur compounds. Even if a slight garlic flavor was still perceived, the combination of coffee, chocolate and garlic flavor was harmonious and the overall sensory impression was positive.

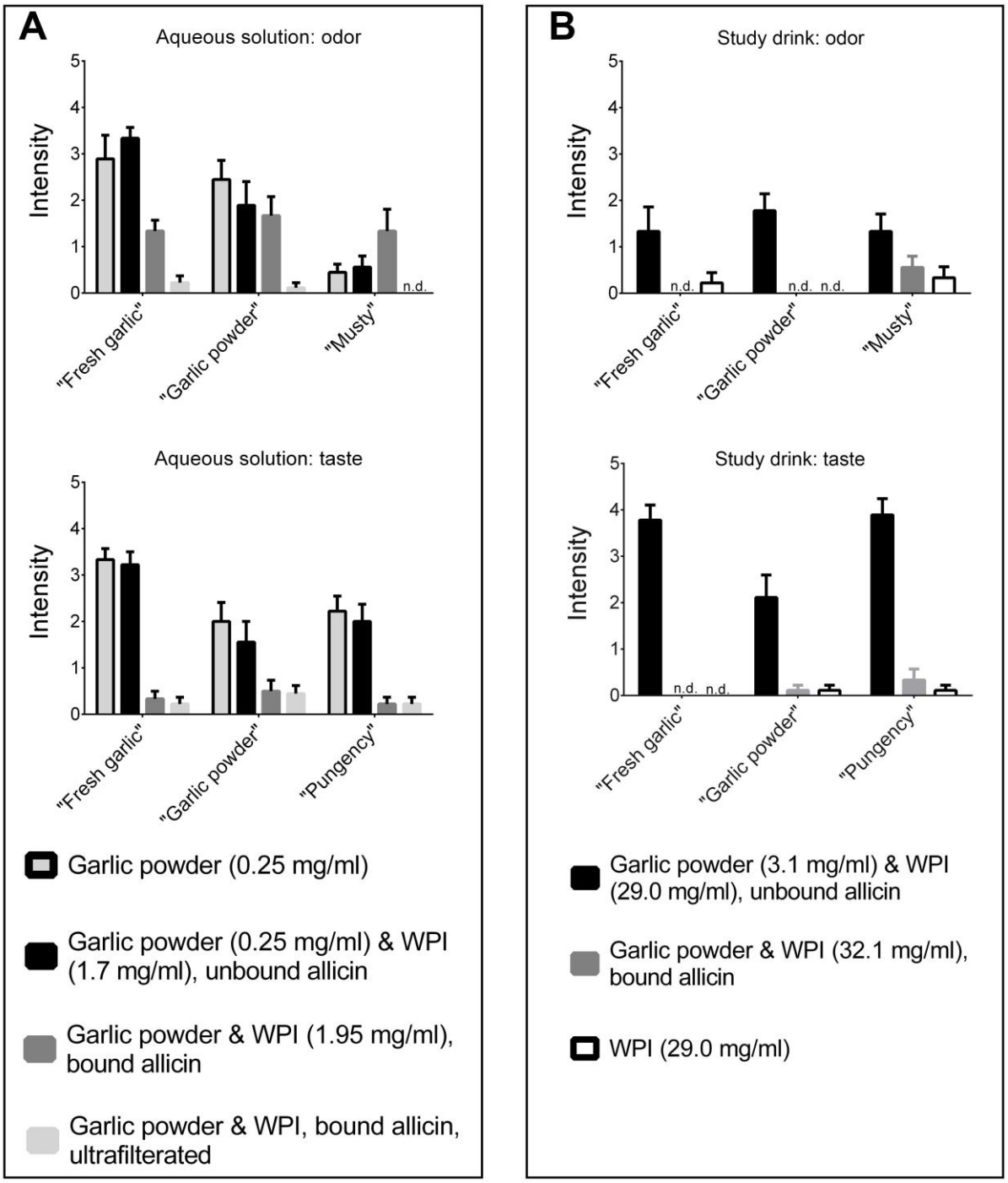

Figure 5-5: Sensory evaluation by quantitative descriptive analysis of WPI with garlic powder (bound) and without (unbound) prior binding reaction. Molar ratio of allicin from garlic powder to β-lactoglobulin from WPI was 1:3 mol/mol. The modified WPI was spray dried at pH 6.0. The intensity of the attributes for odor and taste was measured on a six-point-scale (0 = not perceptible, 5 = strongly perceptible). A) Samples solved in water. B) Samples solved in the study drink. N.d. – not detectable. Error bars indicate standard deviation.

Sensory evaluation of garlic constituents in the study drink

The concentration of garlic powder (bound or unbound) in the study drink (Figure 5-5B) was about twelve times higher than in the analyzed aqueous solution (Figure 5-5A) in order to correspond to the amount of allicin from one garlic clove. Nevertheless, allicin bound the odor intensity of the attribute "fresh garlic" of the drink was lower than of the aqueous solution ($P < 0.05$). In contrast to the aqueous solution containing 0.17% protein, the higher protein concentration of 3% in the study drink probably enabled the retention of volatile compounds. Next to proteins, the presence of fats was shown to reduce the volatility of the hydrophobic organosulfur compounds, like DADS (Hansanugrum & Barringer, 2010). Since milk provides a homogenous mixture of proteins and fat, it reduces the garlic like odor which was also shown by Negishi et al. (2002). The authors reported that the addition of milk decreased the headspace concentration of a DADS solution by 95%.

The sensory panel detected no garlic like odor of the WPI containing modified β-LG. With respect to taste the perceived intensity of all attributes was significantly lower for the samples with modified β-LG compared to the WPI and garlic powder without prior binding ($P \leq 0.0001$). This difference can be attributed to two crucial factors. One is the already mentioned binding of allicin to β-LG. The other is the drying process of the WPI after the binding reaction. Through the spray drying a considerable amount of volatile compounds was removed (Figure 5-2).

5.4.4 Influence of pH and ligand-protein ratio on sensory properties

Figure 5-6 shows the influence of the ligand-protein ratio (1:2, 1:3, 1:4) and the pH-value during spray drying (pH 8, pH 6) on the taste perception. A higher protein excess had no influence on the garlic flavor, which was expected. Since the stoichiometric ratio of allicin and β-LG is 1:2, at a molar ratio of 1:2 allicin was completely bound and a further increase in protein concentration did not enhance the effect. In contrast to the protein-ligand ratio the pH value of the solution during the drying process seemed to have an influence on the sensory perception of the final product. The samples which were dried at an alkaline pH had lower garlic flavor intensity than the sample dried at pH 6.0, especially the intensity of "fresh garlic" was significantly lower ($P < 0.05$). A basic pH of the solution during the drying process promoted the degradation of thiosulfinates (Lawson, 1996). On the other hand a basic pH during drying increased the denaturation of β-LG (Figure 5-4).

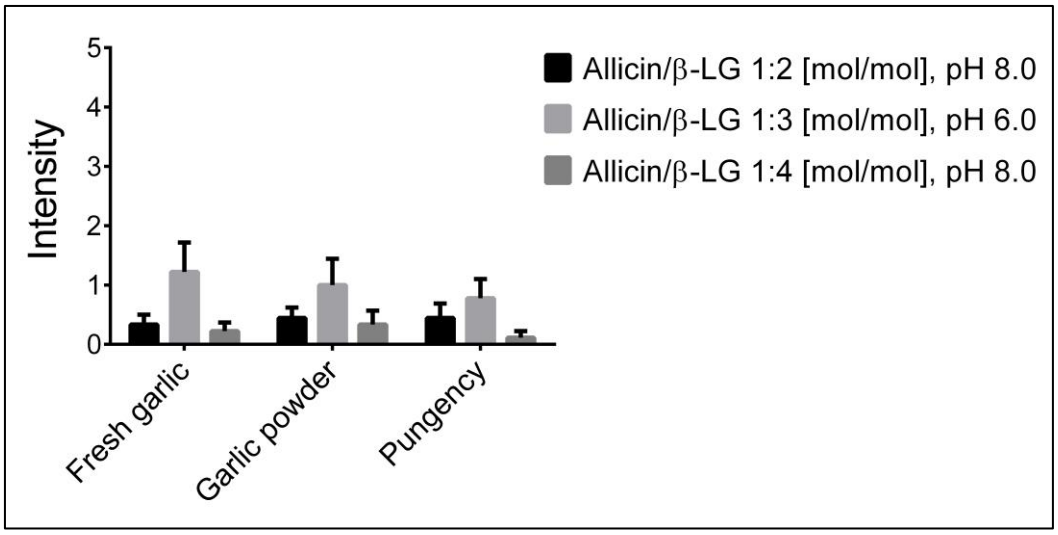

Figure 5-6: Taste evaluation by quantitative descriptive analysis of samples with different ligand-protein-ratios [mol/mol] and different pH values during spray drying (6.0, 8.0). Allicin was added as garlic powde, β-lactoglobulin was added as WPI. The intensity of the attributes for taste was measured on a six-point-scale (0 = not perceptible, 5 = strongly perceptible). Samples were solved in the study drink. Error bars indicate standard diviation.

5.4.5 Influence of sugar content on sensory properties

Different sugar concentrations of the study drink were examined. As expected, with increasing sugar content the intensity of sweetness increased ($P \leq 0.01$) and the intensity of bitterness decreased ($P \leq 0.05$). There was no clear effect on the garlic related taste attributes (Supplementary Figure 5-3). In contrast, Lee & Kim (2013) reported that the combination of fat and sugar enhances the reduction of the pungent sensation.

5.5 Conclusions

β-LG from WPI was covalently modified with allicin from garlic powder. The used protein and ligand source were suitable for the application in food and enabled an efficient binding reaction. The modified β-LG was stable during drying processes and showed a high solubility which enables the enrichment in a wide range of food products. Due to the modified thiol group heat induced aggregation was prevented which resulted in an increased thermal stability compared to the native protein. In addition, β-LG modified by allicin was shown to be nearly free of any garlic odor and taste. An ultrafiltration process before drying of the WPI containing modified β-LG would enable to produce the product without any residual garlic flavor compounds and thus with a relatively neutral taste. The developed beverage was an appropriate food matrix for the enrichment of modified β-LG without prior purification, since remaining flavor-active ingredients were considerably masked by the protein rich emulsion. β-LG proved to be a suitable transporter for allicin, particularly the stability,

the absence of undesired sensory properties and the simple, low-cost process enables the application in food systems.

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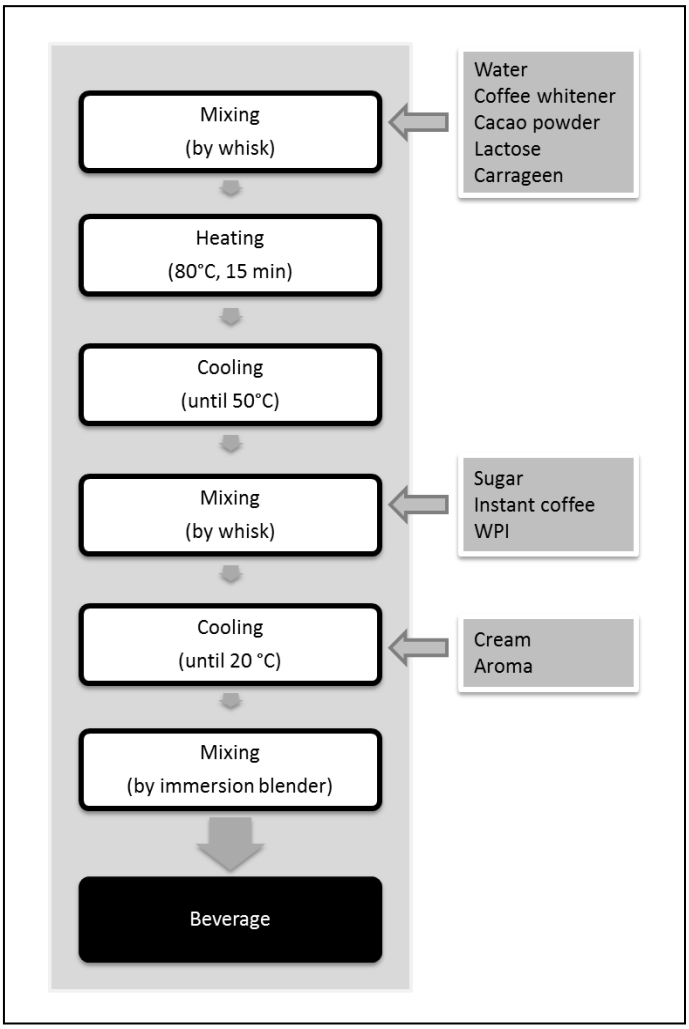
5.6 Supplementary

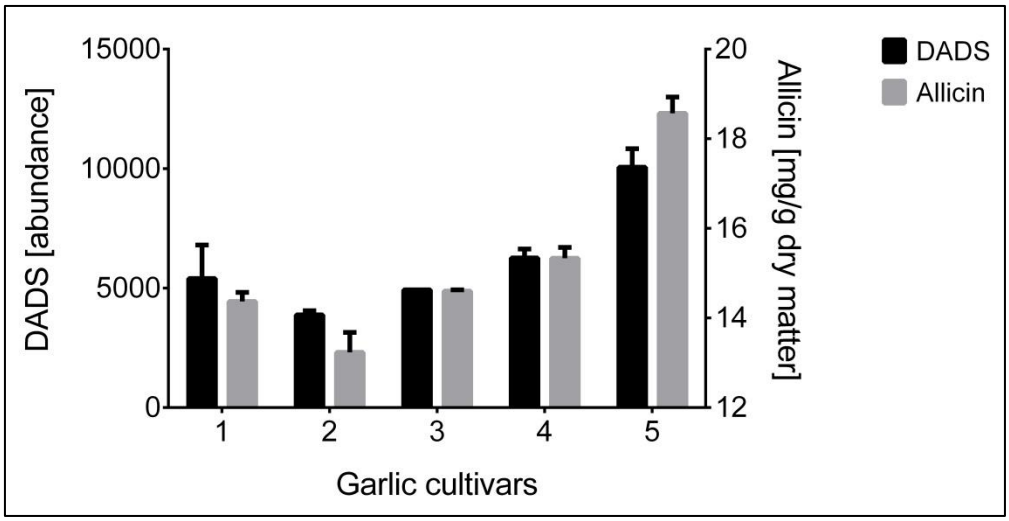
Supplementary Table 5-1: Composition of the developed beverage.

