

# Peroxidase-mediated cross-linking of bovine $\alpha$ -lactalbumin

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# Peroxidase-mediated cross-linking of bovine $\alpha$ -lactalbumin

W.H. Heijnis

## **Thesis**

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## **Abstract**

The research presented in this thesis aimed at controlling the horseradish peroxidase-catalyzed cross-linking of bovine  $\alpha$ -lactalbumin and the implications of this cross-linking for the foam stabilizing properties. Attention is also given to microreactors and their potential to control the enzymatic cross-linking of proteins.

The proportion of cross-linked  $\alpha$ -lactalbumin dimers, oligomers and polymers could be directed by variations in ionic strength, pH,  $H_2O_2$ , and temperature.

Covalent  $\alpha$ -lactalbumin dimers were proteolytic digested. FTMS analysis of the peptide mixture resulted in the unambiguous identification of a Tyr18-Tyr50 dityrosine cross-link. Structural modeling of the  $\alpha$ -lactalbumin dimer indicated that favorable electrostatics direct the selectivity of the cross-linking reaction and, hence, the formation of an intermolecular cross-link. The formation of the Tyr18-Tyr50 cross-link suggests that further cross-linking of  $\alpha$ -lactalbumin dimers enables the formation of linear polymers.

A microreactor system was set up to obtain control over the reaction conditions to cross-link proteins. The enzymatic cross-linking of  $\alpha$ -lactalbumin was analyzed as a function of enzyme and substrate(s) feed. The increase in absorption at 318 nm due to dityrosine formation was found to be directly correlated to the decrease in monomeric  $\alpha$ -lactalbumin and was shown to be a good tool to monitor the cross-linking reaction.

The  $\alpha$ -lactalbumin oligomers produced were investigated for their foam stabilizing properties. Cross-linked  $\alpha$ -lactalbumin oligomers did not stabilize foams, whereas  $\alpha$ -lactalbumin polymers acted as an anti-foam, destabilizing other protein films.



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A grayscale microscopic image of plant cells, likely onion skin, showing large, rectangular cells with prominent cell walls. The image is used as a background for the chapter title.

# Chapter I

## General Introduction

## Enzymatic cross-linking of food proteins

The ability to change the functional properties of proteins has made the cross-linking of food proteins a continuous topic for investigation (1-3). The cross-linking of proteins can result in a different solubility, foam stability, whip ability, and in different emulsification properties (2). The most common method to cross-link food proteins is enzymatic modification (1, 4). The enzymatic treatment is considered to be more mild and specific than chemical modifications, such as maillardation (5). In this thesis we focus on the enzymatic cross-linking of proteins for food ingredient applications.

**Table I:** Enzymes previously described as food protein cross-linkers and their sources.

Cross-linking enzymes	EC number	Sources
Transglutaminase	2.3.2.13	Animal, Microbial
Laccase	1.10.3.2	Microbial, Plant, and Fungal
Tyrosinase	1.14.18.1	Animal, Plant, and Fungal
Peroxidase	1.11.1.7	Animal, Microbial, Plant, and Fungal
Lipoxygenase	1.13.11.12	Animal, Plant, and Fungal
Protein disulfide reductase	1.8.1.8	Animal
Protein disulfide isomerase	5.3.4.1	Animal and Plant
Sulfhydryl oxidase	1.8.3.2	Animal

Enzymes that have been used to cross-link (food) proteins are listed in **Table I**. These enzymes differ in their ability to cross-link proteins with various types of molecules. Due to their broad specificity, the oxidative enzymes laccase, tyrosinase, and peroxidases can form cross-links between carbohydrates, peptides, phenolic compounds, and/or proteins, while the more specific transglutaminases can only cross-link proteinaceous material to proteins (**Table II**). The formation of a protein-protein cross-link is usually defined as a homo cross-link whereas the formation of a cross-link between a protein and another type of molecule is defined as a hetero cross-link. A cross-link can also be formed with a mediator, acting as a bridging agent. The most commonly used cross-linking enzymes and their properties are discussed below.

Very often, in research dealing with cross-linking of proteins, modification of their (techno)functional properties is described as a reason for conducting the research. So far, most of these studies have focused on the formation of products. A main exception concerns the gelling properties of cross-linked whey protein isolate (WPI; **Table II**). More information on the relation with functionality can be found later in this introduction.

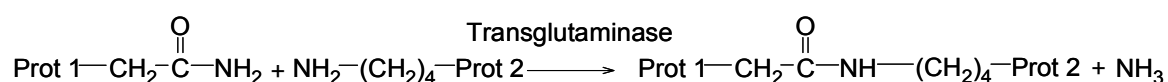
**Table II:** Enzymatic cross-linking of food proteins and related peptides.

Enzyme (source)	Protein (Peptide)	Homo XL	Mediator	Added chemicals	Functionality	Ref.
<b><i>Trans-glutaminase</i></b>						
Microbial	WPI	√		DTT, ± Ca <sup>2+</sup>	ND	(6)
	WPI	√		DTT & Ca <sup>2+</sup> or sulfite & Ca <sup>2+</sup>	Gelling	(7)
	WPI	√		DTT	Gelling	(8)
	α-LA	√		EDTA or SDS or GuHCl	ND	(9)
	α-LA	√		EDTA		(10)
	α-LA	ND	Primary amines	Ca <sup>2+</sup> or EDTA	ND	(11)
Guinea pig liver	WPI	√		DTT, ± Ca <sup>2+</sup>	ND	(6)
	WPI	√		DTT, Ca <sup>2+</sup>	ND	(12)
Unknown	WPI	√			Gelling	(13)
<b><i>Laccase</i></b>						
Fungal	Peptides	√			ND	(14)
	α-Casein	√	Oat spelt xylan, FA, <i>p</i> -CA		ND	(15)
	WPI	√		± DTT	Gelling	(16)
<b><i>Tyrosinase</i></b>						
Fungal	Tyr peptides, β-Casein	√	L-Dopa		ND	(17)
	α-Casein	√	Oat spelt xylan FA, <i>p</i> -CA		ND	(15)
	α-LA, β-LG, Lysozyme	√	Caffeic acid		ND	(18)
<b><i>Peroxidase</i></b>						
Microbial	WPI	√			Gelling	(16)
Fungal	Tyr peptides	√			ND	(19)
Horseradish	BSA, Casein, and Gliadin	√			ND	(20, 21)
	Ovalbumin, BSA, Lysozyme, and β-LG	-	Catechol		ND	(22)
	β-Casein	√	Arabinoxylan		ND	(23)
	Gly-Tyr-Gly	√	FA		ND	(24, 25)
	BSA	-	(Gly-Ala-Tyr) <sub>n</sub>		ND	(26)
Horseradish and milk	Tyr	√			ND	(27)

Abbreviations; ND: Not determined, XL: cross-link, WPI: Whey protein isolate, α-LA: α-lactalbumin, β-LG: β-lactoglobulin, BSA: bovine serum albumin, Tyr: Tyrosine, Gly: glycine, Ala: alanine, FA: ferulic acid, *p*-CA: *p*-coumaric acid, L-Dopa: L-3,4-dihydroxyphenylalanine, DTT: dithiothreitol, EDTA: ethylenediaminetetraacetate, SDS: sodium dodecyl sulfate, GuHCl: guanidium hydrochloride.

**Transglutaminase (EC 2.3.2.13)**

Transglutaminases catalyse the formation of a covalent bond between the gamma-carboxamide group of glutamyl residues and the  $\epsilon$ -amino groups of lysine in proteins and peptides (28). The formation of an isopeptide bond between a glutamine and lysine residue is shown in **Figure 1**.

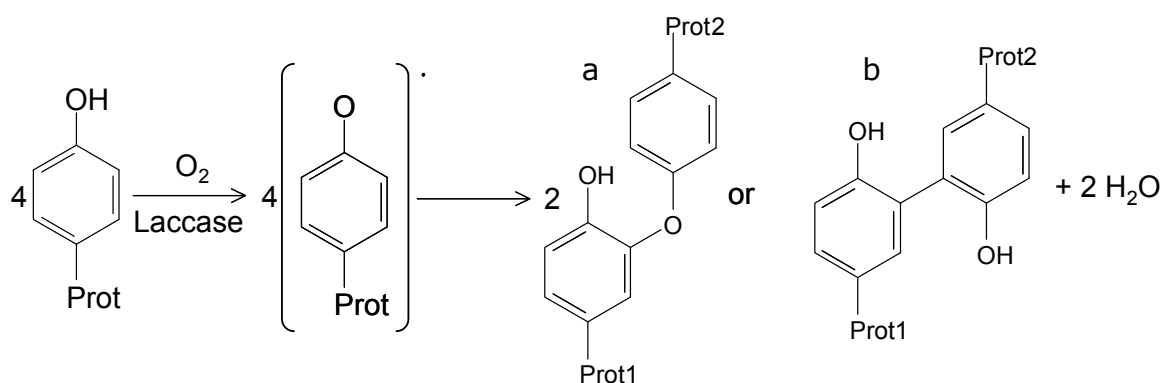


**Figure 1:** Transglutaminase-catalyzed cross-linking reaction between a glutamine and lysine residue.

Transglutaminase has been shown to catalyze the cross-linking of casein,  $\beta$ -lactoglobulin, soybean protein (2), WPI, and  $\alpha$ -lactalbumin (6-10). Studies with WPI and  $\alpha$ -lactalbumin revealed that the structures of the proteins need to be slightly unfolded to induce cross-linking. Partial unfolding of the whey proteins can be achieved by the addition of dithiothreitol (DTT) or ethylenediaminetetraacetate (EDTA). The addition of EDTA to  $\alpha$ -lactalbumin creates the  $\text{Ca}^{2+}$ -depleted more flexible apo form and facilitates cross-linking (9). This can be explained by the increased number of accessible positions for modification by primary amines of apo  $\alpha$ -lactalbumin compared to its holo form (11).

**Laccase (EC 1.10.3.2)**

Laccases are copper-containing monooxygenases that catalyze the one-electron oxidation of a wide range of organic and inorganic components (29, 30). Their use spans from the textile to the pulp and paper industries, and from food applications to bioremediation processes (31). Laccases show differences with respect to their reactivity towards amino acids. For instance, the fungal laccase from *Trametes hirsu* shows a dominant activity on tyrosine compared to tryptophan and cysteine (32). The initial one-electron oxidation of tyrosine can result in the subsequent formation of either an *ortho-ortho* dityrosine (**a**, 3,3'-dityrosine) or an isodityrosine (**b**, 3,O'-dityrosine) bond (**Figure 2**). For tyrosine (Y) and short tyrosine containing peptides (GY and GLY) the formation of homo oligomers up to 9 peptides was shown (14). When protein tyrosines act as laccase substrates, protein cross-links are formed.



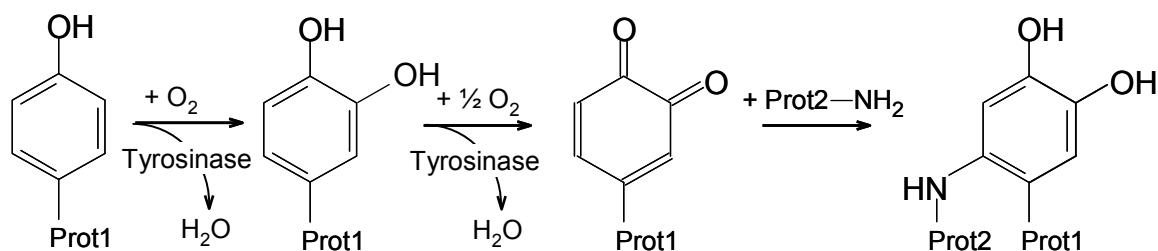
**Figure 2:** Laccase-catalyzed formation of an (iso)dityrosine cross-link; (a) *ortho-ortho* dityrosine; (b) isodityrosine.

Just as for transglutaminase, a compact globular structure of proteins is hindering their cross-linking by laccases. This is illustrated by the small protein coactosin. This protein contains four tyrosine residues of which one is located in a flexible tail. Upon treatment with laccase, the native protein was oligomerized to a tetramer, while upon truncation of the flexible tail the cross-linking reaction was stopped (32). This indicated that the tyrosine located on the flexible tail is involved in the formation of coactosin oligomers and the formation of a tetratyrosine cross-link. The laccase-catalyzed cross-linking of WPI was stimulated by the unfolding of the protein via the DTT-mediated reduction of disulfide bonds (16). Another possibility to increase cross-linking is the addition of small phenolic mediators like ferulic acid (FA) and *p*-coumaric acid (*p*-CA). These mediators, as well as hydrolyzed feruloylated xylan, increased the extent of cross-linking of  $\alpha$ -casein by acting as bridging agents (15). The use of hydrolyzed feruloylated xylan opens the possibilities for heteroconjugate cross-linking of carbohydrates and proteins.

### ***Tyrosinase (EC 1.14.18.1)***

Tyrosinases are copper-containing monooxygenases that catalyze the *ortho*-hydroxylation of monophenols to *o*-diphenols and the subsequent oxidation to *o*-quinones. These *o*-quinones can react further with amino, sulfhydryl, and pyrrolidine side chains of proteins (18). When tyrosine residues present in proteins act as substrates, protein cross-links are formed (**Figure 3**).

Food proteins, which have been homo cross-linked with tyrosinase include apo  $\alpha$ -lactalbumin,  $\alpha$ -casein, and  $\beta$ -casein (15, 17, 18). More food proteins can be cross-linked with phenolic compounds, like caffeic acid, acting as mediators (18). Compared to laccase, tyrosinase is less active in heteroconjugate formation with hydrolyzed feruloylated xylan (15).

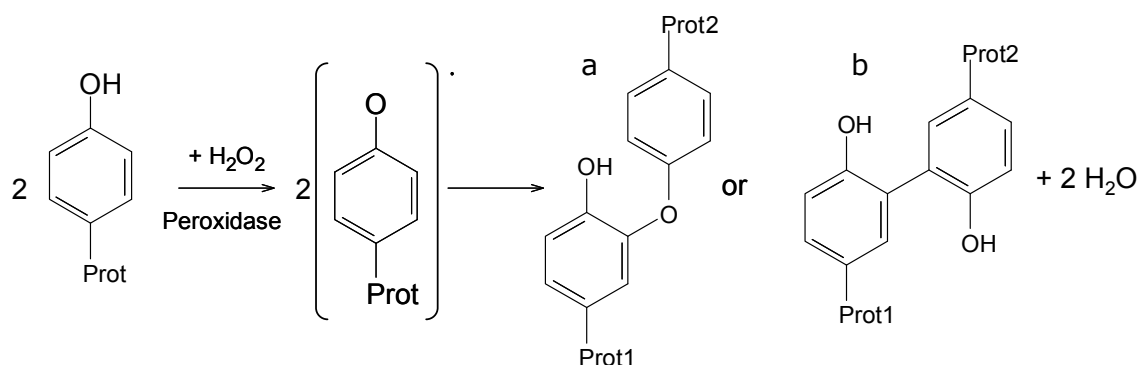


**Figure 3:** Tyrosinase-catalyzed formation of a dopa *o*-quinone and the subsequent formation of a protein cross-link.

### ***Peroxidase (EC 1.11.1.7)***

Peroxidase is the main cross-linking enzyme discussed in this thesis. In contrast to laccase and tyrosinase, this oxidative enzyme uses hydrogen peroxide as oxidation agent. Peroxidases are widespread and several isoforms are often found in individual plants (33, 34). In the next paragraph the heme-containing peroxidase extracted from horseradish is discussed for its mode of action and applications.

Peroxidases can be rather aspecific concerning their substrates (35) and they oxidize several amino acids. Horseradish peroxidase (HRP) does react with Tyr, Phe, Trp, His, and Cys, with Tyr being oxidized the best (36). After the initial one-electron oxidation step an *ortho-ortho* dityrosine (**a**, 3,3'-dityrosine) or an isodityrosine (**b**, 3,O'-dityrosine) cross-link can be formed (**Figure 4**).



**Figure 4:** Peroxidase-catalyzed formation of tyrosine radicals and the subsequent formation of an (iso)dityrosine protein cross-link; (**a**) *ortho-ortho* dityrosine; (**b**) isodityrosine.

Several food proteins and related peptides have been homo cross-linked with peroxidases (**Table II**). This does not imply that peroxidases are able to oxidize every protein regardless of their structure.  $\alpha$ -Lactalbumin, for instance, is not cross-linked in the presence of calcium ions (16, 37). The cross-linking of BSA at neutral pH is only successful with the addition of catechol, while at pH 9.5 BSA could form a cross-link without the addition of a mediator (20,

22). This indicates that also for peroxidase, the conformation of the protein substrate is of influence in the success of cross-linking.

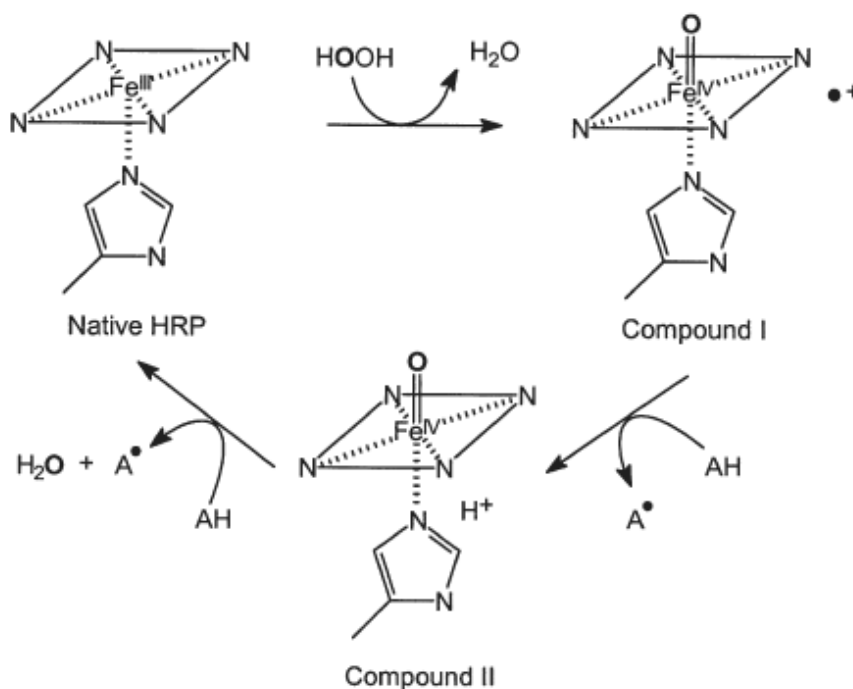
Studies towards heterologous cross-linking of proteins with feruloylated carbohydrates have been performed in a stepwise way: First tyrosine-containing peptides and ferulic acid were heterologous cross-linked (24, 25). This was then followed by the cross-linking of  $\beta$ -casein to feruloylated arabinoxylans (23).

The success of enzymatic cross-linking of a natively folded protein depends on two main factors. Firstly, the target amino acids should be accessible for reaction with the enzyme and secondly, the size and shape of the protein substrate must not hinder the formation of a cross-link. By selection of a different enzyme different amino acids will be involved in the cross-linking process. Glutamine and lysine for transglutaminase; aromatic amino acids, histidine, and cysteine for laccase and peroxidase; and tyrosine, cysteine, and proline for tyrosinase. (Un)folding of a protein upon changes in pH, temperature or by the addition of a denaturant can result in an increased availability of the target amino acids. Besides intermolecular cross-linking the cross-linking can also be intramolecular, as was shown for the non-food protein calmodulin (38, 39). This all indicates the complexity of the enzymatic cross-linking of proteins, but also the possibilities for directing this reaction. In this thesis we have studied the HRP (type VI-a)-mediated cross-linking of the bovine whey protein  $\alpha$ -lactalbumin.

## **Horseradish Peroxidase**

### ***Mode of action***

The heme-containing glycoprotein horseradish peroxidase (HRP) is a widely used oxidative enzyme. Seven peroxidase isozymes have been extracted from the roots of the horseradish, each of them has its own physical and catalytic properties (40, 41). The basic catalytic mechanism is the same for each of these isozymes. With  $\text{H}_2\text{O}_2$  as oxidizing co-substrate, the enzyme can catalyse the oxidation of a wide variety of donor substrates, such as phenols and aromatic amines. The reaction follows a three step cycle as schematically depicted in **Figure 5**. First, the heme group of the native HRP is oxidized to Compound I ( $\text{Fe}^{\text{III}} \rightarrow \text{Fe}^{\text{IV}}=\text{O}$ ). Compound I is then capable of reducing two donor substrates (AH) in a two-step reaction, with an intermediate formed called Compound II. The radical products formed out of the two oxidized donor substrates can subsequently react further. This will be described later.



**Figure 5:** Reaction cycle of HRP, showing the enzyme intermediates, Compounds I and II. (42)

An overdose of  $\text{H}_2\text{O}_2$  results in the inactivation of HRP. There are two pathways that lead to the inactivation of HRP, one in the absence and one in the presence of donor substrate (43,44). In the absence of donor substrate, HRP is irreversibly inactivated in a time- and  $\text{H}_2\text{O}_2$ -concentration dependent process. The suicide inactivation results from the reaction of  $\text{H}_2\text{O}_2$  with Compound I, which leads to a modification of the heme. In the presence of a donor substrate, the same inactivation mechanism applies, but in addition the enzyme may become irreversibly damaged by radical intermediates generated during turnover.

### ***Applications of HRP mediated oxidation***

HRP is used in a wide spectrum of applications and is considered to be the mostly used enzyme in analytical systems (42, 45). HRP catalyzes the oxidation of numerous chromogenic substrates and is, therefore, widely used in colorimetric or fluorimetric assays. Biosensors and immunoassays are the most common applications for HRP in many disciplines (42).

### ***Dityrosine***

When the donor substrate ( $\text{AH}$  of **Figure 5**) is tyrosine in the reaction with HRP, the reaction product is dityrosine. This compound can subsequently react further to trityrosine and tyrosine oligomers. Dityrosine was first recognized 50 years ago (46) and has thereafter been



the topic in various studies (39, 47-49). The formation of dityrosine cross-links in a protein is shown in **Figure 4**. The dityrosine bond can be either a isodityrosine (3,O'dityrosine) or a *ortho-ortho* dityrosine (3,3'dityrosine), of which the latter is most abundant (47, 48). Throughout this study we do not discriminate between the two forms of dityrosine.

Besides enzyme-catalyzed, dityrosine can also be formed upon UV irradiation,  $\gamma$ -irradiation, and upon contact with radicals (48, 49). The dityrosine cross-link formed is resistant against reduction, heat treatment, and proteolysis (47, 48). At room temperature, the influence of this cross-link on the secondary and tertiary structure of some cross-linked proteins (calmodulin, bovine pancreatic ribonuclease A, and bovine eye lens  $\gamma$  B-crystallin) is minimal (50). However, the melting temperature of these proteins can be decreased by cross-linking.

Dityrosine is part of biological elastic complexes functioning as a protection for proteolysis and physicochemical trauma. Examples of these complexes are elastin, collagen, human lens protein, ligaments of insects, and the gluten network in wheat flour (47, 49, 51).

Oxidation by free radicals and UV irradiation are related to aging processes and the dityrosine formed can be used as a biomarker in the aging processes. For example, dityrosine formation is found in age-related diseases as Parkinson's disease, atherosclerotic lesions (47, 52).

#### *Determination of dityrosine*

The detection of the presence of dityrosine is most often regarded as a quantitative analysis, resulting from the fact that dityrosine is a biomarker for oxidative stress. In these situations it is not that relevant to investigate the exact location of the cross-link. The analyses described below are, therefore, mostly focused on dityrosine quantification.

Upon cross-linking the conjugated system of tyrosine is extended and a shift in UV-spectrum is observed. The absorption maximum of dityrosine is centered around 318 nm (53). A more specific determination is reached with fluorescence spectroscopy. Upon excitation at 315 nm (alkaline conditions) or 284 nm (acidic conditions) an intense fluorescence signal is obtained at 420 nm (39, 54).

In addition to the spectroscopic techniques, amino acid analysis is used for the detection of dityrosine. This analysis can be performed with (47, 51, 55), or without (48, 49, 52, 53) subsequent analysis by mass spectrometry (MS). For this, the completely hydrolyzed protein is separated with high-performance liquid chromatography (HPLC). Retention times of tyrosine and dityrosine are known and their concentrations can be quantified with standards

(49). MS analysis is added to this system to find other oxidation products of tyrosine, like acetyl dityrosine and nitrotyrosine (47). Although the dityrosine can be quantified in this way, it is not known which tyrosines are involved in the dityrosine cross-link.

Amino acid analysis has also been used to assign the tyrosines involved in cross-linked calmodulin (38). Protein digestion followed by MS analysis was used to identify the tyrosines involved in the peroxide-induced intra- and intermolecular cross-linking of sperm whale metmyoglobin (56, 57). Site-directed mutagenesis has been used for the indirect assignment of tyrosines involved in the cross-linking process. Lack of cross-linking after mutation was observed for the cross-linking of coactosin by laccase (32) and the cross-linking of  $\alpha$ -synuclein by cytochrome c (58).

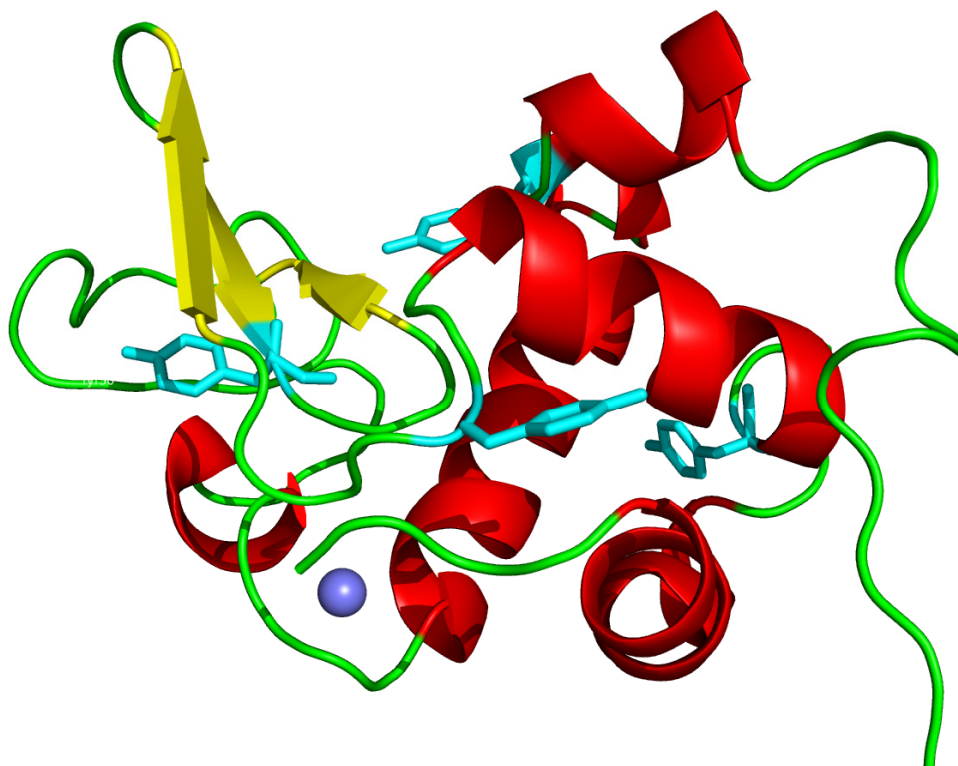
### Bovine $\alpha$ -lactalbumin

$\alpha$ -Lactalbumin is one of the whey proteins in milk. The physiological function of  $\alpha$ -lactalbumin is the interaction with the lactose synthesis enzyme system (EC 2.4.1.22) in mammary glands (59, 60). In the human diet,  $\alpha$ -lactalbumin is a rich source of essential amino acids (61, 62). The total proportion of essential amino acids is 63 % (mol basis), compared to 52 % for the total milk protein. **Figure 6** depicts the amino acid sequence of bovine  $\alpha$ -lactalbumin (59). The four tyrosine residues, depicted bold, are the primary targets for oxidation by HRP.  $\alpha$ -Lactalbumin contains 8 cysteines, which are involved in four disulfide cross-links: Cys6-Cys120, Cys28-Cys111, Cys61-Cys77, and Cys73-Cys91.