Ingredient	Content [%]
water	85.6
coffee whitener*	4.3
WPI**	3
sugar	2.1
lactose	2.5
cream	0.9
instant coffee	0.8
cocoa powder	0.4
carrageen	0.3
aroma	0.1

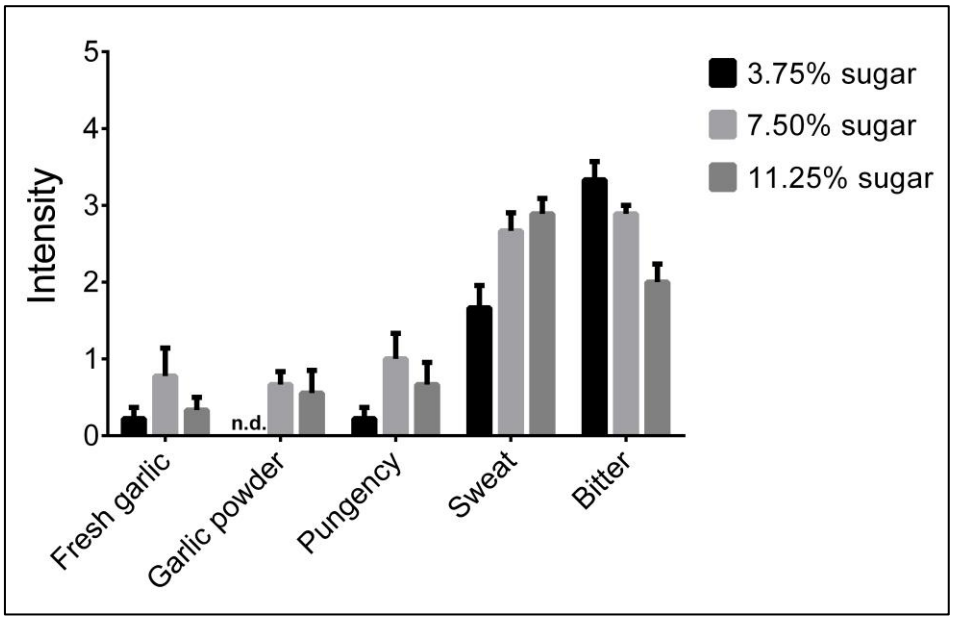
*Main ingredientens: glucose syrup, vegetable fat.
**Modified or unmodified form.



Supplementary Figure 5-1: Schematic production process for the developed beverage.



Supplementary Figure 5-2: Allicin concentration ± SD analyzed by HPLC and diallyl disulfide (DADS) abundance ± SD analyzed by headspace GC-MS of dissolved garlic powders from five different cultivars. Garlic samples: 1 –white garlic from China; 2 – purple-type single bulb garlic from China; 3 – white garlic from Spain; 4 white garlic from China; 5 – purple-type garlic from France. 1.2 mg garlic powder was dissolved in 2 ml distilled water. DADS signal from GC-MS analysis correlates with allicin concentration of the sample (pearson correlation r=0.993).



Supplementary Figure 5-3: Taste evaluation by quantitative descriptive analysis of modified WPI (ligand-protein-ratio 1:3) solved in the study drink with different sugar concentrations. The intensity of the attributes for taste was measured on a six-point-scale (0 = not perceptible, 5 = strongly perceptible). N.d. – not detected. Error bars indicate standard diviation.

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6. Bioavailability of S-allylmercaptocysteine transported by with allicin modified β-lactoglobulin in healthy individuals

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6.1 Abstract

The whey protein β-lactoglobulin was proposed as nanotransporter for allicin, a small bioactive compound from garlic. However, the bioavailability of the covalently bound ligand was not examined so far. In the present study, the bioavailability of S-allylmercaptocysteine from β-lactoglobulin modified with allicin was investigated in a double-blind, randomized, diet-controlled cross-over study. Nine male volunteers ingested a single oral dose of β-lactoglobulin modified with allicin (111 µM allicin use), garlic powder in capsules (111 µM allicin potential), and aged garlic extract in capsules (111 µM S-allyl cysteine). The concentration of allyl methyl sulfide in breath gas, N-acetyl-S-allyl cysteine in urine as well as the S-allyl cysteine in plasma were analyzed. The ally methyl sulfide excretion caused by the modified β-lactoglobulin was significantly higher than for the other treatments which indicated the efficient release of S-allylmercaptocysteine. In contrast, the allicin release from garlic powder in acid-resistant capsules (15%) and thus the excretion of allyl methyl sulfide was low. β-Lactoglobulin delivered S-allylmercaptocysteine, a stable allicin derivate, without impairing its bioavailability due to the incorporation in the protein chain.

6.2 Introduction

Garlic is known for its therapeutic and health promoting properties which are mainly provided by allicin (S-allyl 2-propene-1-sulfinothioate), the major thiosulfinate in freshly crushed garlic (Borlinghaus et al., 2014; Butt et al., 2009; Miron et al., 2000). It has been suggested that allicin exerts many of its bioactivities by transforming the thioallyl-moiety via SH/SS exchange reactions with intracellular thiol containing molecules, resulting in the formation of the corresponding S-allylmercapto-conjugates (Miron et al., 2010; Rabinkov et al., 1998).

Due to the manifold beneficial effects a regular intake of garlic is recommendable. However, many people avoid garlic because of the pungent taste and the malodorous breath after garlic consumption (Rosin et al., 1992). Therefore, different garlic supplements are commercially available, the most prevalent forms are coated tablets filled with garlic powder or dried aged garlic extract (AGE). Garlic powder supplements do not contain any allicin until disintegration in the gastrointestinal tract, where alliin can be converted to allicin by alliinase (Lawson & Hughes, 1992). AGE contains mainly the stable, water-soluble, non-volatile organosulfur compounds S-allyl cysteine (SAC) and S-allylmercaptocysteine (Ried et al., 2010). It is claimed to be odorless, which means free of garlic taste and typical garlic breath. Furthermore, it was suggested that AGE is safer and causes less adverse effects due to the absence of irritating compounds (Amagase, 2006). However, supplements are not part of a natural balanced diet and large quality differences between garlic supplements have been revealed, because most garlic powder products release far less allicin than it

ought to be (Arnault et al., 2005; Lawson & Wang, 2001). Furthermore, the production of AGE is very time consuming, as it takes about 20 month (Butt et al., 2009).

To enable the regular intake of allicin within the usual diet but without the pungent smell and taste, the enrichment in functional foods through an appropriate transporter could be a solution. Delivery systems like liposomes, microcapsules, and chitosan complexes have been tested to improve the stability and solubility of allicin (Lu et al., 2014; Wang et al., 2012; Pirak et al., 2012). Even if the stability was improved, it was still insufficient for the demands of processed foods. Further, the sensory properties of the mentioned delivery systems have not been assessed.

The binding of allicin to the whey protein β-lactoglobulin (β-LG) is an innovative approach to stabilize the organosulfur compound and mask the strong flavor and pungency. β-LG has been suggested as a suitable transporter, since it has the GRAS (generally recognized as safe) status, a high nutritional value, desired technological and versatile functional properties. According to previous studies allicin can be bound to the free thiol group of β-LG by forming the stable S-allylmercaptocysteine which is free of garlic odor and flavor (chapter 3-5).

The aim of the present study is to investigate the systemic availability of S-allylmercaptocysteine from with allicin modified β-LG in comparison with free allicin and free S-allylmercaptocysteine. Garlic powder in acid-resistant capsules was used to provide free allicin and AGE capsules were used to deliver S-allylmercaptocysteine and the structural similar SAC. Since different metabolic pathways of these organosulfur compounds have been reported, it was necessary to analyze various metabolites. The excretion products N-acetyl-S-allyl cysteine (NASAC) in urine and allyl methyl sulfide (AMS) in breath gas were detected. Further, the concentration of SAC in plasma was determined.

6.3 Materials and Methods

6.3.1 Subjects

Nine male non-smoking volunteers aged 20-29 years participated in the study. They were normal weight (BMI in range of 19-25 kg/m^2) and in good health conditions, confirmed by anamnesis form and blood baseline characteristics (Table 6-1). Exclusion criteria were overweight, metabolic and endocrine diseases, allergies, malabsorption syndromes, smoking, alcohol abuse, use of dietary supplements or any form of medication. All subjects were asked to maintain their usual lifestyles throughout the study. The study was approved by the ethics committee of the Medical Faculty of the Christian-Albrechts-University of Kiel, Germany. Written informed consent was obtained from all subjects.

Table 6-1: Baseline characteristics of subjects (*n* = 9).

Variable	Mean	SD
Age (years)	26.0	2.92
Body weight (kg)	73.1	9.02
Body height (m)	1.81	0.06
BMI (kg/m^2)	22.2	1.74
Haematocrit (%)	42.0	2.06
Blood Hb (g/dl)	142.1	7.10
Fasting plasma glucose (mg/dl)	85.6	11.47

6.3.2 Study design

The study was conducted in a double-blinded, diet-controlled cross-over design comprising three treatment days at intervals of two weeks. The treatments had following composition:

- A: garlic powder in acid-resistant capsule, allicin yield: 18 mg (111 µM) + placebo beverage
- B: dried aged garlic extract in gelatin hard capsule, SAC content: 18 mg (111 µM) + placebo beverage
- C: placebo capsule + beverage with WPI, containing 3.7 g with allicin modified β-LG (189 µM)

For each subject the treatments A, B and C were randomly assigned to the different treatment days, in order that every participant ingested each capsule-drink combination once after the three treatment days. One week before each treatment day, participants were instructed to avoid the consumption of vegetables and spices of genus *Allium* and foods containing them (wash-out period). A list of corresponding food items (e.g. garlic, onion, leek, numerous processed foods) was provided for detailed information. Compliance was controlled by a self-completed three-day dietary record. Inspection of records showed no deviation from the *Allium*-restricted diet. Between treatment days was a break of one week without any diet restrictions, followed by the next wash-out period.

At treatment days, the capsule-drink combinations were given in the morning after a 12 h overnight fast. Each time the subjects ingested five capsules with a glass of water (200 ml) plus the chilled coffee drink (300 ml). A standardized *Allium*-free diet was offered during the whole day. The first meal was served one hour after treatment intake. A total amount of 2.6-2.8 l water was drunk over the whole day allocated to glassful portions at regular time points. Other drinks were not allowed.

6.3.3 Breath gas sampling and analysis

Breath gas samples were collected from six subjects (same subjects at each treatment day). At treatment days the subjects used the provided toothpaste that was free of herbs and other strong flavors and they were not allowed to use mouthwashes. For breath-sampling the subjects were introduced to breathe naturally and relaxed (avoiding deep inhaling and exhaling), hold their breath for 15 s and then exhale into the sample bag until it was mostly full (up to 80% of maximum volume). The breath samples were collected in 1.0 l Tedlar bags (polyvinylfluoride bags, Restek GmbH, Bad Homburg, Germany) containing a polypropylene valve with a septum fitting and a mouthpiece. Breath gas samples were taken prior consumption of the capsule-drink treatment and 2, 4, 6, 8, and 24 hours after ingestion. AMS concentration was analyzed by GC-MS within 24 hours after sample collection. A 6890 gas chromatograph equipped with a MS detector 5975 and a DB-5MS column, 60 m x 0.32 mm i.d., 0.25 µm film thickness was used (all from Agilent Technologies, Santa Clara, US). The tedlar bag and a gastight syringe (2.5 ml) were incubated at 50 °C for 30 min before 2 ml breath gas sample was manually transferred into the injector. Between samples the syringe was manually washed with air three times to remove potential residues. The injector worked at 230 °C in the splitless mode with a purge flow of 7 ml/min for 0.4 min. Helium flow rate was 1 ml/min and column temperature was programmed from 40 °C for 3 min, increase to 60 °C at a rate of 10 °C/min, then increase to 180 °C at a heating rate of 20 °C/min, held for 2 min at 180 °C. The MS detector worked in the electron ionization mode at 70 eV, the source temperature was maintained at 230°C and the GC-MS interface at 250 °C. Mass spectrometer worked in the selected ion mode (SIM) and monitored target ions were *m/z* 61, 71, 73 and 88 at dwell time of 100 ms.

Before usage, the bags were pre-conditioned with nitrogen to reduce the background emission of contaminants. Therefore, bags were flushed with pure nitrogen, incubated at 50 °C for 1 h. After removing of the gas, the bags were again flushed with nitrogen and evacuated by a vacuum pump. Calibration was done by standard reagent of AMS (98%, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) at each day of analysis twice. Therefore, AMS was diluted with ethanol to give a solution of 500 µg/l (solution 1), 1000 µg/l (solution 2) and 2000 µg/l (solution 3). Tedlar bag was filled with 500 ml nitrogen and 10 µl of AMS solution 1 were added by a gastight syringe through the septum. The sample bag was incubated (50 °C, 30 min) to ensure complete evaporation, and 2 ml gas sample was taken and analyzed. Then, the AMS concentration was increased by the next injection of standard solution 1. The procedure was repeated several times with solution 1 and afterwards with 2 and 3 to get a calibration curve ranging from 6 ng/l to 400 ng/l, which provided a linear response (R^2 in range of 0.998-0.999). For calculation of AMS concentration the volume change due to sample taking was corrected. Three independent measurements were performed of each concentration level. LLOQ was at 5 ng/l.

6.3.4 Blood sampling, processing and analysis

Blood samples were collected before treatment administration (baseline) and then hourly until 8 hours after the dose plus once after 24 hours. Blood was drawn into tubes containing lithium-heparin (Sarstedt, Nümbrecht, Germany) and immediately centrifuged (2000 x *g*, 10 min, 4 °C). The plasma fraction was collected and stored at -80°C until analysis. Baseline parameters (haematological: leucocyte count, erythrocyte count, platelet count, Hb concentration, haematocrit, mean corpuscular volume, mean corpuscular Hb and mean corpuscular Hb concentration) were analyzed from additional fasting blood at the first treatment day.