The three-dimensional structure of  $\alpha$ -lactalbumin consists of two domains ( $\alpha$ -helical and  $\beta$ -sheet domain) separated by a cleft (**Figure 7**). The  $\alpha$ -helical domain contains four  $\alpha$ -helices and two  $3_{10}$  helices. The  $\beta$ -sheet domain contains a triple  $\beta$ -sheet, a  $3_{10}$  helix and loops (63, 64). Tyr<sup>50</sup> is located in the  $\beta$ -domain, Tyr<sup>36</sup> at the domain interface, and both Tyr18 and Tyr103 in the  $\alpha$ -domain (65).  $\alpha$ -Lactalbumin is a calcium binding metalloprotein. The calcium binding site is located on the junction of the  $\alpha$  and  $\beta$  domain and composed of Lys79, Asp82, Asp84, Asp87, and Asp88 (66). The binding of calcium is an important factor in the stability of the protein. Holo (with calcium) and apo (without calcium)  $\alpha$ -lactalbumin have a melting temperature of 66 °C and 39 °C, respectively (67).

1	10	20
H-Glu-Gln-Leu-Thr-Lys-Cys-Glu-Val-Phe-Arg-Glu-Leu-Lys-Asp-Leu-Lys-Gly- <b>Tyr</b> -Gly-Gly-		
21	30	40
Val-Ser-Leu-Pro-Glu-Trp-Val-Cys-Thr-Ala-Phe-His-Thr-Ser-Gly- <b>Tyr</b> -Aap-Thr-Gln-Ala-		
41	50	60
Ile-Val-Gln-Asn-Asn-Asp-Ser-Thr-Glu- <b>Tyr</b> -Gly-Leu-Phe-Gln-Ile-Asn-Asn-Lys-Ile-Trp-		
61	70	80
Cys-Lys-Asp-Asp-Gln-Asn-Pro-His-Ser-Ser-Asn-Ile-Cys-Asn-Ile-Ser-Cys-Asp-Lys-Phe-		
81	90	100
Leu-Asp-Asp-Asp-Leu-Thr-Asp-Asp-Ile-Met-Cys-Val-Lys-Lys-Ile-Leu-Asp-Lys-Val-Gly-		
101	110	120
Ile-Asn- <b>Tyr</b> -Trp-Leu-Ala-His-Lys-Ala-Leu-Cys-Ser-Glu-Lys-Leu-Asp-Gln-Trp-Leu-Cys-		
123		
Glu-Lys-Leu-OH		

**Figure 6:** Amino acid sequence of bovine  $\alpha$ -lactalbumin (59).



**Figure 7:** Crystal structure of bovine holo  $\alpha$ -lactalbumin (PDB accession code 1f6s) (66). The calcium ion is depicted as a purple sphere.

### ***$\alpha$ -Lactalbumin folding***

The folding states of  $\alpha$ -lactalbumin have been the topic of numerous studies. Besides the holo and apo form,  $\alpha$ -lactalbumin can form a folding intermediate called the molten globule.

#### *Molten globule state*

The molten globule is defined as a folding intermediate of a globular protein in between the native and denatured protein. Due to a high stability of its molten globule,  $\alpha$ -lactalbumin is considered as a model in protein folding studies (68). The  $\alpha$ -lactalbumin molten globule can be formed at pH values in between pH 3.0-4.2 and above pH 9.5 (the so called A-state) and upon the moderate addition of strong denaturants (64, 68, 69). All molten globule states can be defined as a '*compact globule with native-like secondary structure and with slowly fluctuating tertiary structure*' (70). This compact globular structure can be described by the different Stokes radii (71). The largest radius is found for unfolded  $\alpha$ -lactalbumin (2.5 nm), followed by the molten globule (2.0 nm), and apo and holo  $\alpha$ -lactalbumin (1.9 nm). The increase in radius upon the transition to the molten globule state implies a loss of tertiary structure and, therefore, an increased flexibility.

#### *Apo $\alpha$ -lactalbumin*

The  $\text{Ca}^{2+}$ -depleted apo form of  $\alpha$ -lactalbumin at low ionic strength and elevated temperature is a similar state to the molten globule A state (68, 71). This can not be deduced from the Stokes radii in which holo and apo  $\alpha$ -lactalbumin do not differ (1.88 vs. 1.91 nm (71)). Despite the small difference in radii, the removal of calcium from  $\alpha$ -lactalbumin leads to a less compact protein structure. Holo  $\alpha$ -lactalbumin has a stable tertiary structure in solutions of various pH's and ionic strength. Apo  $\alpha$ -lactalbumin shows a large variety of conformational states in these conditions (67). Apo  $\alpha$ -lactalbumin is far more sensitive to cross-linking by HRP than the holo form (37). Therefore, we used apo  $\alpha$ -lactalbumin in this thesis to be subjected to oxidation by HRP.

### **Functionality of cross-linked proteins**

Within food science, there is a certain interest to use enzymatic crosslinking of proteins to improve the (techno-)functional properties of these proteins. In a recent overview (4), some examples are given of the use of crosslinking enzymes in commercial processes (*e.g.* bread making). Others have studied the effect of crosslinking on foam, emulsion or gel properties in

more simplified systems. (72-78). While there are clear effects observed in some cases, the observations sometimes seem contradicting. As a result of the cross-linking of WPI by transglutaminase, the WPI solution increased in viscosity and gel strength (7). Extensive cross-linking resulted in too large proteins to form a network, resulting in an increase in gel point and a decrease in gel strength (8).

Crosslinking of  $\beta$ -lactoglobulin to soy or sesame seed globulins increased the emulsifying activity, while no effect was found on the foam forming capacity (72). Another study compared the crosslinked products formed after incubation of caseinate, whey protein powder and total milk protein with transglutaminase, lactoperoxidase, laccase or glucose oxidase (73). The resulting physical properties tested (*e.g.* viscosity, foam stability) showed in some cases large improvements, but in other cases deterioration. This illustrates that cross-linking can be used to increase the functionality of the proteins. However, it also illustrates that in order to obtain the desired functionality the reaction conditions should be controlled. Under such controlled conditions, the crosslinking reaction can be used to produce pre-processed protein samples with specific functionality. For this, first the enzyme is incubated with the proteins to produce protein aggregates of specific size (-distribution). These can then be added in a food product to provide a better functionality.

In recent years several articles have been published that discuss the effect of the presence of heat induced protein aggregates on foam stability (79-83). While foam properties are typically considered to be mainly the result of interfacial properties (*e.g.* surface pressure, dilatational modulus, adsorbed amount), it has been shown that in the presence of aggregated proteins this relation may not be that dominant. There are several studies in which the aggregation was found to have significant effects on the foam properties, while little or no effects on the interfacial properties are observed (82-84). The mechanism by which these protein aggregates could contribute to foam stability is the bridging effect known as pickering stabilisation. This mechanism is known to occur for particle stabilised foams (85, 86). In such systems the particles bridge the distance between the two adsorbed interfaces of neighbouring air-bubbles in foam.

Depending on the properties of the particles used (*e.g.* size, shape, hydrophobicity) the particle can stabilize or de-stabilize the thin films between bubbles (84, 85). This is the mechanism by which anti-foams destabilize foams (87). While in food science often the aim is to obtain more stable foams, there is also a wide range of systems in which (controlled) instability of foam is needed. In industry many processes are hindered by unwanted foam

formation. In such cases anti-foams can be applied to destabilize the foam as described above. There are different classes of anti-foam, but many are based on silicon oil, or similar components.

The effect of protein aggregation on the foam stability has been found to depend on the aggregate type or size (82, 84, 88). It was shown that, while short time heating lead to increased foam stability, longer heating times resulted in the formation of larger aggregates with poorer foam stabilizing properties. From this it was concluded that the size of the protein aggregates is an important determinant for the resulting foam properties. To test the effect of aggregate size on the foam properties, in this thesis the enzymatic cross-linking was used to produce different protein aggregates.

### **Microreactor technology**

The previous paragraphs mention the importance of control of the HRP-mediated cross-linking reaction of  $\alpha$ -lactalbumin. A relatively new tool to control enzymatic processes is the microreactor. We will discuss the major advantages and developments in microreactor technology below.

#### ***History and basic principles***

The term microreactor technology refers to ‘miniature’ continuous reactors with channels of 10-300 microns in diameter and volumes in the sub-microliter scale. Liquid microreactors or microfluidic devices were first used in the 90’s of the last century. The development of microtechnology was the driving force for the miniaturization of reactors, as was shown before for *e.g.* electronics.

In a basic setup of a microreactor the reagents flow through a channel. The residence time is dependent on the flow rate and the channel dimensions. Over the years, more types of reactors have been added to the list of microfluidics. Examples are multi-layer flow reactors, membrane reactors, and reactors designed for mixing or separation. Together with the trend to immobilize enzymes in the reactor these reactors facilitate multi-enzyme catalysis, cascade reactions, and purification. However, most of these systems are aimed at analysis (micro total analysis systems,  $\mu$ TAS) and not for production purposes.

The advantage of microfluidics over conventional reactors, that is often mentioned, is their ease to scale up. By placing the multiple reactors parallel the throughput of a system can be increased. This has already been performed successfully (94, 95). With the parallelization an

increase in throughput is achieved as large as the number of parallel reactors. Although parallelization is an attractive concept, it is not yet widely applied, due to some practical problems. The small dimensions of the microreactor facilitate clogging by large particles like dust or air bubbles, which results in differences in flow rates in the channels that are not blocked. Also, leakage occurs, although this is hard to observe due to the small volumes used. The control of temperature will be more difficult in parallel channels (96).

The miniaturization of reactors results in more control over the systems and reduces waste streams. The beneficial control over the system by microfluidics is obtained by the factors listed below (97, 98):

- Localized control of concentration gradients. Typically two reagents are brought together in a Y or T shaped channel and left to react for a specific time, dependent on flow-rate and channel length. Due to the dimensions of the microreactor there is only a laminar flow and transport of the reagents is diffusion driven. The reagent concentration in every place in the microreactor can be calculated and predicted. This makes it possible to determine the microreactor dimensions and reactor conditions where the enzyme conversions leads to the highest productivity (99).
- High heat transfer coefficients. Microreactors have high surface/volume ratios, which results in a high heat transfer coefficient. Due to the high heat transfer, solutions can be heated or cooled faster compared to large scale reactors (100). This is favorable for reactions at elevated temperatures when one of the components is only stable at low temperatures, for instance in the case of  $\text{H}_2\text{O}_2$  (**Chapter IV**).  $\text{H}_2\text{O}_2$  can than be stored at a low temperature before entering the microreactor. The reaction temperature is reached very fast and the reaction takes place before  $\text{H}_2\text{O}_2$  destabilizes. Another application is to perform extreme exothermic reactions in a microreactor to lower the risk for explosion (101). The cooling of the exothermic reaction lowers the probability and the little sample volumes lower the impact of an explosion.
- Separation of reaction products and elimination of unwanted side products. A microreactor enables a continuous flow reaction. With the continuous flow a separation can be obtained, most often through diffusion. A three-phase flow system is an example of a simple separation system (102, 103). For the separation of peroxidase-catalyzed cross-linked proteins, FricDiff (friction difference) (89), free-flow electrophoresis on a chip (90, 91), pinched flow fractionation (92), and deterministic lateral displacement (93) might be of interest. Separation can be obtained by differences in diffusion coefficients, electrophoretic mobilities, and particle sizes of the molecules of interest.

### ***Enzymatic oxidation of proteins in a microreactor***

The list of enzymes used in microfluidics is quite extensive. However, enzymatic cross-linking reactions in microreactors have thus far only been used for enzyme immobilization (106, 107). There they facilitate the cross-linking process, via aldehydes or polylysine as linkers, to let the polymerized enzymes precipitate in the microreactor.

Despite the fact that HRP is considered a model enzyme for use in microfluidics (105), controlling the HRP-mediated oxidation of proteins in microreactors is hardly described. Peroxidases are used for example in the determination of glucose by a reaction with a dye (108). Peroxidase-catalyzed cross-linking in a microreactor was first shown in the polymerization of phenols (109). This reaction was later incorporated in multienzyme catalysis and multistep synthesis in the polymerization of a phenol (110, 111).

### **Aim and outline of the thesis**

Oxidative enzymatic cross-linking is an attractive tool to change the functionality of food proteins. However, up to now it is far from clear what conditions are needed for a particular enzyme-protein system to optimize the process in terms of product formed. In this thesis we describe the HRP-mediated cross-linking of bovine  $\alpha$ -lactalbumin. This milk protein was selected as a target protein substrate because of its well-documented folding and functional properties.

Therefore, our aims were to:

- obtain control over the cross-linking process to produce more defined oligomers
- assign the amino acid residues involved in cross-link formation
- determine the foam properties of the cross-linked protein oligomers

In **Chapter I** an overview is given of the prerequisites for the homologous enzymatic cross-linking of proteins. Special attention is paid to the cross-linking with horseradish peroxidase. In **Chapter II** the influence of reaction conditions on the  $\alpha$ -lactalbumin oligomer formation is determined. The effect of ionic strength, pH, temperature and H<sub>2</sub>O<sub>2</sub> additions were analyzed by size exclusion chromatography. The covalent dimer was fractionated and subsequently digested by proteolysis (**Chapter III**). Mapping of the peptides fragments by LC-MS resulted in the assignment of the dityrosine cross-link. This finding will help in understanding the formation of various oligomers and finally in an increased control over the cross-linking process.



A recent development in the control over enzymatic processes is the microreactor. One of the bottlenecks in using a microreactor system for the cross-linking of  $\alpha$ -lactalbumin is the lack of a detection system. Therefore, in **Chapter IV**, a microreactor system with an in-line monitoring step was developed. Finally we studied the implications of  $\alpha$ -lactalbumin cross-linking for its functionality in food applications (**Chapter V**). With foam and thin film experiments, we found that the cross-linking affects  $\alpha$ -lactalbumin stabilized foams. The effect on thin film stability was further tested in thin films stabilized by  $\beta$ -lactoglobulin. The results of the different chapters are discussed in **Chapter VI** including the possible application and future extensions of the research.

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## Chapter II

# Directing the oligomer size distribution of peroxidase-mediated cross-linked bovine $\alpha$ -lactalbumin

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### **Abstract**

Enzymatic protein cross-linking is a powerful tool to change protein functionality. For optimal functionality in gel formation, the size of the cross-linked proteins needs to be controlled, prior to heating. In the current study, we addressed the optimization of the horseradish peroxidase-mediated cross-linking of calcium-depleted bovine  $\alpha$ -lactalbumin. To characterize the formed products, the molecular weight distribution of the cross-linked protein was determined by size exclusion chromatography. At low ionic strength, more dimers of  $\alpha$ -lactalbumin are formed than at high ionic strength, while the same conversion of monomers is observed. Similarly, at pH 5.9 more higher oligomers are formed than at pH 6.8. This is proposed to be caused by local changes in apo  $\alpha$ -lactalbumin conformation as indicated by circular dichroism spectroscopy. A gradual supply of hydrogen peroxide improves the yield of cross-linked products and increases the proportion of higher oligomers. In conclusion, this study shows that the size distribution of peroxidase-mediated cross-linked  $\alpha$ -lactalbumin can be directed towards the protein oligomers desired.

## Introduction

Enzymatic cross-linking of whey proteins, prior to heating, has been used to decrease gel point temperature and to improve gel strength upon gelation. Because only limited cross-linking showed these beneficial effects (1, 2), there is a need to direct the enzymatic cross-linking process from polymer formation into oligomer formation to produce cross-linked proteins of desired functionality.

Enzymes such as transglutaminase, laccase, and peroxidase have been applied to form cross-linked whey proteins (2-6). Transglutaminase (EC 2.3.2.13) induces isopeptide bond formation between glutamyl and lysine residues in proteins. Laccase (EC 1.10.3.2) and peroxidase (EC 1.11.1.7) catalyze the one-electron oxidation of tyrosine residues (7-9). The tyrosyl radicals generated induce (iso)dityrosine conjugation and, as a consequence, covalent protein cross-links are formed. Which tyrosines are radicalized depends on the accessibility of the tyrosine groups (3).

Bovine  $\alpha$ -lactalbumin has been thoroughly investigated for its structural and folding properties (10-13). Removal of the calcium co-factor generates apo  $\alpha$ -lactalbumin, which has a native-like, but more dynamic structure than holo  $\alpha$ -lactalbumin (10, 12-14). Apo  $\alpha$ -lactalbumin is less thermostable than holo  $\alpha$ -lactalbumin and more susceptible to pH- and ionic strength-induced structural changes (14). The plasticity of apo  $\alpha$ -lactalbumin allows peroxidase-catalyzed intermolecular cross-linking (8), resulting in a range of protein oligomers (6, 8), while the holo form is not reactive. The more flexible tertiary structure of the apo form leads to increased exposure of the tyrosine residues. However, as discussed elsewhere (8), no single tyrosine was identified as becoming more reactive. Thus, changes in temperature, pH and ionic strength are expected to cause conformational perturbations of tyrosine residues in apo  $\alpha$ -lactalbumin. This suggests that changes in apo  $\alpha$ -lactalbumin conformation will influence the cross-linking reactivity and hence the type of reaction products formed. Due to its flexibility, apo  $\alpha$ -lactalbumin has the potential to direct the oligomer formation.

The catalytic mechanism of horseradish peroxidase (15) and the biological implications of peroxidase-mediated protein aggregation (16) have been addressed in detail. However, little attention has been paid to how the size of the cross-linked products can be specifically altered. In this research, we have addressed the product specificity of the peroxidase-mediated cross-linking of apo  $\alpha$ -lactalbumin. Using different reaction conditions (pH, ionic strength and hydrogen peroxide ( $H_2O_2$ ) concentration), the extent of cross-linking

and type of products formed were monitored by size exclusion chromatography (SEC). Fluorescence spectroscopy, circular dichroism (CD), and differential scanning calorimetry (DSC) were used to investigate the protein conformation of the  $\alpha$ -lactalbumin substrate. Future research will aim at the study of the structural and techno-functional food properties of the generated  $\alpha$ -lactalbumin oligomers and their mode of cross-linking.

## Materials and methods

### *Materials*

A commercial  $\alpha$ -lactalbumin powder (BioPURE, Davisco Foods International Inc., Le Sueur, MN, USA) was used. According to the manufacturer, the protein content was 95 % (w/w) of which 90 % (w/w)  $\alpha$ -lactalbumin. As  $\alpha$ -lactalbumin is the only present protein, which can cross-link we refer to it as pure  $\alpha$ -lactalbumin (6). The calcium content of the  $\alpha$ -lactalbumin powder was 0.55 % (w/w). When indicated, excess calcium chloride (20 mM) or EDTA (20 mM) were added to create 100 % holo, or 100 % apo  $\alpha$ -lactalbumin, respectively. Horseradish peroxidase (HRP) type VI-a (P6782), and catalase (C30) were obtained from Sigma (Sigma Chemical CO, St Louis, MO, USA). All other (bio)chemicals were of analytical grade and purchased from Sigma or Merck (Darmstadt, Germany).

### *Enzymatic cross-linking*

$\alpha$ -Lactalbumin solutions of 1 % (w/v) with a volume of 1 mL were incubated at 20 and 37 °C for one hour in different ammonium acetate buffers (0.1 mM-100 mM, pH 5.9 and 6.8). HRP (50  $\mu$ L, 10 mg/mL) and hydrogen peroxide (2-96  $\mu$ L, 0.5 M H<sub>2</sub>O<sub>2</sub>) were added to induce cross-linking. A catalytic amount of catalase (20  $\mu$ L, 150  $\mu$ g/mL) was added to quench the reaction after the desired incubation time. Incubation conditions were varied by changing the pH (5.9-6.8), the salt concentration (0.1 mM-100 mM ammonium acetate), or H<sub>2</sub>O<sub>2</sub> concentration (1-48 mM). The H<sub>2</sub>O<sub>2</sub> cosubstrate was added either all at once or in aliquots of 2  $\mu$ L at 10 minute intervals.

### *Peroxidase activity*

ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), 9.1 mM) in 0.1-100 mM ammonium acetate buffer pH 5.9 and 6.8, was incubated in a 1 mL cuvette at 37 °C in a UV-1601 spectrophotometer equipped with a CPS-240A cell positioner (Shimadzu SI, Columbia, MD, USA). After addition of 16  $\mu$ L HRP (0.5  $\mu$ g/mL) to 1 mL ABTS solution, the mixture was left to equilibrate for 5 minutes. H<sub>2</sub>O<sub>2</sub> (33  $\mu$ L, 0.3 % (w/w)) was added to start the reaction and the increase in absorption at 405 nm was monitored for 10 minutes. The initial slope of  $\Delta A_{405\text{nm}}/\text{minute}$  was used to calculate the enzymatic activity via the UVProbe software (Shimadzu).

### *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

Covalent protein cross-linking was analyzed by SDS-PAGE using a Phast-system (GE Healthcare, Uppsala, Sweden) according to the supplier's instructions. PhastGels Gradient 8-25 gels (Amersham) were used for protein separation. Before application, protein samples (5 mg/mL) were heated for 5 minutes at 100 °C in the

presence of 1.25 % (w/v) SDS and 1.25 % (v/v)  $\beta$ -mercapthoethanol. Proteins were stained with Coomassie Brilliant Blue and a protein molecular weight marker (GE Healthcare) was used for calibration.

#### ***Size-exclusion chromatography (SEC)***

Peroxidase-mediated cross-linking of  $\alpha$ -lactalbumin was also analyzed by SEC. Diluted protein samples (20  $\mu$ L, 5 mg/mL) in 0.1 M ammonium acetate buffer pH 6.8 were applied to a Superdex 75 10/300 GL column (GE Healthcare, Uppsala, Sweden) connected to an Äkta Purifier system at room temperature. The column was equilibrated and eluted with 0.1 M ammonium acetate buffer pH 6.8 at a flow rate of 0.9 mL/min. The eluate was monitored at 280 nm. Calibration of the column was performed with a low molecular weight SEC calibration kit (GE Healthcare). The kit contained blue dextran (2000 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

To describe the size distribution of the products formed, the SEC chromatogram was divided in four regions (>60 kDa, 40-60 kDa, 25-40 kDa, and 10-25 kDa). For each region the amount of material under the peaks eluted was calculated with Unicorn software (GE Healthcare). From this information the decrease in monomeric  $\alpha$ -lactalbumin and the increase in oligomers was estimated, arbitrarily assuming similar weight absorption coefficients for monomeric and oligomeric forms. The increase in oligomer fractions was calculated to obtain the size distribution of reaction products.

#### ***Circular Dichroism (CD)***

CD spectra were recorded at 37 °C on a J-715 (Jasco, Tokyo, Japan) CD spectrometer. Near UV CD spectra (250–350 nm, slit width 2 nm) were determined as an average over 20 repetitive scans in 10 mm quartz cuvettes with protein concentrations of 1 mg/mL. All spectra were corrected for reference spectra recorded in the absence of protein.

#### ***Tryptophan fluorescence***

Protein samples were diluted in 0.1-100 mM ammonium acetate buffer pH 6.8 to a final concentration of 0.05 mg/mL and equilibrated for 1 h at 20 or 37 °C. After this incubation, the sample was transferred to a thermostatted 1 ml quartz cell. Tryptophan emission was recorded between 300 and 400 nm upon excitation at 295 nm with slit widths of 5 nm.

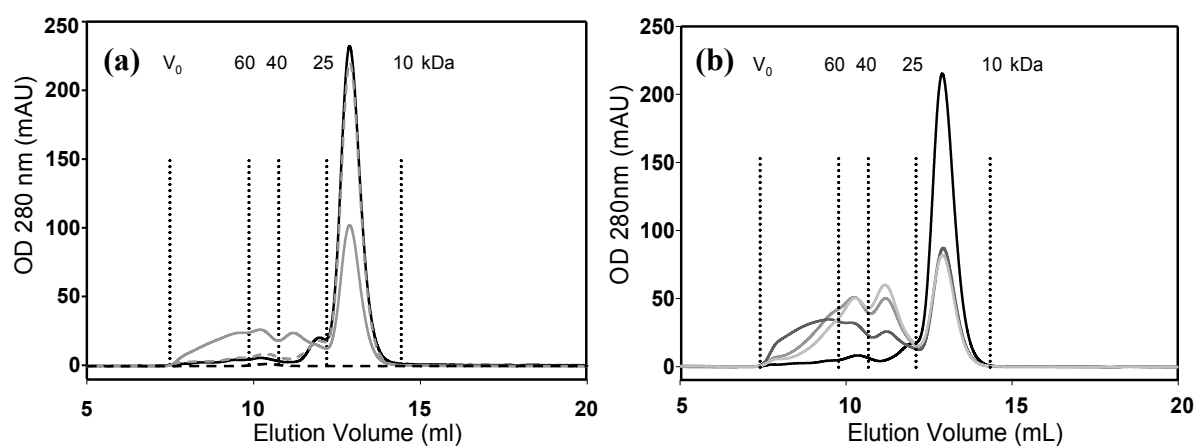
#### ***Differential scanning calorimetry (DSC)***

DSC measurements were performed on a VP-DSC MicroCalorimeter (Microcal Inc., Northhampton, MA, USA). Protein samples (2.0 mg/mL, in 0.1-100 mM ammonium acetate buffer pH 6.8) were heated from 15 to 100 °C with a heating rate of 60 °C/h. All samples were thermostatted and degassed before analysis.

## Results and Discussion

### *Peroxidase-catalyzed cross-linking of apo $\alpha$ -lactalbumin*

Commercial  $\text{Ca}^{2+}$ -depleted  $\alpha$ -lactalbumin exists for at least 80 % in the  $\text{Ca}^{2+}$ -free apo-form, according to the suppliers specification. The influence of pH and ionic strength on the peroxidase-mediated cross-linking of apo  $\alpha$ -lactalbumin was monitored by size-exclusion chromatography (SEC). **Figure 1a** shows a typical size-exclusion chromatogram of untreated apo  $\alpha$ -lactalbumin, and apo  $\alpha$ -lactalbumin cross-linked with 1 mM hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in 100 mM ammonium acetate buffer, pH 6.8 at 37 °C, either in the absence or presence of  $\text{Ca}^{2+}$ . Apo  $\alpha$ -lactalbumin, cross-linked without added  $\text{Ca}^{2+}$  shows a decrease in monomeric  $\alpha$ -lactalbumin (10-25 kDa) and an increase in oligomers. As reported before (8), binding of  $\text{Ca}^{2+}$ -ions inhibits the peroxidase-catalyzed cross-linking of  $\alpha$ -lactalbumin and almost no cross-linking products are observed. The three major fractions of cross-linked  $\alpha$ -lactalbumin in **Figure 1a** correspond to dimer (25-40 kDa), trimer (40-60 kDa), and higher oligomers (>60 kDa) as determined by SDS-PAGE. The relative yield per fraction, for various reaction conditions at 37 °C, are summarized in **Table I**.