Every plasma sample was prepared in triplicate. Plasma concentrations of SAC were analyzed by RP-HPLC after o-phthalaldehyde (OPA)-labeled precolumn derivatization and with fluorescence detection. The OPA reagent solution was prepared by mixing 2 ml OPA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) solution (40 mM, in methanol) with 10 µl MPA and addition of 7.99 ml borate buffer (0.1 M, pH 10.5). The reagent solution was stored at -80 °C until usage. Plasma analysis was conducted without the knowledge of the treatment assignment. S-carboxymethyl-L-cysteine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as internal standard. To precipitate proteins, 300 µl cooled ethanol (-80 °C) were added to 100 µl plasma sample, vortexed for 20 s and incubated at -80 °C for 30 min. After repeated vortexing the sample was centrifuged (10000 x *g*, 10 min, 4 °C) and 250 µl supernatant were collected and lyophilized by vacuum drying. Derivatization of the sample was performed by adding 100 µl of reaction solution to the residue and vortexing vigorously for at least 20 s. The sample was analyzed by RP-HPLC equipped with a C18 column (Nucleoshell RP 18, particle size 2.7 µM, 3 x 150 mm, Macherey-Nagel, Düren, Germany) at 30 °C and eluted with 50 mM sodium acetate buffer, pH 5.5, 0.5 % THF (eluent A) and methanol (eluent B) at a flow rate of 0.3 ml/min. The samples were placed in a refrigerated autosampler (4 °C) and were analyzed within 24 h. The injection volume was 10 µl and detection was performed at 235 nm and 455 nm as excitation and emission wavelength, respectively.

Method validation was conducted with SAC standard in accordance with the FDA "Bioanalytical Method Validation, Guidance for Industry" (Food and Drug Administration, 2001). Validation was performed on three separate days, each day including nine non-zero calibration standards and five replicates of samples at low (1 µM), middle (5 µM) and high levels (8 µM). Stock solution of SAC was made by dissolving appropriate amounts in water and dilution to concentrations ranging from 0.5-60 µM. Stock and working solutions were stored at -80 °C. The calibration standards were prepared by spiking SAC-free plasma with SAC standard solution. The lower limit of quantification (LLOQ) of SAC was set at 100 nM. Accuracy and precision (intra- and inter-day) of the method were determined

from the calibration curves and responses from five replicates of each calibration sample (low, middle and high level) on each day of validation. The coefficient of variation (CV) of the inter-analysis and inter-day precision for SAC was < 10%. The recovery was determined by comparing the peak area of SAC spiked to plasma before protein precipitation with the peak area of the same concentration of pure standard (low, middle and high standard level). The recovery of SAC was 52 ± 5.1% and after correction by the internal standard the recovery was 95 ± 15.1 %. The stability of the processed samples was assessed by repeated measurements of SAC spiked plasma at low (1 µM) and high (8 µM) concentrations in triplicate. The detected SAC concentration was within 15% of the nominal concentration during 30 h after processing. For method verification a mobile phase blank, a quality control sample (plasma spiked with known concentration of SAC and internal standard) and a zero-sample (plasma without SAC and SAMC, internal standard added after protein precipitation) were prepared and measured with every run.

6.3.5 Urine sampling, processing and analysis

At treatment days subjects took a sample of their first void urine and collected afterwards the urine over 24 hours. After 24-h collection, urine volume was determined and an aliquot of 10 ml was transferred into a plastic tube and stored at -80°C until analysis. First void urine samples were stored at -80 °C as well. Analysis was conducted without the knowledge of the treatment assignment.

Every urine sample was prepared in triplicate. Urine concentration of N-acetyl-S-ally cysteine (NASAC) was analyzed by OPA-labeled precolumn derivatization and RP-HPLC with fluorescence detection. At first, NASAC was deacetylated by acylsase I (from porcine kidney, 572 units/mg protein, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to SAC. Therefore, 200 µl urine were mixed with 200 µl acyslase solution (0.5 mg/ml in 66.7 mM potassium phosphate buffer, pH 7.4) and incubated at 37 °C for 30 min. Then, 800 µl methanol were added, followed by vortexing and incubation at room temperature for 10 min. The sample was centrifuged (14000 rpm, 5 min) and 1000 µl supernatant was collected and lyophilized by vacuum drying. Derivatization of the sample was performed by adding 100 µl of OPA reaction solution to the residue and vortexing for 20 s. The RP-HPLC analysis was the same as mentioned above for SAC.

Method validation was conducted in accordance with the FDA “Bioanalytical Method Validation, Guidance for Industry” and conducted in the same way as described for metabolites in plasma (Food and Drug Administration, 2001). Stock solution of NASAC was made by dissolving appropriate amounts in water and dilution to concentrations ranging from 1-40 µM. Stock and working solutions were stored at -80 °C. The calibration standards were prepared by spiking NASAC-free urine (control

urine) with NASAC standard solutions. The lower limit of quantification (LLOQ) was at 140 nM NASAC. The coefficient of variation (CV) of the inter-analysis and inter-day precision for NASAC was < 10%. The recovery (70 ± 8.4%) was determined by comparing the peak area of with NASAC spiked control urine (low middle and high standard level) with the peak area of control urine samples where SAC was spiked to the supernatant after enzyme treatment. For method verification a mobile phase blank, a zero-sample (urine without SAC and NASAC) and quality control sample (urine spiked with known concentration of NASAC) were prepared and measured with every analytical run.

Creatinine analysis was based on the Jaffe reaction according to the method of the German Research Foundation (DFG) (Blaszkewicz & Liesenhoff-Henze, 2002), forming a yellow-orange complex of creatinine and picric acid under alkaline conditions and measurement of absorption at wavelength of 492 nm by a spectrophotometer (Helios Gamma, UV-Vis, Thermo Spectronic, Cambridge, UK). Quantification was done by a linear calibration function. The performance of creatinine analysis was controlled by commercial reference material (Duotrol Urin Liquid Level 1, Biomed Labordiagnostik GmbH, Oberschleissheim, Germany) in each analysis series.

6.3.6 Biokinetic calculations

The following pharmacokinetic parameters were estimated for the metabolites in plasma and breath gas: time to reach maximum metabolite concentration (t_{max}), maximum metabolite concentration (c_{max}), and the area under the plasma concentration–time curve (AUC) (by using the trapezoidal rule). The calculations were performed by using GraphPad Prism (version 6.00, GraphPad Software, San Diego, USA).

Due to the variability of urine volume and consequently of metabolite concentrations, the concentration of NASAC in 24-h collected urine was standardized by creatinine excretion rate according to (Garde et al., 2004). The excreted amount of NASAC within 24 hours was expressed as $\Delta NASAC_{24} = ([NASAC]_{urine}/[creatinine]) \cdot K_{creatinine}$, whereas the excretion creatinine rate $K_{creatinine}$ (mM creatinine per day) is defined as $K_{creatinine} = ([creatinine] \cdot \Delta V)/\Delta t$.

6.3.7 Study product preparation

Preparation and analysis of garlic powder

Garlic cloves were manually peeled and cut into 3-4 mm thick slices. In a perforated plastic bag the slices were frozen in liquid nitrogen and freeze dried afterwards (laboratory freeze dryer, Gamma 1-16 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). The dried slices

were ground by an analysis mill. The allicin content of the garlic powder was measured by RP-HPLC with an Agilent 1200 Series system (Agilent Technologies, Santa Clara, US). Therefore, the powder was solved in water (5 mg/ml) and filtrated by a syringe filter (0.2 µm pore size). RP-HPLC analysis was conducted on a C-18 column Nucleodur Gravity (100 mm x 2 mm i.d., 1.8 µm particle size, Macherey-Nagel GmbH & Co. KG, Düren, Germany). The mobile phase consisted of 5 mM ammonium acetate dissolved in water, pH 6.6 (eluent A) and acetonitrile with 0.1% formic acid (eluent B) at a flow rate of 0.2 ml/min with a gradient program as follows: 40% B (0-10 min), 100% B (15-19 min), 5% B (20-22 min), 40% B (25-35 min). The injection volume was 5 µl, the UV detector operated at 205 nm and the column temperature was 25 °C. Quantification of allicin was done by calibration with allicin standard. Allicin was synthesized according to Small et al. (1947), as described in chapter 3. The allicin potential was 1.9% and was constant for at least 3 month.

Preparation and analysis of dried aged garlic extract

Liquid aged garlic extract (Kyolic liquid, Wakunaga of America Co., Ltd., Madero, USA) was freeze dried and pulverized. SAC content was analyzed by RP-HPLC after precolumn derivatization with OPA and as descripted above. The SAC content was 0.62%.

Capsule preparation

952.4 mg of garlic powder (allicin yield 18 mg) was filled into acid resistant hard capsules (DRcaps™, composed of hypromellose), where for aged garlic extract (2640.0 mg, containing 18 mg SAC) hard gelatin capsules (both Capsugel, Colmar, France) were used. The placebo capsules contained maltodextrin.

In vitro release of allicin

In vitro dissolution test for solid dosage forms was conducted according to the European Pharmacopoeia (method 2.9.3) by using a dissolution tester (PT-DT70, Pharma Test AG, Hainburg, Germany) with a rotation speed of 100 rpm at 37 °C (Council of Europe, 2005). One capsule was placed in a basket with stirrer adapter in a covered round bottom glass vessel containing 750 ml medium. The dissolution test comprised four different media: first acidic solution pH 3.0 (0.1 M HCl, 0.2% NaCl) for 2 h, then sodium acetate buffer pH 4.5 for 0.5 h, phosphate buffer pH 6.8 for 2 h and finally phosphate buffer pH 7.4 for 1 h. The basket was transferred from one vessel to another after each incubation step. The allicin concentration of each medium was determined by RP-HPLC as

described above. As reference the maximum allicin yield was determined by stirring vigorously one capsule in 100 ml phosphate buffer (pH 6.8) for 30 min. Complete disintegration of the capsule and solvation of the content was assured. The allicin concentration of the solution corresponded to 100% allicin release.

To analyze the release behavior of the acid resistent capsules compared to the gelatin capsule (used for AGE) without the formation difficulties of allicin riboflavin was used as an easily detectable model substance. The capsules were filled with a mixture of maltodextrin (200 mg) and riboflavin 5'-monophosphate sodium salt hydrate (68 mg, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Dissolution test was conducted as described above. Additionally, preprandial gastric fluid (pH 1.2) was used for comparison. Every 30 min a sample of the dissolution medium was taken and riboflavin concentration was measured by the absorbance at 445 nm using a spectrophotometer (Helios Gamma UV-Vis, Thermo Spectronic, Cambridge, UK). Quantification was done by using a calibration curve of the riboflavin standard. All tests were done in triplicate.

Preparation of modified WPI

For the production of the modified β-LG only food and pharmaceutical grade ingredients were used. First, the β-LG content of the WPI (BiPRO, Davisco Foods International, Inc., Eden Prairie, US) was determined. Therefore, WPI was solved in water (0.3 mg/ml) and filtered through 0.2 µm syringe filters (regenerated cellulose membrane, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). RP-HPLC was performed as described in chapter 3. Quantification of β-LG was done by calibration with β-LG AB standard. The WPI contained 97.7% protein and 74% β-LG in dry matter.

The covalent binding of allicin from garlic powder to β-LG from WPI was done by dissolving WPI and garlic powder separately. Garlic powder of the same batch as applied for the garlic powder capsules was used. The garlic powder was filtered before it was added to the protein solution. The pH of the mixture was adjusted to 8.5 by 0.1 M NaOH and the final concentration of WPI and garlic powder was 26 g/l (corresponds to 1000 µM β-LG) 2.9 g/l (corresponds to 333 µM allicin), respectively. The solution was stirred for one hour and incubated at 4 °C for 24 h. Afterwards the pH was adjusted to 6.0 by 0.1 M HCl, followed by spray drying on a pilot plant spray dryer (Mobile Minor 2000, Niro A/S, Copenhagen, Denmark) using a rotating atomizer disc at a flow rate of 47 ml/min, at 180 °C/70 °C inlet/outlet temperature and an outlet pressure of 4 bar. The degree of modification was determined by RP-HPLC, as described elsewhere (chapter 3). The treated WPI contained 57% of modified β-LG (based on total β-LG amount).

Beverage production

The beverage was proposed to be like a chilled ready to drink coffee drink, but without using the usual main ingredient milk. Milk was replaced by the unmodified or modified WPI in order that β-LG was the most prevalent protein in the drink. Table 6-2 shows the ingredients of the final product. All ingredients were purchased from a local grocery store, except carrageen (Satiagum ADC 25, Cargill Deutschland GmbH, Krefeld, Germany) and vanilla flavor (Symrise AG, Holzminden, Germany), which were generous gifts. At first coffee whitener, cacao powder, lactose and carrageen were solved in water by vigorous stirring. The solution was heated to 80 °C, hold for 15 min and cooled down in a water bath. As temperature was below 30 °C, sugar, instant coffee, cream, aroma and unmodified (placebo) or modified WPI were added under stirring. Finally, the drink was mixed by an immersion blender and filled in plastic bottles (300 ml per bottle). The test drink contained 10.7 g modified WPI and the placebo drink 9.7 g unmodified WPI per serving size (300 ml). The placebo drink was comparable with the drink containing the modified β-LG with respect to appearance, texture and taste confirmed by sensory analysis chapter 5. The beverage was produced one day before consumption and stored at 4 °C.

Table 6-2: Composition of the developed beverage.

Ingredient	Content [%]
water	85.6
coffee whitener*	4.3
WPI**	3
sugar	2.1
lactose	2.5
cream	0.9
instant coffee	0.8
cacao powder	0.4
carrageen	0.3
aroma	0.1

*Main ingredientens: glucose syrup, vegetable fat.

**Modified or unmodified form.

6.3.8 Synthesis of metabolites

S-allyl-cysteine

SAC was synthesized as described by Besada et al. (2005) (Figure 6-1). To an ice cooled solution of L-cysteine (0.5 g, 4.132 mM) in ethanol (10 ml) allyl bromide (0.54 ml, 6.198 mM) was added, followed

by sodium ethoxide (0.28 g, 4.132 mM). The reaction mixture was stirred for an hour. Ethanol was removed by rotary evaporation and the residue was diluted with water, acidified with 1M HCl and extracted with ethyl acetate. After drying over anhydrous sodium sulfate, the solvent was removed by rotary evaporation to provide the product as white powder (801.1 mg yield). ^{1}H NMR (500 MHz, CD3OD) 5.81-5.89 (m, 1H); 5.14-5.26 (m, 2H); 3.68 (dd, 1H, J = 3.85, 8.51); 3.21-3.29 (m,2H); 3.12 (dd, 1H, J = 3.95, 14.6); 2.87(dd, 1H, J = 8.55, 14.55). HRMS: m/z 162.0592 $[M+H]^{+}$, calculated: m/z 162.0583 $[M+H]^{+}$.