**Figure 1:** Size exclusion chromatograms of: **(a)** Untreated apo  $\alpha$ -lactalbumin (black line), HRP (dashed black line), and apo  $\alpha$ -lactalbumin after peroxidase incubation (grey lines). Apo  $\alpha$ -lactalbumin in 100 mM ammonium acetate pH 6.8 was incubated at 37 °C with 1 mM  $\text{H}_2\text{O}_2$  in the absence (solid line) and presence (dashed line) of 20 mM  $\text{CaCl}_2$ . **(b)** Apo  $\alpha$ -lactalbumin cross-linked by peroxidase treatment at different ionic strength (grey lines). Darker grey is indicating a higher ionic strength (respectively 0.1 mM, 10 mM and 100 mM ammonium acetate pH 6.8). Untreated  $\alpha$ -lactalbumin (black line).

**Table I:** Quantitative size distribution of  $\alpha$ -lactalbumin products present in the reaction mixture at different ionic strength and pH at 37 °C.

incubation conditions	integration section (kDa) <sup>a</sup>			
	10-25	25-40	40-60	>60
pH 6.8				
reaction blank	86	7	4	3
100 mM NH <sub>4</sub> Ac + 20 mM CaCl <sub>2</sub>	83	8 (32) <sup>b</sup>	4 (17)	5 (51)
100 mM NH <sub>4</sub> Ac	38	14 (15)	16 (25)	32 (60)
10 mM NH <sub>4</sub> Ac	35	22 (29)	22 (36)	22 (35)
0.1 mM NH <sub>4</sub> Ac	34	26 (37)	23 (38)	16 (25)
pH 5.9				
100 mM NH <sub>4</sub> Ac	39	13 (14)	13 (21)	34 (66)
10 mM NH <sub>4</sub> Ac	33	14 (13)	15 (22)	38 (65)
0.1 mM NH <sub>4</sub> Ac	31	15 (14)	17 (25)	37 (61)

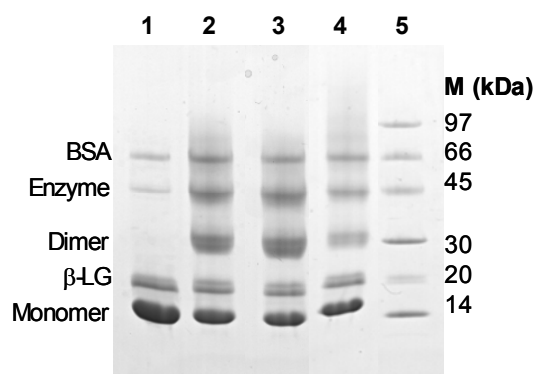
<sup>a</sup> The relative protein absorbance in each size range, given as percentage of the total absorbance at 280 nm, as measured by SEC. <sup>b</sup> Size distribution of oligomers.

At low ionic strength and pH 6.8 relatively more dimers and trimers are formed, whereas at a high ionic strength at pH 6.8 more higher oligomers are formed (**Figure 1b**). At all ionic strengths and pH 5.9 the yield of cross-linked product is comparable to pH 6.8, but at pH 5.9 more higher oligomeric  $\alpha$ -lactalbumin is formed (>60 kDa, **Table I**). The size distribution for each ionic strength does not show a clear difference at pH 5.9. The same reactions are performed at 20 °C as Griko and coworkers (14) and this research (**Figure 3**) showed a decrease in disruption of tertiary structure at lower temperatures. At 20 °C the cross-linking shows a lower total conversion of monomeric  $\alpha$ -lactalbumin (**Table II**). A low ionic strength causes a small increase in monomer conversion as compared to the high ionic strength. At 20 °C and 37 °C, similar observations in size distribution correlated to ionic strength are observed at pH 6.8. To verify that the oligomers observed are formed by covalent bonds and not by non-covalent aggregation, the samples were studied by SDS-PAGE under dissociating and reducing conditions. **Figure 2** shows dimers and trimers of  $\alpha$ -lactalbumin with a decreasing intensity under high ionic strength reaction conditions. This confirms that the aggregates observed in SEC are a result of covalent bonds. With SEC under reducing conditions the conversion of monomeric  $\alpha$ -lactalbumin was comparable to the native SEC (not shown).

**Table II:** Quantitative size distribution of  $\alpha$ -lactalbumin products present in the reaction mixture at different ionic strength and pH at 20 °C.

incubation conditions		integration section (kDa) <sup>a</sup>			
		10-25	25-40	40-60	>60
pH 6.8					
	100 mM NH <sub>4</sub> Ac	79	8 (19) <sup>b</sup>	5 (23)	8 (58)
	10 mM NH <sub>4</sub> Ac	72	14 (49)	8 (30)	7 (21)
	0.1 mM NH <sub>4</sub> Ac	70	17 (63)	8 (26)	5 (11)
pH 5.9					
	100 mM NH <sub>4</sub> Ac	72	12 (36)	6 (19)	10 (45)
	10 mM NH <sub>4</sub> Ac	56	20 (45)	20 (24)	13 (32)
	0.1 mM NH <sub>4</sub> Ac	52	14 (21)	13 (27)	21 (51)

<sup>a</sup> The relative protein absorbance in each size range, given as percentage of the total absorbance at 280 nm, as measured by SEC. <sup>b</sup> Size distribution of oligomers.

**Figure 2:** SDS-PAGE of apo  $\alpha$ -lactalbumin cross-linked by peroxidase treatment at different ionic strength. 1: Blank reaction without H<sub>2</sub>O<sub>2</sub>. 2, 3 and 4: Reactions with 1 mM H<sub>2</sub>O<sub>2</sub> in 10, 0.1, and 100 mM ammonium acetate buffer pH 6.8. 5: Marker proteins with their molecular masses indicated at the right. Enzyme = peroxidase.  $\beta$ -LG =  $\beta$ -lactoglobulin.

### Changes in enzyme performance

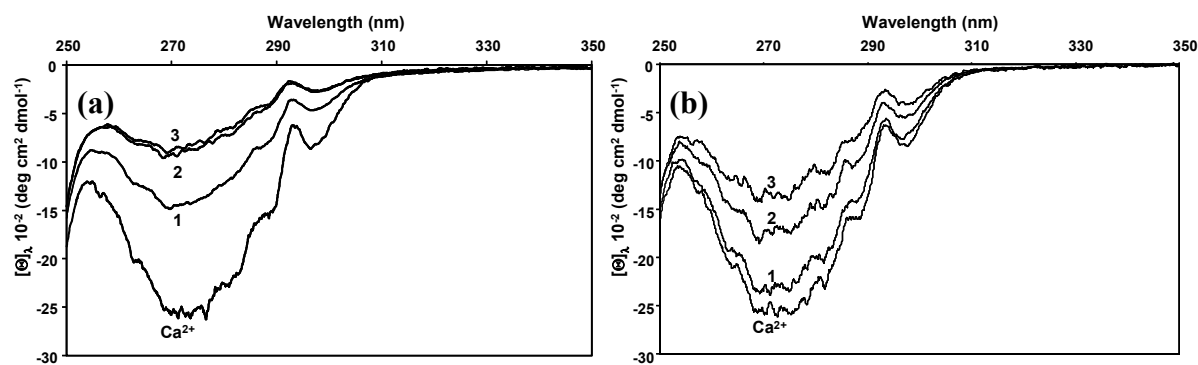
The protein conformation of apo  $\alpha$ -lactalbumin might be the main cause of different cross-linking behavior with various buffer conditions, but the peroxidase catalyst might also behave differently (17). The enzyme activity was tested using a spectrophotometric assay with ABTS performed, using the same reaction conditions. Horseradish peroxidase (HRP) shows a 4 to 5 times higher oxidizing activity with ABTS at pH 5.9 compared to pH 6.8 and a 1.0 to 1.1 times higher activity at high ionic strength compared to low ionic strength (no further data shown). Especially at pH 5.9, the higher activity might be an explanation for the preferred formation of  $\alpha$ -lactalbumin higher oligomers (Table I). However, with more higher oligomers formed an increased conversion of monomeric  $\alpha$ -lactalbumin is also expected. Since at all conditions the consumption of  $\alpha$ -lactalbumin was similar, the reactivity of



monomeric  $\alpha$ -lactalbumin is considered not to be a limiting factor. Most likely, protein conformational changes at the incubation conditions are causing different cross-linking products. Therefore, the conformational state of  $\alpha$ -lactalbumin, at the cross-linking conditions, is investigated.

### Conformational state of $\alpha$ -lactalbumin

A change in the accessibility of tyrosine residues within the  $\alpha$ -lactalbumin molecule likely causes a difference in cross-linking pattern. To understand this, we analyzed the near-UV circular dichroism (CD) and tryptophan fluorescence spectra of apo and holo  $\alpha$ -lactalbumin at different salt concentrations.

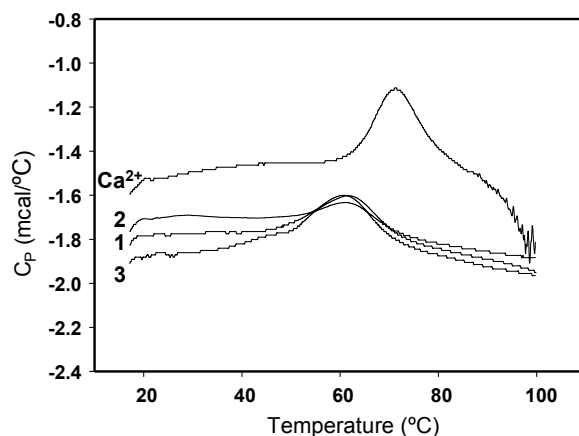


**Figure 3:** Near-UV CD spectra of apo  $\alpha$ -lactalbumin in solutions of different ionic strength at 37 °C (a) and 20 °C (b). 1: 100 mM, 2: 10 mM, 3: 0.1 mM ammonium acetate buffer pH 6.8.  $\text{Ca}^{2+}$ : apo  $\alpha$ -lactalbumin in 100 mM ammonium acetate buffer pH 6.8 with 20 mM  $\text{CaCl}_2$ .

**Figure 3** shows the near-UV CD spectra for untreated  $\alpha$ -lactalbumin at 20 and 37 °C at different ionic strengths at pH 6.8. In the absence of  $\text{Ca}^{2+}$ -ions, the ellipticity around 270 nm is weaker at low ionic strength, indicating a conformational change around the tyrosine and phenylalanine residues (18). The decreasing magnitude of ellipticity resembles the gradual chemical denaturation of  $\alpha$ -lactalbumin by guanidium hydrochloride (19), indicating  $\alpha$ -lactalbumin to be slightly unfolded at low ionic strength. Furthermore, the ellipticity is lower at 37 °C and the effects of ionic strength on ellipticity are less profound compared to 20 °C. Similar temperature and ionic strength effects on the ellipticity of  $\alpha$ -lactalbumin have been reported before (14).

Lowering the ionic strength (both at 20 and 37 °C) does not change the wavelength of the tryptophan fluorescence emission maximum of apo  $\alpha$ -lactalbumin (not shown). Furthermore, no change is observed in the melting temperature of  $\alpha$ -lactalbumin (**Figure 4**). A small

change in enthalpy is observed, but this is negligible compared to the holo and apo  $\alpha$ -lactalbumin enthalpy difference. Together with the unaltered far-UV CD properties of apo  $\alpha$ -lactalbumin at different ionic strengths and temperatures (20, 21), this indicates that apo  $\alpha$ -lactalbumin retains much of its native structure at low ionic strength.

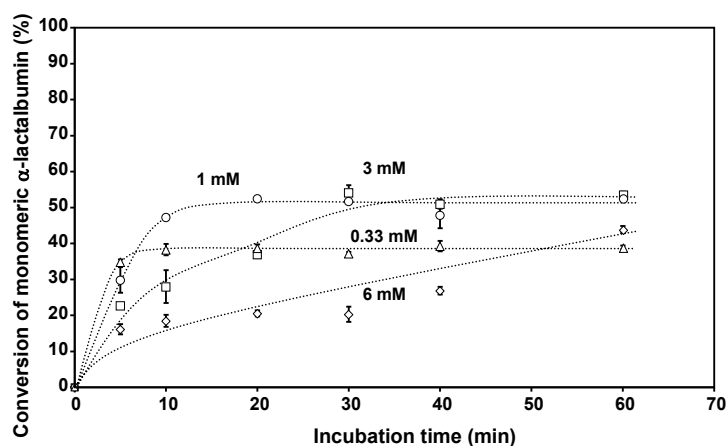


**Figure 4:** DSC-profiles of the temperature-dependent unfolding of  $\alpha$ -lactalbumin at different ionic strength in the absence and presence of  $\text{CaCl}_2$ . Apo  $\alpha$ -lactalbumin in (1) 100 mM; (2) 10 mM and (3) 0.1 mM ammonium acetate buffer pH 6.8;  $\text{Ca}^{2+}$ : apo  $\alpha$ -lactalbumin in 100 mM ammonium acetate buffer pH 6.8 with 20 mM  $\text{CaCl}_2$ .

The tryptophans do not become exposed, but the near-UV CD data indicate some change in the orientation of tyrosines (18). When the orientation of the tyrosines shows minor ellipticity changes (100 mM at 20 °C; **Figure 3**), no cross-linking can occur. When major ellipticity changes occur (0.1 mM at 20 °C), cross-linking is observed. Local conformational changes of  $\alpha$ -lactalbumin at low ionic strength might, therefore, increase the tyrosine availability for oxidation and cross-linking. With the increased availability, cross-links can be formed easier and multiple cross-links per molecule are formed. Quantitative analysis of dityrosine formation, via the absorption increase at 318 nm (6), supports this hypothesis: At high ionic strength more higher oligomers are formed than at low ionic strength, but no increase in the extent of dityrosine formation is observed. This suggests that the dimers and trimers formed at low ionic strength (cf. **Figure 1b**) contain a higher number of cross-links per molecule than the oligomers formed at high ionic strength. Interestingly, the same conversion speed of monomeric  $\alpha$ -lactalbumin is observed in 10 and 100 mM ammonium acetate (results not shown).

### Role of cosubstrate

The concentration of the cosubstrate  $\text{H}_2\text{O}_2$  is critical for the peroxidase-catalyzed protein cross-linking process. Besides from increasing the reaction rate, an increase in the amount of  $\text{H}_2\text{O}_2$  can inhibit the mode of action of HRP (22). To study this, the peroxidase-mediated cross-linking of apo  $\alpha$ -lactalbumin at 100 mM ammonium acetate buffer pH 6.8 with different  $\text{H}_2\text{O}_2$  concentrations was monitored by SEC. Since there are no changes in ionic strength and pH, the conversion of monomeric apo  $\alpha$ -lactalbumin was taken as a measure of cross-linking. **Figure 5** shows the time dependence of reacted monomer with 0.33, 1, 3, and 6 mM  $\text{H}_2\text{O}_2$  in 100 mM ammonium acetate pH 6.8. With 0.33 mM  $\text{H}_2\text{O}_2$  the highest initial conversion rate is observed. The highest degree of conversion (50-60 %) of monomer is observed at 1 and 3 mM  $\text{H}_2\text{O}_2$ . All the conditions result in the same size distribution of oligomers (**Table III**). At higher ( $\geq 12$  mM)  $\text{H}_2\text{O}_2$  concentrations, a lower conversion was obtained. This is likely due to (ir)reversible enzyme inhibition by excess  $\text{H}_2\text{O}_2$  (22) as was also observed with the ABTS assay (not shown).



**Figure 5:** Time-dependent conversion of fraction monomeric  $\alpha$ -lactalbumin upon treatment with peroxidase and various  $\text{H}_2\text{O}_2$  concentrations in 100 mM ammonium acetate pH 6.8. Indicated is the molarity of  $\text{H}_2\text{O}_2$  after addition.

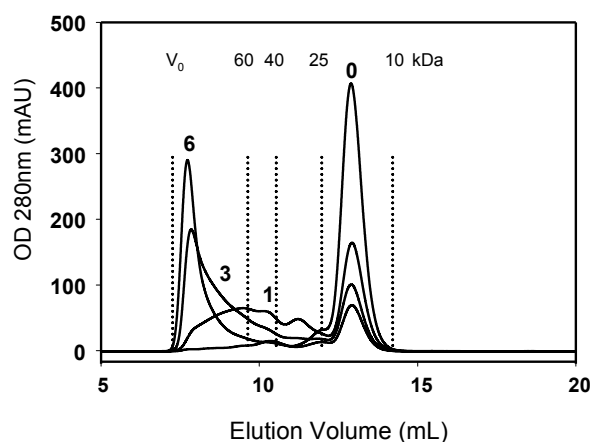
When the amount of  $\text{H}_2\text{O}_2$ , equivalent to 6 mM, was added in aliquots of 2  $\mu\text{L}$  (1 mM) at 10-min intervals, apo  $\alpha$ -lactalbumin was almost completely (80 %) converted into higher oligomers (**Figure 6** and **Table III**). The high conversion of monomeric apo  $\alpha$ -lactalbumin upon multiple additions of  $\text{H}_2\text{O}_2$  is independent of ionic strength and a total conversion of monomeric  $\alpha$ -lactalbumin of 80 % is reached (**Table III**). However, at low ionic strength smaller oligomers are formed, compared to high ionic strength, even after multiple additions of  $\text{H}_2\text{O}_2$ . When EDTA is added to scavenge the residual  $\text{Ca}^{2+}$ -ions, conversion of monomers is increased to at least 90 %, and no changes in size distribution are observed. The addition of

H<sub>2</sub>O<sub>2</sub> in aliquots shows the great potential of modulating the H<sub>2</sub>O<sub>2</sub> concentration to increase monomer conversion.

**Table III:** Quantitative size distribution of  $\alpha$ -lactalbumin products present in the reaction mixture at different ionic strength and H<sub>2</sub>O<sub>2</sub> concentrations at 37 °C.

buffer properties <sup>a</sup>	H <sub>2</sub> O <sub>2</sub> conc	integration section (kDa) <sup>b</sup>						
		10-25		25-40		40-60		>60
Single Addition; <i>n</i> mM H <sub>2</sub> O <sub>2</sub>								
100 mM	0.33 mM	45	20	(31) <sup>c</sup>	18	(35)	18	(34)
	1 mM	33	17	(20)	20	(30)	30	(50)
	3 mM	29	16	(16)	20	(29)	35	(55)
	6 mM	35	18	(22)	20	(31)	27	(46)
	12 mM	66	16	(46)	10	(32)	8	(23)
Multiple Additions; 1 mM H <sub>2</sub> O <sub>2</sub>								
100 mM	1x	38	14	(15)	16	(25)	32	(60)
	3x	23	7	(0)	9	(8)	61	(92)
	6x	19	4	(0)	4	(1)	73	(99)
10 mM	1x	35	22	(29)	22	(36)	22	(35)
	3x	18	10	(5)	18	(22)	53	(73)
	6x	15	6	(0)	11	(10)	68	(91)
0.1 mM	1x	34	26	(37)	23	(38)	16	(25)
	3x	18	15	(12)	25	(31)	42	(57)
	6x	15	9	(3)	17	(19)	60	(79)
100 mM + 20 mM EDTA	3x	10	7	(1)	11	(9)	72	(90)
	6x	6	6	(0)	9	(6)	79	(95)

<sup>a</sup> Concentration ammonium acetate. <sup>b</sup> Relative protein absorbance in each size range, given as percentage of the total absorbance at 280 nm, as measured by SEC. <sup>c</sup> Size distribution of oligomers.



**Figure 6:** Peroxidase-mediated cross-linking of apo  $\alpha$ -lactalbumin in 100 mM ammonium acetate pH 6.8 with multiple additions of 1 mM H<sub>2</sub>O<sub>2</sub> (number of additions indicated) as measured by SEC.

## Conclusions

Peroxidase-mediated cross-linking of apo  $\alpha$ -lactalbumin yields a wide range of oligomer products. The size distribution of the cross-linked products can be directed by changing the ionic strength and pH. With the obtained knowledge it is possible to direct the peroxidase-mediated cross-linking of  $\alpha$ -lactalbumin to form biomacromolecules with a different number of cross-links and altered properties.

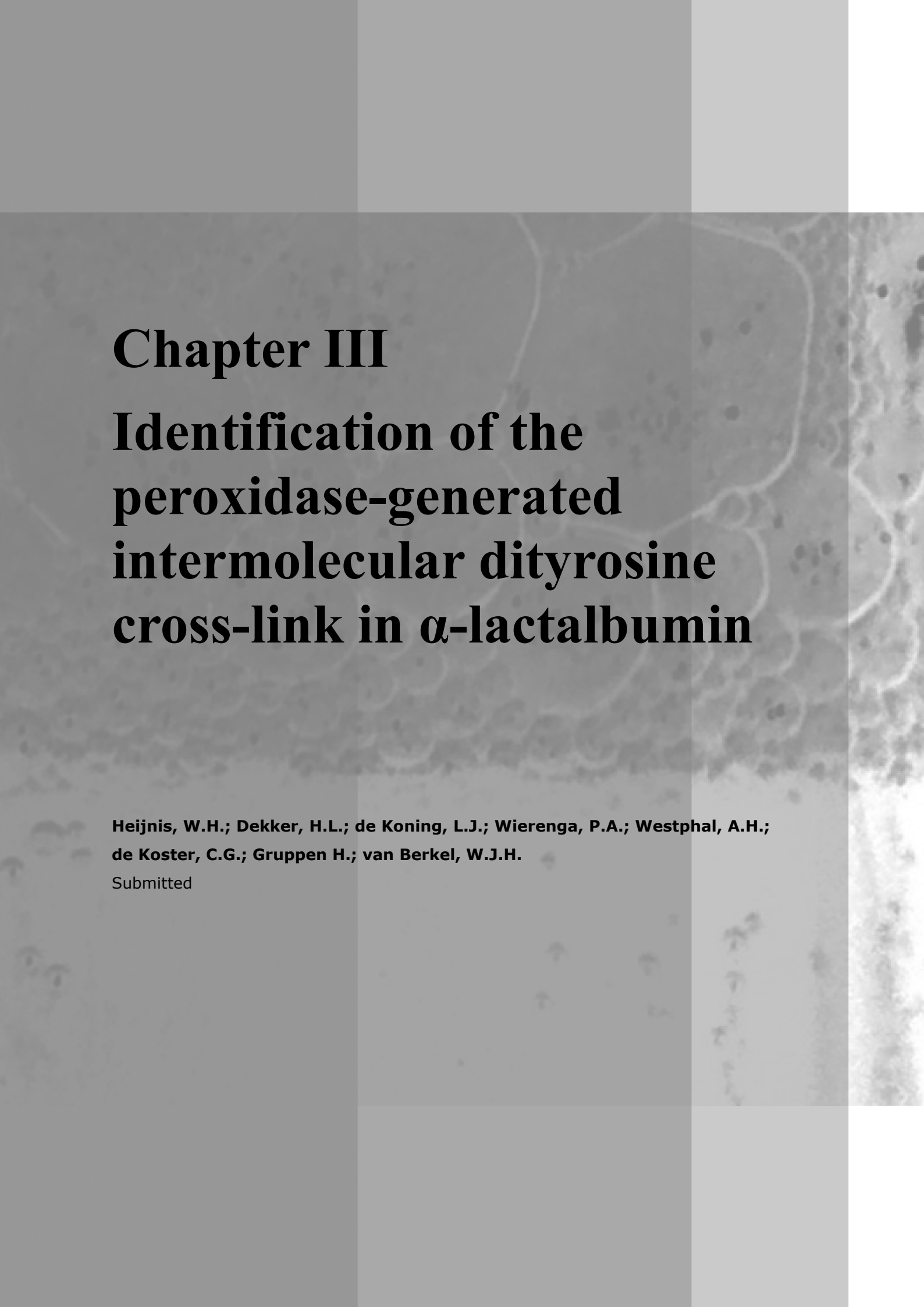
## Abbreviations used

HRP, horseradish peroxidase; EDTA, ethylenediaminetetraacetic acid; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; CD, circular dichroism; V<sub>0</sub>, void volume; DSC, differential scanning calorimetry;  $\beta$ -LG,  $\beta$ -lactoglobulin; BSA, bovine serum albumin.

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A grayscale microscopic image of cells, likely showing a network of thin, branching structures (possibly neurons or fibroblasts) against a darker, textured background. The image is used as a background for the title page.

# **Chapter III**

## **Identification of the peroxidase-generated intermolecular dityrosine cross-link in $\alpha$ -lactalbumin**

**Heijnis, W.H.; Dekker, H.L.; de Koning, L.J.; Wierenga, P.A.; Westphal, A.H.;  
de Koster, C.G.; Gruppen H.; van Berkel, W.J.H.**

Submitted

### **Abstract**

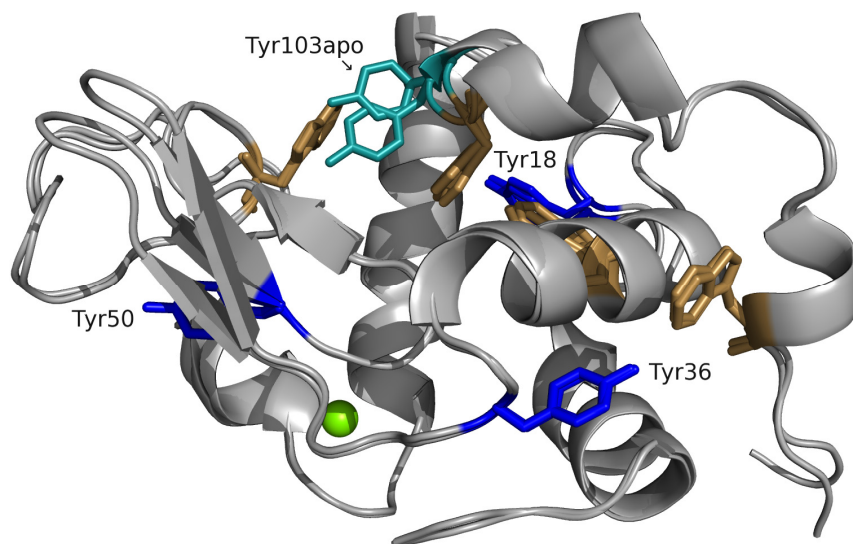
The peroxidase-mediated oxidation of calcium-depleted bovine  $\alpha$ -lactalbumin generates a mixture of covalently bound protein oligomers with interesting foaming properties. Here, we separated the initially formed covalent  $\alpha$ -lactalbumin dimer and studied its mode of cross-linking. Liquid Chromatography-Fourier Transform Mass Spectrometry (LC-FTMS) of proteolytic digests revealed the unambiguous identification of a peroxidase-catalyzed covalent link between Tyr18 and Tyr50. This shows that, although the radical reaction is often regarded as a random reaction, the initial product formation is specific. Protein structural modeling indicates that the conjugation reaction between these tyrosines is sterically favored and involves initial non-covalent protein complex formation through charge compensation, supporting an intermolecular cross-link. The identification of the Tyr18-Tyr50 cross-link supports the view that the peroxidase-mediated oxidation of apo  $\alpha$ -lactalbumin is a sequential process, involving the formation of linear trimers and higher-order oligomers.



## Introduction

Enzymatic cross-linking is an emerging tool to change the functionality of food proteins (1-4). One such cross-linking procedure involves the peroxidase-mediated one-electron oxidation of tyrosine residues with the subsequent generation of (iso)dityrosine bonds (5-7).

Recently, it was demonstrated that the holo form of the regulatory bovine whey protein  $\alpha$ -lactalbumin is insensitive to peroxidase-catalyzed cross-linking, whereas the apo form was cross-linked (8). The cross-linking of the apo form was quantified in-line with UV-detection of the dityrosine bond. Furthermore, from studying the reaction as a function of pH and ionic strength, it was established that the size distribution of the cross-linked  $\alpha$ -lactalbumin products can be directed towards the protein oligomers desired (9). Extensive cross-linked  $\alpha$ -lactalbumin resulted in the formation conjugates with anti-foam properties (10).