N-acetyl-S-allylcysteine

N-acetyl-S-allylcysteine (NASAC) was synthesized according to the method described by Jandke & Spiteller (1987) (Figure 6-1). Briefly, to a solution of N-acetyl cysteine (0.5 g, 3.068 mM) in water (10 ml) allyl bromide (0.29 ml 3.374 mM) was added and the system was brought to pH 10 by addition of 2M NaOH. Ethanol was added to the above mixture until it became clear solution. The reaction mixture was stirred for four hours at room temperature. Ethanol was removed by rotary evaporation and the residue was diluted with water (20 ml), acidified with 1M HCl and extracted with ethyl acetate (3 x 50 ml). The organic layer was dried over anhydrous sodium sulfate, filtered and the solvent was removed in rotary evaporator to provide the product as a white powder (510.9 mg yield). ^{1}H NMR (500 MHz, CD3OD) 5.76-5.82 (m, 1H); 5.10-5.17 (m, 2H); 4.57 (dd, 1H, J = 4.75, 8.20); 3.14-3.22 (m,2H); 2.97 (dd, 1H, J = 4.90, 13.95); 2.75 (dd, 1H, J = 8.15, 13.95); 2.03 (s, 3H). HRMS: m/z 202.0500 $[M-H]^{-}$, calculated: m/z 202.0532 $[M-H]^{-}$.

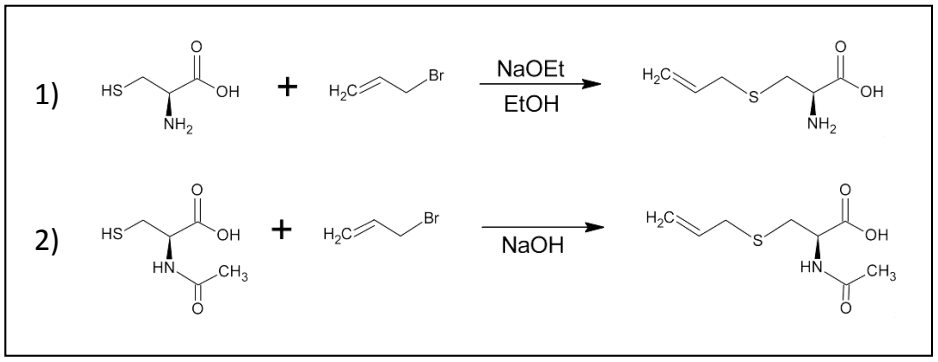

Figure 6-1: Synthesis of S-allyl-cysteine (1) and N-acetyl-S-allyl cysteine (2).

6.4 Results and discussion

6.4.1 Composition of study products

The concentration of the bioactive compounds of interest was analyzed in each study product and the results are summarized in Table 6-3. About 60% of β-LG present in WPI incubated with garlic powder was modified by allicin which is in line with the reported reaction of allicin and β-LG (chapter 3). The applied amount of allicin (111 µM) produced 189 µM SAMC which was less than the maximal possible amount (222 µM) according to the stoichiometric ratio of 2:1 (allicin/β-LG). This was probably caused by partial degradation of allicin previously to the binding reaction, favored by the incubation conditions (pH 8.5). The SAC content (0.68%) of the dried AGE was in accordance with the findings of other studies (Colín-González et al., 2012; Lawson, 1996). The allicin potential of the garlic powder (2.03%) used in the present study was relatively high compared to garlic powder used in other studies (allicin potential: 0.9-1.3%) (Lawson & Gardner, 2005; Lawson & Wang, 2005). To minimize the necessary amount of garlic powder a garlic cultivar yielding a particularly high allicin amount was chosen (chapter 5).

To determine the allicin release from the garlic powder in acid resistant hard capsules under simulated gastrointestinal conditions, an *in vitro* dissolution test was applied using a dissolution tester with the rotating basket method and simulated gastric fluid for 2 h followed by sodium acetate buffer at pH 4.5 for 0.5 h, phosphate buffer at pH 6.8 and pH 7.4 for 2h and 1 h, respectively. The pH value of the simulated gastric fluid was set to 3.0 because the capsules were ingested immediately after the protein rich beverage, resulting in an increased gastric pH value (Koziolek et al., 2013). About 15.4 ± 1.10 % of the potential allicin was released in total after 5.5 h of *in vitro* digestion, of which a proportion of 48% were already released during gastric stage (i.e. after 2 h) and the residual allicin was detected in the phosphate buffers simulating the intestinal stage.

Gastric resistant capsules were used in the present study for garlic powder to maintain the activity of the inherent alliinase as a limited formation of allicin from garlic powder during digestion is a known problem: Since garlic powder contains only alliin and no allicin itself, the enzymatic activity of the inherent alliinase is crucial for the formation of allicin from alliin when the powder is dissolved. However, alliinase is irreversibly inactivated below pH 3.5, the usual range of gastric fluid, and even at a natural pH value the enzyme activity decreases fast at body temperature (Lawson & Gardner, 2005). Therefore, gastric resistant capsules are required. However, the allicin release of most of the garlic powder supplements is less than 15% of their claimed allicin potential, despite their enteric-coating. It has been suggested that an impaired alliinase activity and slow tablet disintegration are the main reasons for the low release (Lawson & Wang, 2001). In the present study the low allicin release of the garlic powder capsules was caused by the insufficient integrity of the capsule under

gastric conditions. Since half of the released allicin concentration was already detected in the simulated gastric fluid, solvent had to be able to penetrate into the capsule.

In addition, the disintegration behavior of the capsules was investigated by using riboflavin as model substance. Figure 6-2 illustrates the release of riboflavin from the gelatin hard capsule and the acid resistant capsule in preprandial (pH 1.2) and postprandial (pH 3.0) gastric fluid. The riboflavin release of the gelatin capsule started rapidly (< 15 min) under both conditions and the capsule dissolved and disappeared completely after 2 h of incubation. The release from the acid resistant capsule started after 30 min and was delayed under fed conditions. Nevertheless, the majority of riboflavin was released at the end of the gastric stage under both conditions. Even though the capsule material remained intact it became soft and instable, leading to leaks between the body and the cap where the acid solution can penetrate into the inner of the capsule.

Marzorati et al. (2015) investigated the dissolution behavior of the same acid resistant capsule material (DRcaps™, Capsugel) as used in the present study. In contrast to our results, they reported that this capsule was able to protect the viability of probiotics and stability of enzymes across gastric passage. However, they observed that the capsules were partially damaged after the simulated gastric stage under and released their content quite soon during intestinal simulation. The simulated gastric stage used by Marzorati et al. (2015) comprised 1 h instead of 2 h which was used in this study. The authors noted that the longer digestion time had a significant impact on the capsule stability. Thus, the limited protection of capsule content against gastric fluid observed in this study were probably due to the longer incubation time applied during *in vitro* dissolution and caused by postprandial conditions *in vivo*.

Table 6-3: Composition of study products.

Study product		Main bioactive compounds	
Product	Administered amount	Compound	Amount of substance [µM]
Whey protein isolate with modified β-lactoglobulin	10.7 g	S-allylmercaptocysteine (bound to protein)	189
Garlic powder	952.4 mg	Allicin	111
Aged garlic extract	2640.0 mg	S-allyl cysteine	111
		S-allylmercaptocysteine	24*

* Calculated value based on S-allyl cysteine concentration and S-allylmercaptocysteine/S-allyl cysteine ratio reported by Colín-González et al. (2012).

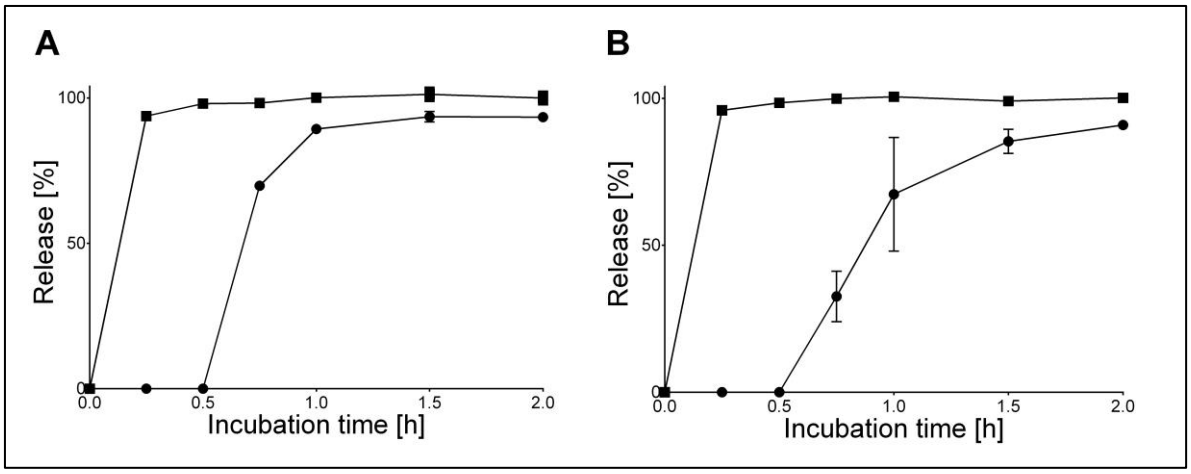

Figure 6-2: Dissolution test of acid resistant capsule (●) and gelatin capsule (■) in simulated gastric fluid under fasted (A) and fed (B) conditions. Values represent mean ± standard error mean.

6.4.2 Breath gas analysis

Breath gas samples were collected from six subjects before and 2, 4, 6, 8 and 24 hours after treatment ingestion. The concentration of the metabolite allyl methyl sulfide (AMS) was analyzed by GC-MS. Figure 6-3 shows the average AMS concentration in breath gas during 24 hours after ingestion and Table 6-4 summarizes the corresponding calculated pharmacokinetic parameters of the three different treatments. Before treatment intake AMS was absent in the breath gas of all subjects at each study day confirming that all subjects followed the *Allium*-restricted diet. The maximum and total AMS concentration after ingestion of the modified β-LG was significantly higher compared to garlic powder and aged garlic extract in capsules ($P < 0.001$). SAMC released from modified β-LG contributed to a nearly ten times higher maximum AMS concentration (C_{max}) and a six times higher area under the AMS concentration-time curve (AUC). 24 hours after each treatment intake low concentrations of AMS were still detectable.

Since neither allicin nor its known metabolites have yet been found in blood or urine, breath AMS is the only established method for determining the bioavailability of allicin or allyl thiosulfinates (Lawson & Gardner, 2005). Several studies confirmed that AMS is a product of the systemic metabolism of allicin and a suitable marker of its bioavailability (Lawson & Gardner, 2005; Lawson & Wang, 2001; Rosen et al., 2001; Suarez et al., 1999; Taucher et al., 1996). Further, Lawson & Wang (2005) observed that equimolar amounts of S-allyl moieties provided by allicin and SAMC contributed to the same amount of AMS, whereas SAC did not produce AMS (Lawson & Wang, 2005). Thus, the dithioallyl group was suggested to be necessary for AMS formation.

The maximum AMS concentration generated by SAMC from modified β-LG in the present study is in line with previous studies. Lawson & Wang (2005) detected a similar ratio between breath AMS

concentration and ingested SAMC amount (C_{max} = 1110 ng/l, after 730 µM SAMC intake). Apparently, SAMC from modified β-LG was completely released and metabolized like pure SAMC in capsules.

Since SAC does not contribute to AMS formation the detected amount of AMS from AGE was probably provided by SAMC (Lawson & Wang, 2005). The AGE in capsules contained 111 µM SAC which is the main organosulfur compound of this product. However, SAMC is present as well, even in a lower extent (about 25% of SAC content) (Colín-González et al., 2012). On the assumption of the reported SAC/SAMC ratio in AGE, 24 µM SAMC were ingested with the administered AGE amount which corresponds to 13% of SAMC from the modified β-LG. The detected amount of AMS per molecule SAMC from AGE was similar to SAMC from the modified β-LG. This result confirms that SAMC of modified β-LG was released and metabolized to the same extent as free SAMC from AGE in capsules. Thus, the bioavailability of SAMC was not reduced through the integration in the polypeptide chain of the protein. To our knowledge the breath gas after AGE intake was not analyzed before, this study provides the first insight into the extent of breath AMS excretion after ingestion of AGE.

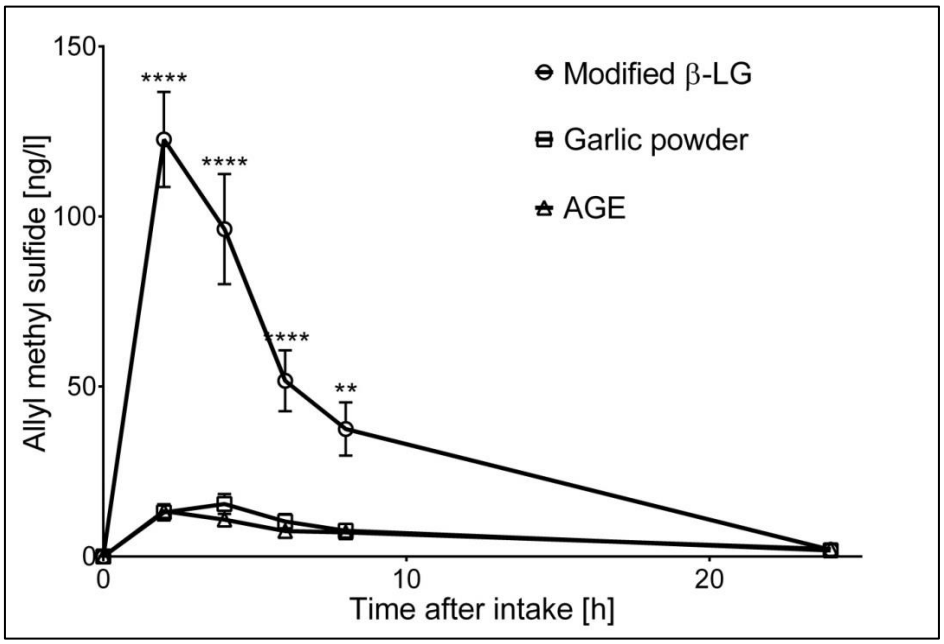

Figure 6-3: Concentration of allyl methyl sulfide in breath gas before and 24 hours after intake of the different preparations. Values represent mean ± standard error mean (*n* = 6). β-LG – β-lactoglobulin.

Table 6-4: Biokinetic variables of allyl methyl sulfide in the breath gas after a single oral dose of the functional beverage containing modified β-lactoglobulin, garlic powder in capsules or aged garlic extract in capsules. Values represent mean ± standard error mean (*n* = 6).

	Modified β-lactoglobulin	Garlic powder	Aged garlic extract
Main delivered organosulfur compounds	S-allymercaptocysteine	Allicin	S-ally cysteine, S-allymercaptocysteine
C_{max} [ng/l]	127.2 ± 15.25	15.8 ± 2.77	14.0 ± 1.61
t_{max} [h]	2.3 ± 0.33	3.6 ± 0.40	2.3 ± 0.33
AUC_{24h} [ng · h/l]	894.5 ± 134.32	160.3 ± 35.93	145.4 ± 13.03

Abbreviations: AUC – area under the allyl methyl sulfide concentration–time curve; C_{max} – maximum allyl methyl sulfide concentration; t_{max} – time to reach the maximum.

The relatively low AMS concentration after the intake of garlic powder capsules was due to the incomplete allicin release confirmed by *in vitro* dissolution testing (ref. 3.1). If the maximum allicin yield (111 µM) would be released an equivalent AMS concentration as formed by 222 µM SAMC of modified β-LG would be assumed. Allicin delivers two S-allyl groups per molecule, hence two molecules of AMS can be formed (Lawson & Wang, 2005). Based on the detected amount of AMS from 189 µM SAMC the expected AMS concentration for the release of 111 µM allicin (AUC_{24h} = 1050 ng · h/l) was calculated by assuming that allicin was metabolized to AMS in the same extent as SAMC. According to this the detected AMS amount from garlic powder corresponded to 15.3% allicin release (Lawson & Wang, 2005). This value is in line with the release determined by the simulated gastrointestinal dissolution test (15.4%) showing a good *in vivo-in vitro* correlation of the dissolution model used.

The interindividual variation of AMS concentration (C_{max}) was about 29% for modified β-LG and AGE, whereas the variation was higher (39%) for garlic powder. Since the formation of allicin and consequently AMS from garlic powder is strongly dependent on gastrointestinal conditions, the individual physiological differences can have a high influence on the allicin formation. Lawson & Gardner (2005) also reported a higher interindividual variation for garlic powder compared to fresh crushed garlic where allicin was already formed before ingestion.

No difference in time needed to reach the maximum AMS level was observed between SAMC from AGE and modified β-LG. Hence, the metabolism of SAMC was not delayed by the integration into the protein chain. After intake of the garlic powder capsules it took about one hour longer to reach the maximum AMS concentration than for the two other treatments. The reason was the delayed release by the acid resistant capsules used for the garlic powder. The dissolution test confirmed that the release of content started about half an hour later under the used simulated gastrointestinal dissolution conditions than the release by the gelatin capsules used for AGE (Figure 6-2).

6.4.3 Plasma analysis

The concentration of SAC in plasma was analyzed before and hourly (1-8 h, 24 h) after treatment intake. As expected, the highest SAC levels were generated by AGE (Figure 6-4, Table 6-5). Garlic powder in capsules and the functional beverage contributed to an increased SAC level in plasma as well. AUC and C_{max} after intake of AGE were about twice as much as after intake of the other treatments. Further, the course of the plasma SAC concentration curve was different between AGE and the other treatments. AGE induced a sharp increase in plasma SAC level, since t_{max} was reached immediately after intake. However, the level decreased relatively fast within three hours after t_{max} and remained stable during the further four hours. In contrast, the SAC plasma curve formed a plateau throughout eight hours after ingestion of the other treatments.

Since fresh garlic (SAC content 0.006% of fresh weight) or garlic powder contains only traces of SAC, the SAC level in plasma induced by garlic powder and the functional beverage was presumably caused by γ-glutamyl-S-allyl-cysteine (Lawson & Wang, 2005). The concentration of γ-glutamyl-S-allyl-cysteine in garlic (fresh/powder) is about 0.3-1.7% of dry matter (Lawson & Gardner, 2005; Mütsch-Eckner et al., 1992). During aqueous extraction γ-glutamyl-S-allyl-cysteine is transformed to SAC causing the relatively high SAC content of AGE (Amagase, 2006). However, the enzymatic transformation by γ-glutamyl transferase can take part *in vivo* as well, leading to the formation of SAC as observed. However, this has not been analyzed before. Assuming an average γ-glutamyl-S-allyl-cysteine concentration of 1% of dry matter garlic, the garlic powder capsules and the functional beverage contained about 9.5 mg γ-glutamyl-S-allyl-cysteine. Accordingly, the detected differences of the AUC between AGE and the other treatments were in line with the different ingested amounts of SAC (18 mg, AGE) and γ-glutamyl-S-allyl-cysteine (10 mg, garlic powder in capsules and in the beverage).

The observed SAC level induced by AGE was in accordance with findings of other human studies (Nantz et al., 2012; Kodera et al., 2002; Rosen et al., 2001). Kodera et al. (2002) observed the maximum SAC concentration one hour after ingestion as well. Further, they observed that SAC was stable in blood and had a half-life of more than 10 h which is in line with the relatively slow decrease of SAC level in the present study. The delayed t_{max} after intake of garlic powder, both in capsules and the functional beverage, was probably due to the transformation reaction of γ-glutamyl-S-allyl-cysteine to SAC. Since the present study is the first one where the SAC level in plasma was analyzed after intake of garlic powder or garlic, there is no comparison.

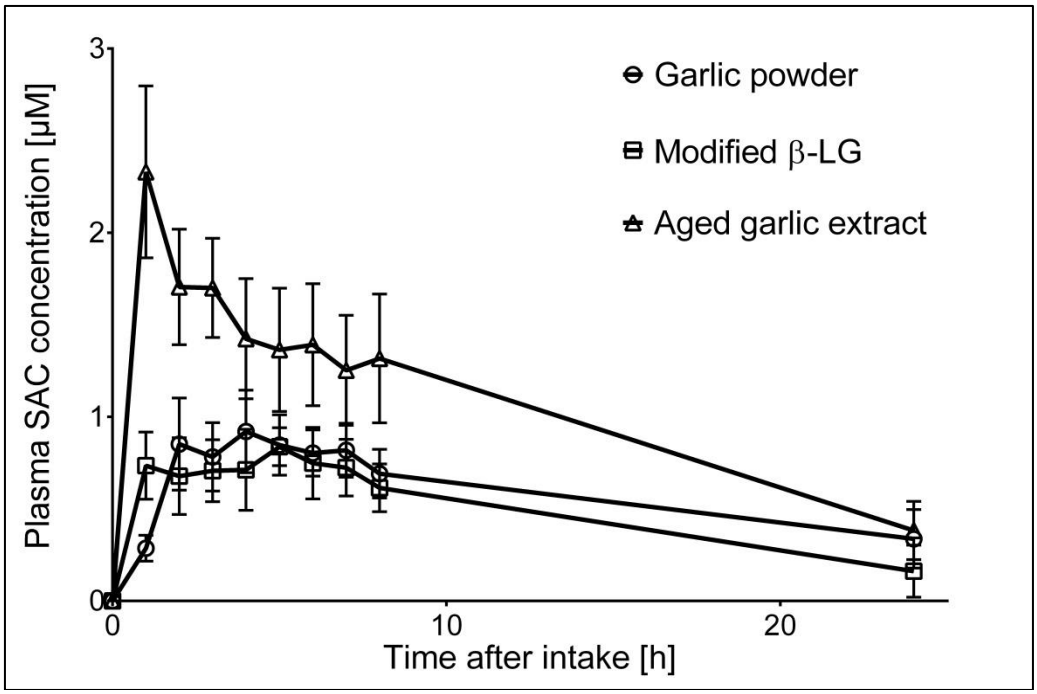

Figure 6-4: Concentration of S-allyl cysteine (SAC) in plasma before and 24 hours after intake of the different treatments. Values represent mean ± standard error mean (*n* = 4). β-LG – β-lactoglobulin.

Table 6-5: Biokinetic variables of S-allyl cysteine (SAC) in plasma after a single oral dose of the functional beverage containing modified β-lactoglobulin, garlic powder in capsules or aged garlic extract in capsules. Values represent mean ± standard error mean (*n* = 4).

	Modified β-lactoglobulin	Garlic powder	Aged garlic extract
C_{max} [µM SAC/l]	0.95 ± 0.316	1.07 ± 0.390	2.35 ± 0.909
t_{max} [h]	4.3 ± 1.71	3.3 ± 1.5	1.5 ± 1
AUC_{24h} [µM SAC · h/l]	11.7 ± 6.26	13.9 ± 6.40	25.4 ± 12.36

Abbreviations: AUC – area under the SAC concentration–time curve; C_{max} – maximum SAC concentration; t_{max} – time to reach the maximum.

6.4.4 Urine analysis

The NASAC concentration of the first void urine and 24h-urine samples was analyzed and standardized by creatinine excretion rate. All first void urine samples did not contain NASAC confirming the compliance with the diet regulations. The ingestion of AGE contributed to the urinary NASAC excretion ranging from 0-0.7 µM NASAC/mM creatinine with an average concentration of 0.17 ± 0.23 µM NASAC/mM creatinine (Figure 6-5). The average total amount of excreted NASAC was 0.83 ± 1.14 mg. Urinary NASAC was detectable in the 24h-urine of 7 out of 9 subjects after ingestion of garlic powder capsules but at a lower level than for AGE (Table 6-6). After intake of the functional beverage NASAC was only detected in the urine of 3 out of 9 subjects. The variation of NASAC concentration between subjects was high for all treatments.

According to animal studies the bioavailability of SAC is > 90% and NASAC has been suggested as a suitable biomarker and excretion product of SAC which is transformed by N-acetyltransferase in the liver and kidney (Amano et al., 2015; Krause et al., 2002; Nagae et al., 1994; Amano et al., 2015). This relation was also observed in the present study. The detected NASAC excretion corresponded to the SAC plasma levels induced by the different treatments. Since AGE provided the highest SAC amount, SAC plasma level and urinary NASAC excretion were higher than for the other treatments. The contribution of fresh garlic or garlic powder to the urinary NASAC excretion has been observed before by deRooij et al. (1996), Verhagen et al. (2001) and also in the present study. Cope et al. (2009) detected an even higher NASAC excretion after ingestion of 5 g fresh garlic compared to 3 g AGE confirming the high bioavailability and transformation of γ-glutamyl-S-allyl-cysteine.

DeRooij et al. (1996) investigated NASAC excretion in humans after ingestion of garlic. The detected total amount of NASAC within 24 hours reached 0.43 mg after intake of 100 mg garlic powder and 1.4 mg NASAC after intake of additional garlic which is in a similar range as in the present study (Table 6-6). Since the elimination half-life of NASAC was reported to be 6 h, the collection of 24h-urine was supposed to be sufficient for a reliable assessment of NASAC excretion. In contrast, the detected plasma SAC levels were remarkable stable until eight hours after ingestion (Figure 6-4). Especially garlic powder and the functional beverage caused a relatively late t_{max} (3-4 h after intake) which could have induced a delayed NASAC excretion as well. Presumably therefore, the NASAC concentration in 24h-urine after intake of garlic powder and the functional beverage was low.

Verhagen et al. (2001) and deRooij et al. (1996) reported that some humans had a deviating excretion of NASAC, i.e. no NASAC excretion after intake of significant amounts of garlic products or an irregular elimination profile. In the present study one out of nine subjects did not excrete NASAC regardless of the treatment. Further, high variations between subjects were observed, whereas intraindividual variations were smaller, i.e. the same subjects excreted the highest and the lowest NASAC amount regardless the treatment. Following this, NASAC analysis in urine is not a reliable quantitative biomarker for bioavailability studies, however it can be used as qualitative marker.

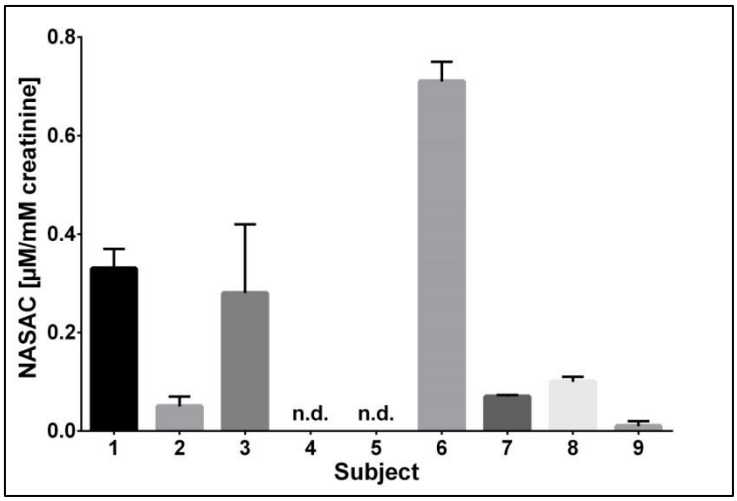

Figure 6-5: Concentration of N-acetyl-S-allyl cysteine (NASAC) in 24 Hour-urine of each subject after a single oral dose of aged garlic extract in capsules. NASAC concentration was corrected by the excretion rate of creatinine. Values represent mean ± standard error mean. N.d. – not detectable.

Table 6-6: Analysis of N-acetyl-S-allyl-cysteine (NASAC) in 24h-urine after intake of the different treatments. Values represent the numbers of subject samples out of nine.

Urinary NASAC	Modified β-LG	Garlic powder	Aged garlic extract
Positive, quantifiable	2	5	7
total $NASAC_{24h}$ [mg]*	0.9 ± 0.88	0.6 ± 0.70	1.1 ± 1.20
Positive, below LLOQ	1	2	1
Negative	6	2	1

* Values express the mean ± standard deviation of samples with quantifiable NASAC concentration. Abbreviations: LLOQ – lower limit of quantification; NASAC – N-acetyl-S-allyl-cysteine; β-LG – β-lactoglobulin.