**Figure 1:** Superimposition of the crystal structures of bovine holo  $\alpha$ -lactalbumin (PDB accession code 1f6s) and bovine apo  $\alpha$ -lactalbumin (PDB accession code 1f6r) (14). Tyr18, Tyr36 and Tyr50 are indicated in blue and Tyr103 is indicated in cyan. Tryptophan residues are shown in gold and the calcium ion in holo  $\alpha$ -lactalbumin is depicted as a green sphere.

Bovine  $\alpha$ -lactalbumin contains four tyrosine residues with different accessibility and reactivity (**Figure 1**). In holo  $\alpha$ -lactalbumin Tyr18 is sufficiently exposed to be photosensitized by chemically induced dynamic nuclear polarization (11). In the flexible acid molten globule state also Tyr36 and Tyr50 were found to become available for photosensitizing (12). NMR measurements of the gradually denaturing acid molten globule showed an increase in availability for tyrosine residues: Tyr36 becomes first available,

followed by Tyr50, Tyr18, and lastly Tyr103 (13). The crystal structure of apo  $\alpha$ -lactalbumin reveals a more open structure at the location of Tyr103 in the  $\alpha$ -domain compared to the structure of holo  $\alpha$ -lactalbumin (14). Thus, it is not obvious which tyrosine residues are exposed and involved in the peroxidase-mediated dityrosine cross-linking process of apo  $\alpha$ -lactalbumin.

To get a better understanding of the mechanism of peroxidase-catalyzed protein cross-linking we have addressed in the present work the mode of cross-linking between  $\alpha$ -lactalbumin monomers. Until now the oxidative formation of protein dityrosine cross-links has mainly been demonstrated by amino acid analysis of complete protein hydrolysates (15, 16) or separated peptides (17), or by indirect evidence from site-directed mutagenesis (18, 19). For the reaction of sperm whale myoglobin with hydrogen peroxide it was established that the radical transfer from the heme cofactor to the protein results in the formation of intra- and intermolecular isodityrosine and dityrosine bonds (20). Here, we isolated a covalent  $\alpha$ -lactalbumin dimer fraction and analyzed its proteolytic digests by Liquid Chromatography-Fourier Transform Mass Spectrometry (LC-FTMS). The mass spectrometric data were subsequently screened for dipeptides allowing the unambiguous identification of the covalent link between Tyr18 and Tyr50. The results are discussed in relation to the structural properties of  $\alpha$ -lactalbumin.

## Materials and Methods

### *Materials*

Apo bovine  $\alpha$ -lactalbumin was obtained from BioPURE, Davisco Foods International Inc., Le Sueur, MN, USA. According to the manufacturer, the material contains 95 % (w/w) protein (of which 90 % (w/w)  $\alpha$ -lactalbumin) and 0.55 % (w/w) calcium. Besides  $\alpha$ -lactalbumin, the commercial preparation was found to contain traces of bovine serum albumin (BSA) and  $\beta$ -lactoglobulin ( $\beta$ -LG) (8). Horseradish peroxidase (HRP) type VI-a (P6782) was obtained from Sigma (Sigma Chemical CO, St Louis, MO, USA). Trypsin (Trypsin Gold, MS grade) was from Promega (Madison, WI, USA) and endoproteinase Glu-C (Protease V8 sequencing grade) was from Roche Biochemical (Basel, Switzerland). The surfactant *RapiGest* was from Waters (Milford, MA, USA). All other (bio)chemicals were of analytical grade and purchased from Sigma or Merck (Darmstadt, Germany).

### *Apo $\alpha$ -lactalbumin cross-linking*

Apo  $\alpha$ -lactalbumin (10 mg dissolved in 1 mL 10 mM ammonium acetate pH 6.8) was incubated for 60 min at 37 °C in the presence of HRP (50  $\mu$ L, 10 mg/mL) and hydrogen peroxide (2  $\mu$ L, 0.5 M). For preparative purposes the cross-linking reaction was repeated 20 times and the products obtained were combined in one stock. The cross-linked proteins were stored at -20 °C and thawed before fractionation.

### ***Six-exclusion chromatography (SEC)***

Cross-linked protein samples (20  $\mu$ L, 5 mg/mL) in 0.1 M ammonium acetate, pH 6.8 were applied to a Superdex 75 10/300 GL column connected to an Äkta Purifier system (GE Healthcare, Uppsala, Sweden) at room temperature. The column was equilibrated and eluted with 0.1 M ammonium acetate, pH 6.8 at a flow rate of 0.9 mL/min. The eluate was monitored at 280 nm. Calibration of the column was performed with a low molecular weight SEC calibration kit (GE Healthcare). The kit contained blue dextran (2000 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

### ***Preparative anion exchange chromatography (AEC)***

Cross-linked apo  $\alpha$ -lactalbumin was fractionated on a preparative scale (4 x 50 mg) using a 6 mL Resource Q column running at 6 mL/min in an Äkta Purifier system (GE Healthcare). The column was equilibrated for 5 min with 20 mM Tris-HCl, pH 7.4. After sample injection (5 mL, 10 mg/mL) the column was washed for 3 min followed by a 20 min linear gradient from 0 to 0.5 M NaCl in 20 mM Tris-HCl, pH 7.4. The column was regenerated with 1.5 M NaCl in 20 mM Tris-HCl, pH 7.4 for 5 min followed by a 5 min re-equilibration of 20 mM Tris-HCl, pH 7.4. The eluate was monitored at 280 and 318 nm and 0.5 mL fractions were collected. Fractions were pooled according to SDS-PAGE and fractions containing dimers and oligomers were dialyzed against water and lyophilized. This resulted in a yield of 30 mg of the dimer pool, which was stored at -20 °C and thawed before digestion.

### ***Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)***

The peroxidase-catalyzed formation of covalently linked apo  $\alpha$ -lactalbumin oligomers was analyzed with SDS-PAGE using a Phast-system (GE Healthcare) according to the supplier's instructions. PhastGels Gradient 8-25 (GE Healthcare) were used. Before application, protein samples (5 mg/mL) were treated with  $\beta$ -mercapthoethanol (1.25 % (v/v)) and heated for 5 min at 100 °C in the presence of SDS (1.25 % (w/v)). Proteins were stained with Coomassie Brilliant Blue and a protein molecular weight marker (article 17-0446-01; GE Healthcare) was used for calibration.

### ***Digestion of $\alpha$ -lactalbumin monomer and dimer***

Stock solutions (1 – 10 mg/mL in water) were made of the commercial apo  $\alpha$ -lactalbumin material and the purified apo  $\alpha$ -lactalbumin monomer and dimer fractions after the cross-link reaction. Both a double digestion was done either starting with trypsin or endoproteinase Glu-C (Glu-C) in the presence of 0.1 % *RapiGest* (a surfactant which can promote digestion and is compatible with mass spectrometry analysis) or a single digestion with Glu-C with and without 0.1 % *RapiGest*.

The samples were diluted between 20 and 100  $\mu$ g of protein with 0.1 M  $\text{NH}_4\text{HCO}_3$  in a volume of 50  $\mu$ L. After the addition of 5  $\mu$ L *RapiGest* from a 1 % stock and 5  $\mu$ L of 100 mM dithiothreitol reduction was started for 1 h at 55 °C. After cooling alkylation was performed by adding 6  $\mu$ L 0.3 M iodoacetamide allowing reaction for 45 min at room temperature.

For the double digestion the samples were divided in a 50/50 (v/v) ratio split. With one part the digestion was started by trypsin with a 1:50 w/w (protease/protein) ratio at 37 °C overnight and with the other part by Glu-C

with the same w/w ratio at room temperature overnight. The next day both samples were again divided in a 50/50 ratio split and one part of the trypsin and Glu-C digestion was boiled for 10 min in a water bath and the other half was acidified with trifluoroacetic acid (TFA) to a final concentration of 1 % (v/v). The second digestion was done with the cooled down boiled samples with a w/w ratio of 1:25 trypsin (37 °C) or Glu-C (room temperature) for 6 h. The reaction was stopped by adding TFA to a final 1 % (v/v) concentration. All samples were depleted from the *RapiGest* following the instruction of Waters and were collected after desalting with a 80 µg Omix C18 tip (Varian) in 20 µL 50 % (v/v) aqueous acetonitrile containing 0.1 % (v/v) TFA. So, for each sample we had 4 different digestions: Trypsin, Glu-C, trypsin followed by Glu-C and Glu-C followed by trypsin.

The single digestion with Glu-C with and without *RapiGest* was done with the same digestion conditions and purification method as described above.

### ***Mass spectrometric analyses***

Mass data were acquired using an ApexQ Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 7T magnet and a CombiSource™ coupled to an Ultimate 3000 (Dionex, Sunnyvale, CA, USA) HPLC system with a 100 µm ID, 150 mm monolithic reverse phase column (Onyx C18, Phenomenex, Torrance, CA, USA).

Samples with up to 1-2 pmol of digest were injected as a 3 µL 0.1 % (v/v) TFA aqueous solutions and directly loaded onto the analytical column. Following injection, a linear gradient was applied in 30 min from water containing 0.1 % (v/v) TFA to water containing 0.1 % (v/v) TFA and 40 % (v/v) acetonitrile at a flow rate of 2 µL/min. During elution a chromatogram based on about 600 high resolution ESI FTMS spectra was recorded using a MS duty cycle of about 3 seconds.

LC tandem mass data were recorded by MS data dependent selection of peptide ions in the Q sector followed by collisional fragmentation in the collision hexapole at an argon pressure of about  $5 \cdot 10^{-6}$  mbar and then detection of peptide fragment ions in the FTMS cell. For this, alternating MS and MSMS experimental sequences were executed with duty cycles of 1 and 3 seconds, respectively.

### ***MS data processing and analysis***

The MS data were processed using the Data Analysis 3.4 software program (Bruker Daltonics). In a batch procedure the 600 mass spectra were extracted from the chromatogram and the monoisotopic masses of the peptides were determined using Brukers peak recognition technology SNAP II™. Mass calibration was achieved by selectively extracting and then summing about 4 mass spectra from the chromatogram showing MSMS confirmed unmodified peptides from trypsin + Glu-C digested  $\alpha$ -lactalbumin. With the theoretical masses of these calibrant peptides the summed spectrum was mass calibrated and the resulting calibration parameters were applied to all spectra in the chromatogram. This resulted in a mass calibration of better than 1.5 ppm over the entire chromatogram for all analyses. For each FTMS analysis the resulting array of up to 600 monoisotopic mass lists was exported as a MASCOT generic file (.mgf). Ion abundances in the exported array of monoisotopic mass lists were the sum of abundances of all isotopes over all charge states for each peptide.

The exported mgf file was imported in the CoolToolBox software program which is a major update of the in-house developed VIRTUALMSLAB program (21, 22).

From the imported array of up to 600 monoisotopic mass spectra the CoolToolBox program constructed up to 500 peptide ion chromatograms. For each peptide ion chromatogram the mass and retention time was taken at the apex of the chromatogram profile and the abundance was summed over the ion chromatogram profile. The ultimate LC-MS data processing resulted in a peptide monoisotopic mass list with abundance and LC retention. Peptide assignments of the ion masses were made by matching the processed LC-FTMS data from the peptides trypsin + Glu-C digested  $\alpha$ -lactalbumin within a mass window of 1.5 ppm with the peptide masses in a database obtained from a corresponding *in silico* digestion/chemical modification of a  $\alpha$ -lactalbumin, with  $\beta$ -lactoglobulin and bovine serum albumin (21). The latter two proteins were detected with tandem MS as minor contaminants in the  $\alpha$ -lactalbumin protein used.

Modified and cross-linked peptides were assigned by matching the experimental ion masses with the masses of all *in silico* generated theoretically assumed peptides and cross-linked peptide pairs from chemical modification and dehydrogenative cross-linking between tyrosine residues (21).

The tandem MS data were processed using the Data Analysis 3.4 software program (Bruker Daltonics). The processed MSMS peptide fragmentation data were individually analysed as peptide residue sequences with chemical modification.

### ***Protein modeling***

Three-dimensional models of the crystal structures of holo bovine  $\alpha$ -lactalbumin (PDB accession code 1f6s) and apo bovine  $\alpha$ -lactalbumin (PDB accession code 1f6r) (14) were visualized with PyMol. The charge distribution of the apo  $\alpha$ -lactalbumin monomer was calculated using the program APBS (23).