6.5 Conclusions

The present study demonstrated that the allicin derivate SAMC was delivered and released by β-LG in the same extent as free SAMC in capsules. Hence, β-LG is a suitable transporter for the bioactive thioallyl moiety of allicin. The reaction of allicin with β-LG generates SAMC in a simple and fast process, enables the enrichment in a functional food and provides a higher level of allicin's bioactive metabolites than the most garlic powder supplements. The breath AMS concentration after ingestion of AGE was analyzed the first time and showed the formation of AMS correspondingly to its SAMC content. Further, it was shown that γ-glutamyl-S-allyl-cysteine from garlic powder in capsules as well as in the functional beverage provided significant amounts of SAC in plasma and NASAC in urine. Conclusively, the beverage enriched with garlic powder delivered physiological relevant amounts of S-allylmercaptocysteine and S-allyl cysteine with an unimpaired high bioavailability but without a

significant perceivable garlic taste due to the binding of allicin to β-LG. The breath AMS was a suitable metabolite to assess the bioavailability of SAMC and allicin. NASAC in urine was shown to be a marker compound for the intake of AGE and garlic powder at least at a qualitative level whereas SAC in plasma was a reliable marker at a quantitative level.

7. General discussion

This thesis presents an innovative concept for the delivery and stabilization of bioactive compounds in food. The central aim was the assessment of the suitability of β-LG as a transporter for covalently bound organosulfur compounds from garlic, i.e. allicin and DADS. This approach comprised the investigation of the protein-ligand interactions, the transfer to a food grade level resulting in the production of a functional food and the assessment of the bioavailability of the transported compound in a human study. Further insights into the structural features of the whey protein were provided. Figure 7-1 illustrates the structure of this work. The following discussion refers to the hypotheses in **chapter 1**.

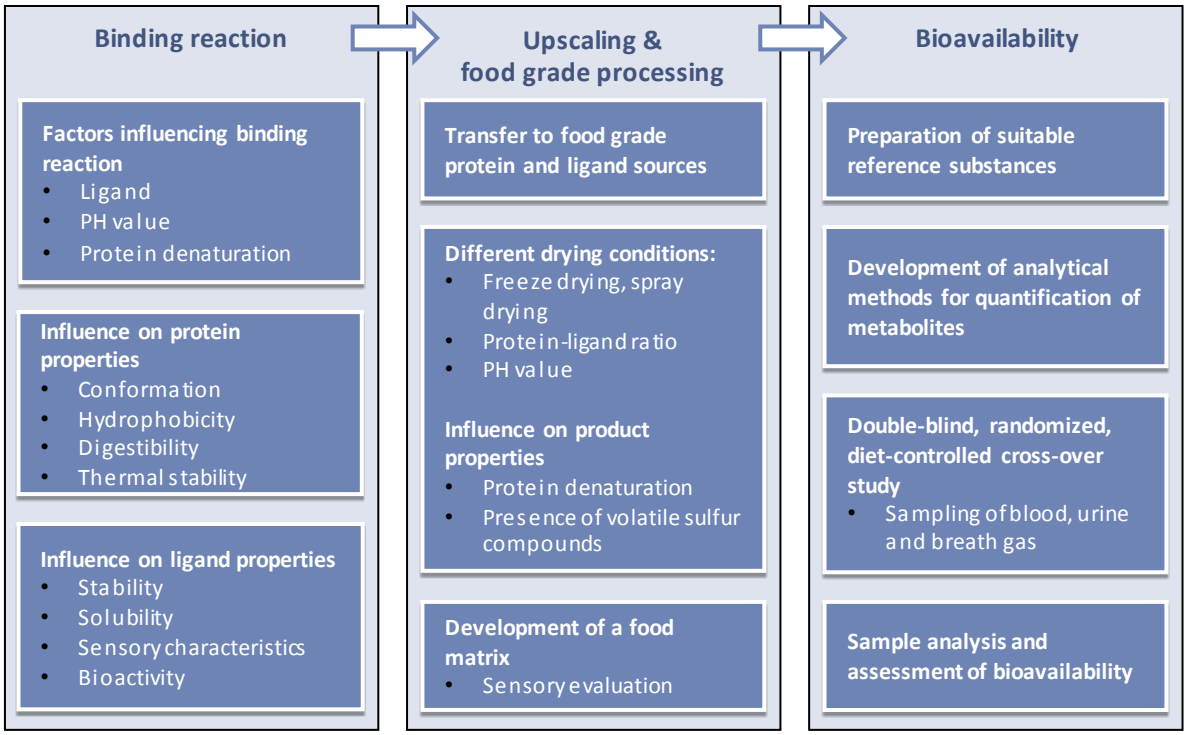

Figure 7-1: Schematic structure of the present thesis.

7.1 Binding of allicin and diallyl disulfide

The interaction of allicin and DADS with β-LG has been comprehensively characterized in **chapter 3** and **4**. Referring to hypothesis 1, it was proved that both ligands, allicin and DADS, can be covalently bound by the free thiol group of β-LG.

The free thiol group is buried in the inner core of the globular structure (**chapter 2.1.1**). Therefore, the thiol group is protected against oxidizing agents except for compounds which are very small and

amphiphilic such as allicin. For many enzymes substrate specificity is mediated by limitation of the access to a thiol containing active center through steric hindrance (Nagy, 2013). Additionally, neighboring functional groups influence the reaction rate. For example, a hydrophobic environment lowers the activation energy of thiol-disulfide exchange reactions (Nagy, 2013). Referring to the structure of β-LG, it can be assumed that the hydrophobic pocket consisting of the α-helix and β-strand H has a catalytic effect on the reaction with allicin and DADS. Figure 7-2 illustrates the hydrophobic environment of Cys^{121}. These structural features known from enzymes contribute to the suitability of β-LG as a transporter for thiol reagents.

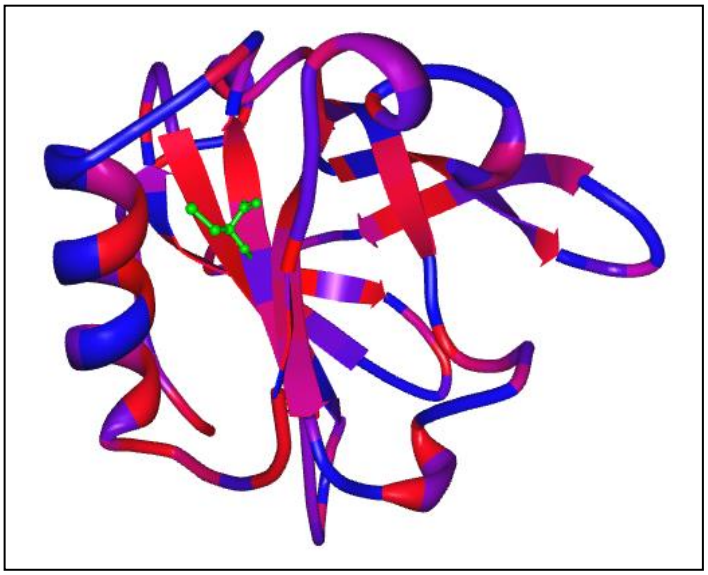

Figure 7-2: Structure of β-lactoglobulin colored according to hydrophobicity. Backbone color indicates highest hydrophobicity in red whereas least hydrophobicity is shown in blue. Side chain of Cys^{121} is shown in green. Diagram was drawn by PDB Protein Workshop 4.2.0 with file 3NPO of Protein Data Bank RCSB provided by Loch et al. (2011) (Moreland et al., 2005).

The deliverance of SAMC by binding of allicin to cysteine containing proteins has been patented by Ott (2005, WO 2005115173 A1), but no application of this patent is known. Miron et al. (2001) patented the binding of allicin to GSH to form S-allylmercaptoglutathione (WO 0136450 A1). Both patents were primarily proposed for the application in pharmaceutical and nutraceutical products.

Beside β-LG, alternative food proteins containing at least one free thiol group are bovine serum albumin (BSA) and ovalbumin (Anand & Mukherjee, 2013; Tatsumi et al., 1998). BSA can form disulfide-linked dimers but the monomeric form is prevalent at neutral conditions (Barbosa et al., 2009). Nevertheless, the covalent binding of a bioactive compound by the free thiol group has not been reported for both proteins, as far as known.

Factors influencing binding reaction

The binding kinetics of the ligands allicin and DADS with β-LG have been analyzed in detail in the present thesis (**chapter 3**). Despite the structural similarity of both organosulfur compounds, the affinity and type of binding were significantly different. From the chemical point of view, the reaction with β-LG is a substitution between the nucleophilic thiolate anion of Cys^{121} and the sulfur of the disulfide moiety (Nagy, 2013). Due to the thiosulfinate group of allicin, its strong electrophilic character accelerates the reaction rate compared to DADS (Block, 1992). On the other side, the lower reactivity of DADS contributes to its higher stability and favors the non-covalent binding.

The interaction between ligand and protein can be categorized in covalent and non-covalent binding reactions (Rawel & Rohn, 2010). The present results revealed that DADS is able to interact in both ways as demonstrated by fluorescence quenching (FQ), RP-HPLC and the concentration of reactive thiol groups (RSH). The simultaneous covalent and non-covalent binding is relatively rare. Prigent et al. (2003) and Ali et al. (2012) reported, that chlorogenic acid was both covalently and non-covalently bound to different proteins. However, it has been indicated that not the chlorogenic acid itself bound covalently but its corresponding quinone.

The versatile binding characteristics of β-LG are of particular interest when it comes to a mixture of potential ligands usually found in food systems. Based on the covalent and non-covalent binding of DADS, it could be expected that β-LG can bind and thereby mask the flavor of other organosulfur compounds from garlic powder as well. Thus, the presence of the protein, even without prior targeted covalent binding at basic conditions, should reduce the garlic like odor and taste. However, this effect was only marginally recognized in the present thesis which demonstrates the high contribution of allicin to the sensory perception of garlic powder (**Figure 5-7**). Referring to isolated ingredients, the sensory perception of allicin could be completely masked through the covalent binding to β-LG. This is in contrast to DADS which remains partly unbound even at a high protein excess.

As expected, the covalent binding reaction was significantly influenced by the pH value (**Figure 3-5**). The thiolate anion is a much stronger nucleophilic than the thiol group, resulting in inhibition of the reaction at pH values below 8 where the protonated form is prevalent (Thurlkill et al., 2006). Since food systems are mostly at a neutral or acidic pH value, a covalent binding reaction is unlikely under these conditions, e.g. when milk and crushed garlic are mixed together. The interaction with native β-LG in milk was not tested yet. Therefore, the present results indicate the necessity of an alkaline pH value (pH 8-9) to produce β-LG modified with allicin or DADS which emphasizes the specificity of the presented reaction. In contrast, once the ligand reacted with the thiol group of the protein, the bonding was stable at a wide pH range as shown by analytical methods at alkaline, neutral and acidic

conditions, i.e. by RSH assay (pH 8), RP-HPLC (pH 7) and LC-MS after peptic digestion (pH 2) (**Figures 3-1, 3-3, Table 4-1**). Conclusively, the covalent binding enables a reliable stabilization of the ligand against various external conditions, e.g. in the food matrix or during digestion, in contrast to non-covalently bound ligands (Ron et al., 2010; Zimet & Livney, 2009).

With respect to thermal denaturation, the applied conditions resulted in an increased affinity but a lower number of covalent binding sites (**chapter 3**). Due to the reduced number of free thiol groups, it can be assumed that covalent aggregation reactions during heating took place. On the one hand, heating leads to the exposure of the free thiol group resulting in a higher reactivity. As a consequence, the affinity of the binding reaction increases, as observed. On the other hand, the thermally induced formation of intermolecular disulfide bonds reduces the number of free thiol groups (**chapter 2.1.2**). Further, the denaturation of β-LG favored the non-covalent interactions as shown by the higher number of binding sites for DADS. Similar observations were made for the non-covalent interaction of β-LG with epigallocatechin gallate (Shpigelman et al., 2010). Due to the thermally induced unfolding of the globular protein, hydrophobic domains are revealed and offer further surface elements for hydrophobic interactions (Tolkach & Kulozik, 2007; Busti et al., 2005).

Influence on protein properties

Referring to ligand binding, desirable and undesirable effects on properties of β-LG have been reported in literature. As an example, the covalent binding of phenols reduced solubility and digestibility of the protein and thus lowered the techno-functional and nutritional value (Rawel et al., 2001). AITC binding improved the emulsifying and foaming properties of β-LG, but it blocked cleavage sites of digestive enzymes resulting in a reduced nutritional value (Rade-Kukic et al., 2011). Since allicin and DADS reacted with the free thiol group only, the digestibility was not interfered (**chapter 4**). Further, the surface hydrophobicity was slightly enhanced due to conformational changes which could lead to improved interfacial absorption behavior without impairing the solubility (Rade-Kukic et al., 2011) (**chapter 3 and 4**).

Additionally, the covalent modification of the free thiol group significantly enhanced the thermal stability of β-LG (**chapter 5, Figure 5-5**). This effect was even more obvious, if the thiol groups of all protein molecules were blocked. The thiol group of Cys^{121} plays a crucial role during heat denaturation. It contributes to thermally induced intermolecular aggregation reactions of β-LG and thus to the irreversibility of denaturation (**chapter 2.1.2**). The disulfide bond can occur between two β-LG molecules, but with respect to milk processing other proteins can be involved as well. For example, β-LG can form aggregates with κ-casein during heating which dramatically reduces the

rennetability of milk and impairs its quality for cheese making (Livney & Dalgleish, 2004; Cho et al., 2003). Further, β-LG containing disulfide linked aggregates are involved in fouling in heat exchangers which is an important issue in dairy processing (Petit et al., 2013). As the modification of the free thiol group inhibits the aggregation reactions of β-LG via disulfide bonds, these heating related problems could be reduced by the covalent binding of allicin or DADS (Hoffmann et al., 1997).

Influence on ligand properties

According to the results of MS analysis, the reaction product of allicin or DADS with cysteine was SAMC, which is in line with the findings of Miron et al. (2010) and Rabinkov et al. (1998) (**chapter 4**). As distinguished from allicin, SAMC is a stable, non-volatile and water-soluble compound (Colín-González et al., 2012; Sigounas et al., 1997b). When incorporated into the protein structure, it does not contribute to sensory perception and its stability enables processing and storage without significant losses. The health related effects of SAMC were suggested to be similar to those of allicin, since SAMC is an allylic disulfide in contrast to the structural similar SAC. According to various *in vitro* and animal studies, SAMC is a metabolite of allicin and acts as a stable reservoir of the S-allyl moiety to mediate and prolong its activity (Liang et al., 2011; Miron et al., 2010; Howard et al., 2007; Pedraza-Chaverrí et al., 2004; Rabinkov et al., 1998) (**chapter 2.2.5**).

7.2 Application of covalently modified β-lactoglobulin in food

Food grade production

In order to obtain a consumable product for the sensory and bioavailability evaluation, the modification of β-LG with allicin had to be transferred to a food grade level. Besides safety and regulatory issues, the resources had to meet technological requirements. The content of the target compounds (i.e. β-LG and allicin, respectively) ought to be as high as possible to minimize the required amounts of resources and reduce possible interferences by other present compounds. Furthermore, the concentration ought to be able to be standardized to a constant level because the reproducible adjustment of the protein-ligand ratio is important. Finally, the resources must be appropriate for processes commonly used in food industry.

Allicin can be generated by fresh garlic or garlic powder only (Trio et al., 2014). Since garlic powder can be stored over month without significant loss of its allicin potential, it was chosen as allicin resource (Lawson & Gardner, 2005). In order to preserve a high allicin potential of the final powder, the fresh garlic was sliced as little as necessary and freeze drying was used. Considering process

efficiency, a garlic cultivar with a particularly high allicin potential was chosen for the production (**Figure 5-2**). WPI (75% β-LG in dry matter) was used as resource for β-LG. The interference of the binding with allicin by other whey proteins was ruled out by RP-HPLC (**Figure 5-3**). Therefore, the complex isolation process of β-LG was not required.

The targeted binding of allicin to β-LG was achieved by incubating dissolved WPI mixed with garlic powder at pH 8.5. Subsequently, the solution was dried wherefore different methods have been tested (**chapter 5**). Under the tested conditions, spray drying at slightly acidic conditions provided the best results with respect to protein denaturation and concentration of volatile organosulfur compounds. Lowering pH to 6 after protein-ligand incubation (at pH 8.5) reduced the degree of denaturation because of the lower reactivity of the thiol groups and thus the lower extent of chemical aggregation (Bauer et al., 1998; Hoffmann & van Mil, 1997) (**chapter 2.1.2**). Additionally, the escape of free volatile organosulfur compounds during spray drying further reduced the garlic like sensory properties of the final product.

In essence, the process comprises the incubation of WPI and garlic powder at an alkaline pH value (8-9), the ultrafiltration to remove remaining low molecular compounds from garlic powder, the acidification of the solution to prevent denaturation during drying and finally spray drying of the protein solution. An online heating step before drying could additionally ensure the microbiological quality of the product. The process does not require any sophisticated equipment and would be feasible for a usual dairy. An upscaling of the process should be possible without special effort .

Sensory characterization

The sensory properties of β-LG modified by allicin from garlic powder were analyzed by quantitative descriptive analysis. With respect to hypothesis 2, it was clearly demonstrated that the binding of allicin to β-LG significantly reduced the garlic like odor, flavor and pungency (**chapter 5, Figure 5-7**). However, the garlic like odor of modified β-LG in aqueous solution was still notable. This was presumably caused by the presence of other organosulfur compounds from garlic powder beside allicin, since this odor was almost absent after ultrafiltration of the product. Nothing is known about the sensory properties of pure SAMC, beside its non-volatile character which prevents the olfactory perception (Kodera et al., 2002). Even if the thiol allyl group contributed to a garlic like flavor, the perception would be prevented by the embedded position within the globular structure of β-LG.

The developed beverage provided a suitable matrix in which the remaining garlic odor and flavor of the enriched modified β-LG was barely perceivable. It was already shown that milk efficiently reduces the concentration of organosulfur compounds in the headspace of crushed garlic (Hansanugrum &

Barringer, 2010; Negishi et al., 2002). This effect is presumably based on the emulsifying effect of whey proteins in combination with the hydrophobic phase provided by triglycerides. Both ingredients were also present in the milk free developed beverage and contributed to the considerable masking effect. Conclusively, products containing milk or milk ingredients seem suitable for the enrichment of the modified β-LG. Further, ultrafiltration of β-LG after the binding reaction with allicin may provide a functional ingredient without any garlic like sensory properties which would enable the application in diverse food products.

Alternative delivery systems for allicin tested so far focused mainly on the stabilization of the compound (**chapter 2.2.3**). Liposomes, microcapsules, and chitosan complexes were shown to improve the stability and solubility of allicin (Lu et al., 2014; Wang et al., 2012; Pirak et al., 2012). However, it has not been demonstrated if these systems are sufficient for the demands of processed foods and the sensory properties have not been assessed yet. Generally, taste is an important factor for customer food choice and also with respect to functional foods, customers are not willing to compromise on taste for health (Verbeke, 2006; Urala & Lähteenmäki, 2004; Grunert et al., 2000). Therefore, one of the main functions of a delivery system is the masking of unpleasant flavors. β-LG considerably masked the odor and flavor of allicin and is hence a suitable transporter from the sensory point of view.

7.3 Bioavailability of S-allylmercaptocysteine from modified β-lactoglobulin

The bioavailability of allicin and its cysteine derivates SAC and SAMC was shown to be higher than 80% (Lawson & Gardner, 2005; Lachmann et al., 1994; Nagae et al., 1994) (**chapter 2.2.4**). Therefore, the function of a delivery system for allicin is not the enhancement of the bioavailability as such. The major limitation of allicin intake is its instability leading to low concentrations in processed foods and the sensitivity of alliinase that causes the low allicin release from garlic powder supplements (Fujisawa et al., 2008b; Lawson & Wang, 2001) (**chapter 2.2.1** and **2.2.2**). For that reason, the aim of the present study was to generate a more stable, bioactive form of allicin without impairing the bioavailability.

As discussed in **chapter 6,** the bioavailability of SAMC from β-LG modified with allicin seems to be as high as that of free SAMC of AGE and that of allicin from garlic powder in capsules which confirmed hypothesis 3 (**Figure 6-3, Table 6-4**). Since no reliable biomarkers for allicin in plasma have been reported so far, breath AMS is the most appropriate metabolite to determine the bioavailability (Lawson & Wang, 2005; Lawson & Gardner, 2005). Due to its stability, it can be presumed that SAMC is detectable in plasma like SAC (Kodera et al., 2002). The detection of SAMC in plasma would provide

a further insight in its bioavailability. The formation of allicin from garlic powder in acid-resistant capsules resulted in lower amounts of absorbable allicin than the amount of SAMC from modified β-LG which limited the comparability of the present results. The formation of allicin from garlic powder in capsules is a known problem and emphasizes the difficulty of allicin delivery (Lawson & Wang, 2001). Nevertheless, considering the different amounts of ingested target compounds, the bioavailability of SAMC from β-LG modified by allicin seemed not to be impaired by the incorporation in the protein chain. This result is additionally supported by the findings of MS/MS analysis of the hydrolyzed protein (**chapter 4**) and by the literature, as SAMC has been reported to be a stable compound. Following this, losses due to SAMC degradation during processing or digestion were not expected (Amagase, 2006). Further, the modification of the free thiol group should not impair the digestibility and thus the release of SAMC, since the pancreatic peptidases do not cleave specifically at cysteine (Rawlings et al., 2012).

The analysis of SAC in plasma and NASAC in urine gave valuable insights into the contribution of γ-glutamyl-S-allyl-cysteine of garlic powder to the formation of SAC that has not been reported before. Garlic powder in capsules as well as in the functional beverage which contained modified β-LG increased the SAC level in plasma significantly (**Figure 6-4, Table 6-5**). Accordingly, the beverage enriched with garlic powder delivered physiological relevant amounts of S-allylmercaptocysteine and S-allyl cysteine with an unimpaired high bioavailability. The enrichment of relatively high amount of garlic powder, without causing a significant perceivable garlic smell or taste, was possible due to the binding of allicin to β-LG (**Figure 5-7**). Since allicin is the main flavor-active compound in garlic powder, the residual intensity of garlic flavor is significantly reduced when allicin is bound. Conclusively, the functional beverage enabled not only the deliverance of the main bioactive compound allicin but also of other bioactive organosulfur compounds from garlic in considerable amounts. According to various studies mixtures of components, like in natural products or in extracts, have shown a higher bioactivity than isolated single active ingredients due to synergistic interactions (Schmidt et al., 2007; Williamson, 2001).

The excretion of AMS in the present study was an indicator of the high bioavailability of SAMC and thereby for the possibility to exert health promoting effects, which is the desired result for the consumer (**Figure 6-3, Table 6-4**). However, AMS contributes largely to the persistent garlic odor in breath that is an unpleasant consequence and would negatively influence the customer's choice (Suarez et al., 1999; Taucher et al., 1996; Rosin et al., 1992). Therefore, the presence of AMS in breath after ingestion of modified β-LG is the main drawback of the presented concept. The formation of AMS by allicin and SAMC seems to be an essential part of their metabolism and thus an unavoidable compromise (Lawson & Gardner, 2005). Additionally, the formation of the same

excretion product is an indication for the same metabolic pathway and in that way probably also for similar bioactive effects of allicin and SAMC.

In contrast, SAC is not metabolized to AMS, but indications for a lower bioactivity have been reported (Lawson & Gardner, 2005; Shirin et al., 2001; Pinto et al., 1997). Studies about the health promoting effects of garlic were mostly focused on the activity of allicin and SAC, since these compounds are the prevalent organosulfur compounds in garlic powder and AGE, respectively. Because of its low concentration in natural products, the bioactivity of SAMC was only tested *in vitro* and in animals so far (Howard et al., 2007; Liang et al., 2011; Pedraza-Chaverrí et al., 2004; Shirin et al., 2001; Sigounas et al., 1997a). Hence, SAMC of modified β-LG would provide an alternative ingredient which can be added to food products and enables the investigation of its bioactivity *in vivo*. Furthermore, the biological effects of SAMC, such as antioxidative and antihypertensive, were also observed for peptides released by β-LG during digestion which could result in an enhanced bioactive effect of the combined compound (Ried et al., 2013; Hernandez-Ledesma et al., 2008; Mullally et al., 1997; Imai et al., 1994).

7.4 Closing remarks and outlook

The covalent interaction between the whey protein β-LG and organosulfur compounds of garlic was investigated in the present thesis for the first time. β-LG was shown to provide the structural features for the covalent binding of allicin and diallyl disulfide. The formed S-allylmercapto-derivative is a stable, non-volatile compound which can be enriched in food without causing a garlic like flavor. The techno-functional properties of the whey protein were not adversely affected by the covalent modification. The bioavailability of SAMC was not impaired by the incorporation into the protein chain. Overall, the results underline the potential of β-LG as a transporter for covalently bound ligands, such as allicin and DADS.

Nonetheless, some further questions arose during this work. The physico-chemical characterization of the modified β-LG indicated a slightly higher surface hydrophobicity and a less rigid protein folding, which is likely to improve the interfacial behavior. An investigation of the emulsifying and foaming properties may clarify how these properties are influenced by the covalent modification. Moreover, the modified thiol group may limit the disulfide-linked aggregation, which is of particular practical importance with respect to denaturation of β-LG, aggregation with κ-casein and fouling on surfaces. Therefore, the effect of the covalent modification on the behavior of β-LG during thermal processes ought to be examined.

Further, it is of interest to accelerate the reaction rate for an up-scaling of the ligand binding. As indicated in the present thesis, the affinity of the binding reaction is increased by unfolding of the protein. Consequently, a gentle preheating (< 80 °C) which favors the unfolding of the globular structure without inducing disulfide linked aggregation or heating in presence of the ligand could significantly shorten the required incubation time. However, the sensitivity of allicin against higher temperatures should be considered. In the present work, the covalent modification was only tested with isolated β-LG and β-LG from WPI. Since milk is a complex matrix, the interaction of allicin with β-LG and the other proteins in milk is interesting as well and would provide a further understanding of the natural occurring interactions between proteins and secondary plant compounds.

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9. Summary

Scientific advances revealed the impact of specific food components on metabolic processes and their contribution to disease prevention and health promotion. The thiosulfinate allicin is an interesting bioactive compound because it is responsible for many of the health promoting effects that have been attributed to garlic. However, allicin is fairly unstable and rapidly degrades during processing and storage. Further, it contributes largely to the strong smell and pungency of garlic. In order to enrich allicin in a functional food, a delivery system is required to stabilize the compound and mask its sensory perception. However, thus far, no appropriate transporter has been proposed. The whey protein β-lactoglobulin (β-LG) has been reported to stabilize small hydrophobic bioactive compounds by non-covalent binding. β-LG is widely available, has diverse techno-functional properties combined with a high nutritional value, contains bioactive peptides and is soluble over a wide pH range. The covalent binding of allicin to β-LG could form an innovative functional ingredient. The aim of the present thesis was to investigate if β-LG is a suitable nanotransporter for organosulfur compounds from garlic, i.e. allicin and diallyl disulfide (DADS). Therefore, the interaction between the protein and the ligands was analyzed, the induced protein modification was characterized and finally the functional ingredient was incorporated into a food product to evaluate the sensory properties and the bioavailability of the bioactive compound.

The overall binding reaction of allicin and DADS with native and heat denatured β-LG was analyzed by fluorescence quenching and reverse phase high performance liquid chromatography (RP-HPLC). Further, the reaction with the amino and thiol groups of the protein was determined by *o*-phthaldialdehyde reagent and Ellman's reagent, respectively (chapter 3). Both ligands were covalently bound by the free thiol group of β-LG, whereby the affinity for allicin was significantly higher than for DADS. Fluorescence quenching and RP-HPLC revealed that DADS was additionally bound through non-covalent interactions. The binding reaction of both ligands was sensitive to the pH value during incubation. The maximum reaction rate was reached at pH 8-9, whereas the rate decreased remarkably below pH 8. The strong influence of the pH value is caused by the pH dependent reactivity of the thiol group. Thermal denaturation of β-LG reduced the maximum number of covalent binding sites due to the lower number of available free thiol groups that might have been caused by heat induced aggregation. In contrast, the exposure of additional hydrophobic surface regions of the denatured protein resulted in an increased number of non-covalent binding sites for DADS. Furthermore, unfolding of the protein structure exposed the free thiol group which resulted in a significantly increased affinity for the reaction with allicin but not for that with DADS.

In order to localize the covalent binding site precisely, the intact protein and the peptides after protein digestion were analyzed by mass spectrometry (MS) (chapter 4). According to the mass difference of the native and the modified β-LG, one cysteinyl moiety was transformed into S-allylmercaptocysteine by allicin and DADS. The stoichiometric ratio of the reaction between allicin and β-LG turned out to be 2:1. The analysis of the hydrolyzed modified protein showed that the major binding site was the free thiol group of Cys^{121}. The modification of the thiol group did not influence the enzymatic digestion by pepsin, trypsin or chymotrypsin. The S-allyl moiety remained stable under digestive conditions. Beside tryptic digestion partially favored the thiol-disulfide exchange with other cysteinyl residues. Conformational changes induced by the binding of allicin have been analyzed by circular dichroism and dynamic light scattering. The relatively small S-allyl moiety caused a moderate loosening of the tertiary structure which was in line with the increased surface hydrophobicity detected by RP-HPLC. The dissociation of β-LG dimers was not induced by the binding of allicin.

In order to obtain a consumable product for sensory and bioavailability evaluation, the production of the functional ingredient, β-LG modified with allicin, was transferred to a food grade level (chapter 5). The process comprised the incubation of whey protein isolate (β-LG source) and garlic powder (allicin source) at an alkaline pH value (8.5), followed by drying. The influence of the drying process (freeze versus spray drying), the pH value during drying (6.0; 8.0) and the protein-ligand ratio on protein denaturation and concentration of volatile organosulfur compounds was investigated. Spray drying at slightly acidic conditions resulted in a low degree of denaturation and a remarkable loss of volatile flavor-active compounds which were measured by headspace gas chromatography-mass spectrometry. Due to the blocked thiol group of the modified β-LG, heat induced disulfide linked aggregation was prevented which resulted in a higher thermal stability than the native protein. The sensory properties of the functional ingredient in aqueous solution as well as within the developed beverage were evaluated by quantitative descriptive sensory analysis. The covalent binding of allicin in combination with spray drying of the modified protein significantly reduced the garlic related organoleptic properties. Further, the pure modified β-LG was nearly free of any garlic taste and smell. The developed beverage provided a suitable matrix for the enrichment of the functional ingredient even without removal of residual compounds of garlic powder.

Finally, the bioavailability of the allicin derivate S-allylmercaptocysteine integrated in the polypeptide chain of modified β-LG was determined in a double-blind, randomized, diet-controlled cross-over study (chapter 6). Nine healthy volunteers ingested three different preparations: garlic powder in acid-resistant capsules (111 µM allicin yield), aged garlic extract in capsules (111 µM S-allyl cysteine) and the modified β-LG (111 µM allicin use) incorporated in a beverage. Different metabolites in

blood, urine and breath gas were analyzed. Allyl methyl sulfide in breath gas turned out to be a suitable marker for the bioavailability of S-allylmercaptocysteine. The excretion of allyl methyl sulfide caused by the modified β-LG indicated that the bioactive compound was completely released from the protein and excreted in the same way as allicin. Free S-allylmercaptocysteine from aged garlic extract contributed in a similar extent to the excretion of allyl methyl sulfide as the S-allylmercaptocysteine from the modified protein. The amount of allicin delivered by garlic powder in capsules was relatively low due to the insufficient capsule stability under gastric conditions. The plasma level of S-allyl cysteine, a further bioactive compound from garlic, showed its high bioavailability from aged garlic extract. Further, S-allyl cysteine was released from γ-glutamyl-S-allyl-cysteine of garlic powder and of the functional beverage which contained residues of garlic powder as well. Urinary N-acetyl-S-allyl cysteine was shown to be a qualitative marker for the bioavailability of S-allyl cysteine from aged garlic extract and from garlic powder. Taken together, the beverage containing the functional ingredient was a consumable product which delivered significant amounts of S-allylmercaptocysteine and S-allyl cysteine without garlic taste.

In conclusion, the present work demonstrated that the free thiol group of β-LG can bind allicin and diallyl disulfide in a specific, targeted manner under alkaline conditions. As a result, β-LG stabilized allicin, masked its organoleptic properties and delivered a highly bioavailable bioactive compound. Thus, β-LG was proved to be a suitable nanotransporter for organosulfur compounds of garlic.

10. Zusammenfassung

Wissenschaftliche Untersuchungen haben gezeigt, dass spezifische Inhaltsstoffe von Lebensmitteln zum Erhalt der Gesundheit und zur Prävention von Krankheiten beitragen können. Das Thiosulfinat Allicin gehört zu dieser Gruppe bioaktiver Substanzen, da es für verschiedene gesundheitsförderliche Wirkungen von Knoblauch verantwortlich ist. Allerdings weist Allicin eine geringe chemische Stabilität auf und wird während der Verarbeitung und Lagerung von Lebensmitteln schnell abgebaut. Des Weiteren trägt es zu dem intensiven Geruch und der Schärfe von Knoblauch bei. Um Allicin dennoch als bioaktive Substanz in einem funktionellen Lebensmittel anreichern zu können, ist ein Transporter zur chemischen Stabilisierung und zur Maskierung der sensorischen Eigenschaften notwendig. Bisher ist kein dafür geeignetes Verfahren bekannt. Das Molkenprotein β-Lactoglobulin (β-LG) wurde bereits häufig als stabilisierender Transporter für nicht-kovalent bindende, hydrophobe Substanzen beschrieben. β-LG ist in großen Mengen verfügbar, besitzt diverse technofunktionelle Eigenschaften sowie einen hohen ernährungsphysiologischen Wert und ist über einen großen pH-Wertbereich löslich. Die gezielte kovalente Bindung von Allicin an β-LG könnte einen innovativen funktionellen Inhaltsstoff darstellen. Das Ziel dieser Arbeit war es, zu untersuchen, ob β-LG ein geeigneter Nanotransporter für die Schwefelverbindungen Allicin und Diallyldisulfid (DADS) aus Knoblauch ist. Zu diesem Zweck wurden die Interaktionen zwischen den Schwefelverbindungen und β-LG sowie die dadurch bedingten Veränderungen umfangreich analysiert. Außerdem wurde der funktionelle Inhaltsstoff (Allicin gebunden an β-LG) in einem Lebensmittel angereichert, um die sensorischen Eigenschaften zu bestimmen und die Bioverfügbarkeit des bioaktiven Stoffes zu untersuchen.

Die Bindungsreaktion von Allicin bzw. DADS an das native und thermisch denaturierte β-LG wurde mittels Fluoreszenzlöschung und Umkehrphasen-Hochleistungsflüssigkeitschromatographie (RP-HPLC) analysiert. Die Reaktion mit Amino- und Thiolgruppen wurde durch die Reagenzien *o*-Phthaldialdehyd und 5,5'-Dithiobis-2-nitrobenzoesäure detektiert (Kapitel 3). Beide Liganden reagierten kovalent mit der freien Thiolgruppe von β-LG, wobei die Reaktion mit Allicin eine signifikant höhere Affinität aufwies als für DADS. Hingegen wurde DADS zusätzlich durch nicht-kovalente Wechselwirkungen gebunden. Die Bindungsreaktion war stark von dem pH-Wert abhängig. Bei pH 8-9 war die Reaktionsgeschwindigkeit maximal, unterhalb von pH 8 sank sie jedoch deutlich ab. Der Einfluss des pH-Wertes wurde durch die pH-abhängige Reaktivität der Thiolgruppe bedingt. Die thermische Denaturierung von β-LG führte zu einer geringeren Anzahl verfügbarer kovalenter Bindungsstellen, was wahrscheinlich durch kovalente Aggregationsreaktionen während der Erhitzung

hervorgerufen wurde. Allerdings wurden hydrophobe Oberflächenregionen durch die Entfaltung des Moleküls freigelegt, die zusätzliche nicht-kovalente Bindungsstellen für DADS boten. Die Denaturierung steigerte auch die Zugänglichkeit der freien Thiolgruppe, wodurch die Affinität der Reaktion mit Allicin signifikant erhöht wurde, nicht aber für DADS.

Zur genaueren Bestimmung der kovalenten Bindungsstelle wurden sowohl das intakte Protein als auch die durch enzymatische Hydrolyse gebildeten Peptide mittels Massenspektrometrie untersucht (Kapitel 4). Anhand der Massendifferenzen zwischen dem nativen und dem modifizierten β-LG konnte geschlossen werden, dass durch die Bindung von Allicin bzw. DADS die Seitenkette eines Cysteins zu S-Allylmercaptocystein derivatisiert wurde. Das stöchiometrische Verhältnis der Reaktion zwischen Allicin und β-LG betrug 2:1. Die Analyse der Peptide des modifizierten Proteins zeigte, dass die freie Thiolgruppe des Cys^{121} die Hauptbindungsstelle darstellte. Die kovalente Bindung von Allicin beeinträchtigte nicht die enzymatische Proteinverdauung durch Pepsin, Trypsin oder Chymotrypsin. Die Cystein-Modifikation blieb während der Verdauung stabil, lediglich während der tryptischen Proteolyse wurde ein Thiol-Disulfid-Austausch mit anderen Cysteinresten des Proteins in begrenztem Umfang beobachtet. Der Einfluss der kovalenten Modifikation auf die Proteinkonformation wurde mittels Zirkulardichroismus-Spektroskopie und dynamischer Lichtstreuung analysiert. Die gebundene S-Allyl-Gruppe führte zu geringen strukturellen Veränderungen, insbesondere auf Ebene der Tertiärstruktur war eine moderate Lockerung der Proteinfaltung zu erkennen. Eine Dissoziation der β-LG-Dimere durch den kovalenten Liganden war nicht zu beobachten.

Für die sensorische und pharmakokinetische Beurteilung des mit Allicin modifizierten β-LGs wurde ein Prozess zur Produktion des funktionellen Inhaltsstoffs in lebensmitteltauglicher Qualität etabliert (Kapitel 5). Der Prozess umfasste die Inkubation von gelöstem Molkenproteinisolat (β-LG-Quelle) zusammen mit Knoblauchpulver (Allicin-Quelle) unter alkalischen Bedingungen (pH 8,5) sowie die anschließende Trocknung. Der Einfluss der Trocknungsmethode (Gefrier- und Sprühtrocknung), des pH-Wertes während der Trocknung (pH 6; 8) sowie des Protein-Liganden-Verhältnisses auf die Proteindenaturierung und den Gehalt flüchtiger Schwefelverbindungen im Endprodukt wurde untersucht. Die Sprühtrocknung bei leicht saurem pH-Wert ermöglichte einen geringen Denaturierungsgrad und gleichzeitig die umfangreiche Entfernung flüchtiger Schwefelverbindungen, die mittels Gaschromatographie gekoppelt mit Massenspektrometrie detektiert wurden. Die kovalente Bindung des Liganden führte dazu, dass das β-LG-Molekül über keine freien Thiolgruppen verfügte. Dadurch wurde die thermisch induzierte Bildung von intermolekularen Disulfidbindungen und somit die Irreversibilität der Denaturierung unterbunden, was zu einer erhöhten Temperaturstabilität des Proteins führte. Die sensorischen Eigenschaften des mit Allicin modifizierten Proteins wurden in wässriger Lösung und in einem speziell entwickelten Getränk durch quantitative,

deskriptive Analyse ermittelt. Durch die kovalente Bindung von Allicin in Kombination mit der Sprühtrocknung des Inhaltsstoffes wurden die Knoblauch-typischen organoleptischen Eigenschaften signifikant reduziert. Das reine, modifizierte β-LG hatte einen nahezu neutralen Geruch und Geschmack. Das entwickelte Getränk bot eine geeignete Matrix für die Anreicherung des funktionellen Inhaltsstoffes, auch in Anwesenheit der übrigen Bestandteile des Knoblauchpulvers.

Abschließend wurde die Bioverfügbarkeit des gebildeten Allicin-Derivates innerhalb der Polypeptidkette von β-LG durch eine doppelt-blinde, randomisierte, Diät-kontrollierte Cross-Over-Studie untersucht (Kapitel 6). Neun gesunden Probanden wurden drei verschiedene Präparate verabreicht: Knoblauchpulver in säureresistenten Kapseln (111 µM Allicin-Potential), fermentierter Knoblauchextrakt in Kapseln (111 µM S-Allylcystein) und modifiziertes β-LG (111 µM Allicin-Einsatz) angereichert in einem Getränk. Verschiedene Metabolite in Blut, Urin und Atemgas wurden analysiert. Allylmethylsulfid im Atemgas erwies sich als geeigneter Marker für die Bioverfügbarkeit von S-Allylmercaptocystein. Die Ausscheidung von Allylmethylsulfid bedingt durch die Aufnahme von modifiziertem β-LG zeigte, dass die bioaktive Substanz scheinbar vollständig aus dem Proteinverband freigesetzt wurde und zu dem gleichen Ausscheidungsprodukt wie auch Allicin metabolisiert wurde. Freies S-Allylmercaptocystein aus fermentiertem Knoblauchextrakt führte in gleichem Maß zu der Ausscheidung von Allylmethylsulfid wie S-Allylmercaptocystein des modifizierten Proteins. Aus den Knoblauchpulver-Kapseln wurde nur eine geringe Menge Allicin freigesetzt, da die Kapseln unter gastrischen Bedingungen nicht ausreichend stabil waren. Wie erwartet, konnte S-Allylcystein im Plasma nach Aufnahme von fermentiertem Knoblauchextrakt deutlich nachgewiesen werden. Des Weiteren konnte jedoch gezeigt werden, dass γ-Glutamyl-S-Allylcystein aus dem Knoblauchpulver und dem funktionellen Getränk ebenfalls zu erhöhten S-Allylcystein-Konzentrationen im Plasma führte. N-Acetyl-S-Allylcystein im Urin stellte sich als qualitativer Marker für die Bioverfügbarkeit von S-Allylcystein und γ-Glutamyl-S-Allylcystein heraus. Schlussfolgernd erwies sich das mit dem funktionellen Inhaltstoff angereicherte Getränk als ein verzehrfähiges Lebensmittel ohne Knoblauchgeschmack, dass physiologisch relevante Mengen S-Allylmercaptocystein und S-Allylcystein mit unbeeinträchtigt hoher Bioverfügbarkeit lieferte.

Zusammenfassend zeigt die vorliegende Arbeit, dass Allicin und DADS gezielt durch die freie Thiolgruppe des β-LGs unter alkalischen Bedingungen kovalent gebunden werden können. Dadurch wird eine Stabilisierung und sensorische Maskierung ermöglicht, ohne die hohe Bioverfügbarkeit zu beeinträchtigen. Demnach erwies sich β-LG als geeigneter Nanotransporter für Schwefelverbindungen aus Knoblauch.

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