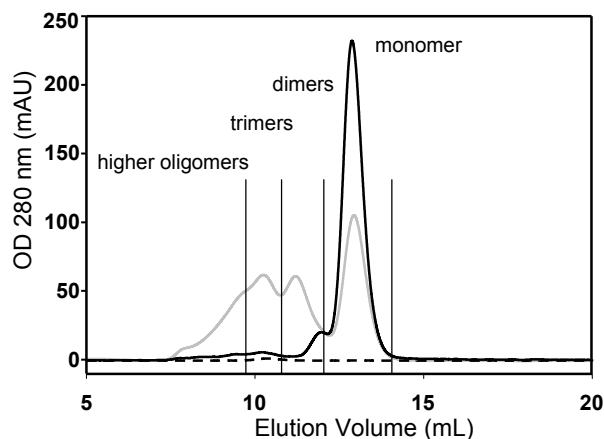
## **Results**

### ***Preparation of dimeric $\alpha$ -lactalbumin fraction***

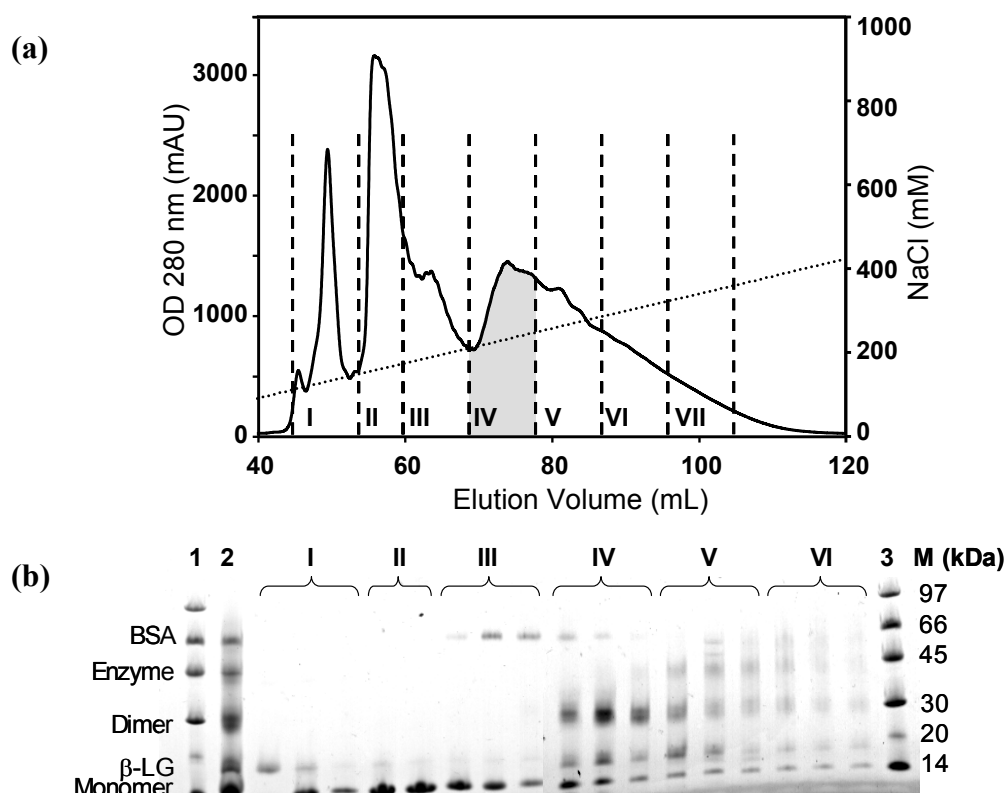
The peroxidase-mediated cross-linking of apo  $\alpha$ -lactalbumin in 10 mM ammonium acetate pH 6.8 results in the apparent molecular mass distribution shown in **Figure 2**. The partition of monomer, dimer, trimer and higher oligomers, as analyzed by SDS-PAGE (no data shown), is about 35-22-22-22 %, taking equal weight-based response factors into account (9).

In order to determine the mode of cross-linking between  $\alpha$ -lactalbumin monomers, peroxidase treated  $\alpha$ -lactalbumin monomers and covalent  $\alpha$ -lactalbumin dimers were fractionated with anion exchange chromatography (**Figure 3a**). As can be seen from the SDS-PAGE pattern in **Figure 3b**, monomeric  $\alpha$ -lactalbumin elutes between 126 mM and 176 mM NaCl in two peaks (Pool I and II). In Pool II a slight increase in OD 318 nm was observed (no data shown), and this might indicate the formation of an intramolecular dityrosine cross-link (8). Most of the  $\alpha$ -lactalbumin dimers elute between 210 mM and 250 mM NaCl (Pool IV). Despite the lack of base-peak separation it was possible to obtain an enriched dimeric  $\alpha$ -lactalbumin preparation (30 mg) in 15 % yield. This preparation contains BSA (2 %),  $\beta$ -LG (29 %) and monomeric  $\alpha$ -lactalbumin (23 %) as determined via protein

standards in SDS-PAGE, but no other  $\alpha$ -lactalbumin oligomers. The protein contaminations present in the dimer pool were, therefore, used as internal standards in further MS experiments.



**Figure 2:** Size exclusion chromatogram of untreated apo  $\alpha$ -lactalbumin (black line) and HRP (dashed black line) in 10 mM ammonium acetate pH 6.8. Apo  $\alpha$ -lactalbumin was incubated at 37 °C for 1 h in the presence of peroxidase and hydrogen peroxide (grey line).



**Figure 3:** Preparative purification of the apo  $\alpha$ -lactalbumin dimer. **(a)** Separation of cross-linked apo  $\alpha$ -lactalbumin by anion exchange chromatography. The Roman numbers indicate the different pools. The grey pool contains the  $\alpha$ -lactalbumin dimer. The dotted line indicates the NaCl gradient. **(b)** SDS-PAGE of the collected pools. 1 & 3: Marker proteins with their molecular mass denoted at the right. 2: cross-linked  $\alpha$ -lactalbumin mixture. BSA = bovine serum albumin. Enzyme = Horseradish peroxidase.  $\beta$ -LG =  $\beta$ -lactoglobulin.

### *Identification of dityrosine cross-Link(s) in dimeric $\alpha$ -lactalbumin*

To identify the mode of cross-linking between  $\alpha$ -lactalbumin monomers, a peptide mapping strategy was developed. Prior to digestion with trypsin and Glu-C, disulfides in treated (pool II; **Figure 3**) and untreated  $\alpha$ -lactalbumin monomer and in the dimer fraction were reduced with dithiothreitol and capped with iodoacetamide. After proteolytic cleavage,  $\alpha$ -lactalbumin peptides were separated by reversed-phase high-performance liquid chromatography (RP-HPLC) and identified by FT-MS.

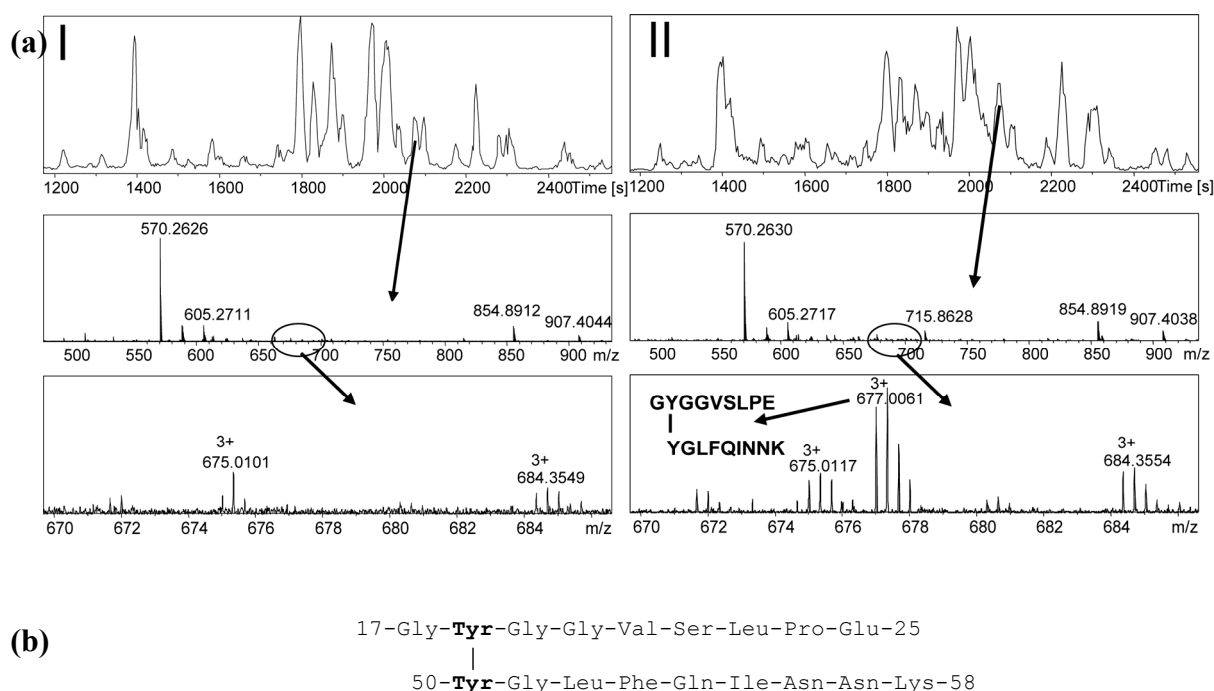
**Table I:**  $\alpha$ -Lactalbumin peptides found in digested  $\alpha$ -lactalbumin monomer and dimer with their retention time on RP-HPLC

Peptide fragment	Retention time (s)	Theoretical mass (Da)	Experimental peptide mass [M + H] <sup>+</sup> (Da)
1-5	926	618.346	618.345
6-10	1344	710.329	710.329
8-10	1000	421.256	421.256
14-16	1028	375.224	375.224
17-25	2003	878.425	878.425
26-49	2027	2774.200	2774.201
50-58	1974	1096.579	1096.578
59-62	1253	606.307	606.307
63-79	1404	2003.818	2003.817
80-93	2443	1699.755	1699.753
95-98	1025	488.308	488.307
99-108	1872	1200.652	1200.652
109-114	930	707.339	707.339
114-121/115-122	1804	1091.519	1091.519

The digestion profiles of both  $\alpha$ -lactalbumin monomers and dimer after the first single digestion showed an increased contamination for the treated monomer: The main difference observed in these digestion profiles are the amounts of BSA and  $\beta$ -lactoglobulin derived peptides (not shown). After a single tryptic or Glu-C digestion the peptides were too large to reveal the location of the dityrosine cross-link. Therefore, a second digestion was performed with the, less contaminated, untreated monomer and dimer. After analysis with RP-FTMS (**Figure 4a**) and applying the CoolToolBox program the peptides were annotated to the different milk proteins. **Table I** lists the theoretical and experimental masses of the  $\alpha$ -lactalbumin peptides found for untreated and dimeric  $\alpha$ -lactalbumin giving a sequence coverage of 96 %. Only the amino acids 11-13 and 94 could not be detected. The presence of

all tyrosine containing peptides can be caused by the presence of residual monomeric  $\alpha$ -lactalbumin. The  $\beta$ -lactoglobulin impurity was used as an internal standard and showed a 100 % coverage with minor miscleavages (no data shown).

Monomeric  $\alpha$ -lactalbumin contains four tyrosines at positions 18, 36, 50 and 103 (24), which in theory can form ten different dityrosine bonds (**Table II**). Out of the possible dipeptides one mass was found that unambiguously matched with a cross-link between Tyr18 and Tyr50 (Table II; **Figure 4**). Due to the low signal it was not possible to perform a MS<sup>n</sup> analysis on this peptide. Extensive searches for the other dityrosine cross-links and for Tyr-Trp, Tyr-Phe, Trp-Trp, Phe-Phe, and Trp-Phe cross-links did not result in any match. This and the identification of even minor modifications in  $\alpha$ -lactalbumin (described below) strengthens the results obtained. In the  $\alpha$ -lactalbumin only a single dityrosine cross-link was formed.



**Figure 4:** (a) Total ion current of untreated (I) and dimeric (II)  $\alpha$ -lactalbumin separated and analyzed on a RP-FTMS system. The mass spectra and extensions thereof at the elution time of the cross-linked peptide is shown for both samples. (b) Sequence of the dityrosine peptide.



**Table II:** Theoretical and experimental masses of  $\alpha$ -lactalbumin dipeptides containing the dityrosine cross-link.

Possible dipeptides	Sequence	Theoretical mass (Da)	Experimental peptide mass $[M + H]^+$ (Da)
Y18-Y18	17-25+17-25	1753.899	ND
Y18-Y36	17-25+26-49	3593.816	ND
Y18-Y50	17-25+50-58	1972.201	1971.981
Y18-Y103	17-25+99-108	2076.356	ND
Y36-Y36	26-49+26-49	5433.733	ND
Y36-Y50	26-49+50-58	3812.118	ND
Y36-Y103	26-49+99-108	3916.273	ND
Y50-Y50	50-58+50-58	2190.503	ND
Y50-Y103	50-58+99-108	2294.658	ND
Y103-Y103	99-108+99-108	2398.812	ND

ND: Not detected

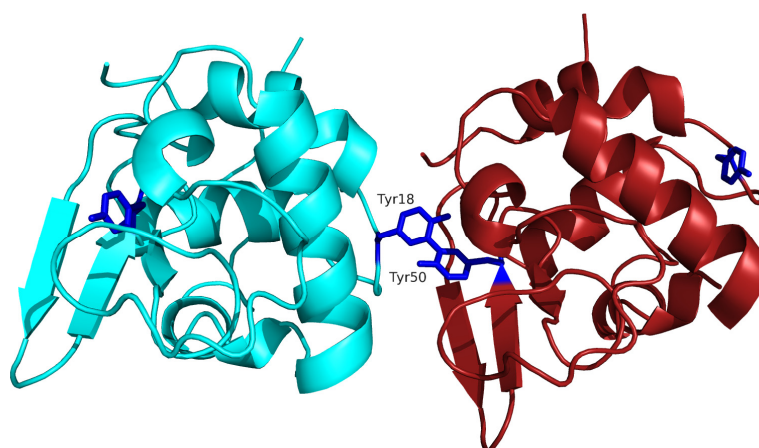
### ***Structural model of the dityrosine cross-linked $\alpha$ -lactalbumin dimer***

The abundance of the Tyr18-Tyr50 containing dipeptide ions and the absence of other possible dityrosine peptide ions in the array of mass spectra indicate that only these two tyrosines are able to come in close vicinity of each other in an apo  $\alpha$ -lactalbumin dimer and form an intermolecular cross-link. To determine if such a cross-link is plausible from a structural point of view, dimer models were made using the crystal structure of apo  $\alpha$ -lactalbumin (1f6r.pdb). All charges on the surfaces of both monomers were labeled, and subsequently one monomer was rotated and translated in space in order to obtain an orientation in which both the distance between the tyrosines was minimal and the charge compensation for both monomers was maximal. Only the Tyr18-Tyr50 combination yields a short inter-tyrosine distance (**Figure 5a**) and a very good charge compensation as shown in **Figure 5b**. It can be seen that the side chain of Tyr18 is in a predominantly positive area (blue), in contrast to that of Tyr50, which resides in a mainly negative region (red) of apo  $\alpha$ -lactalbumin.

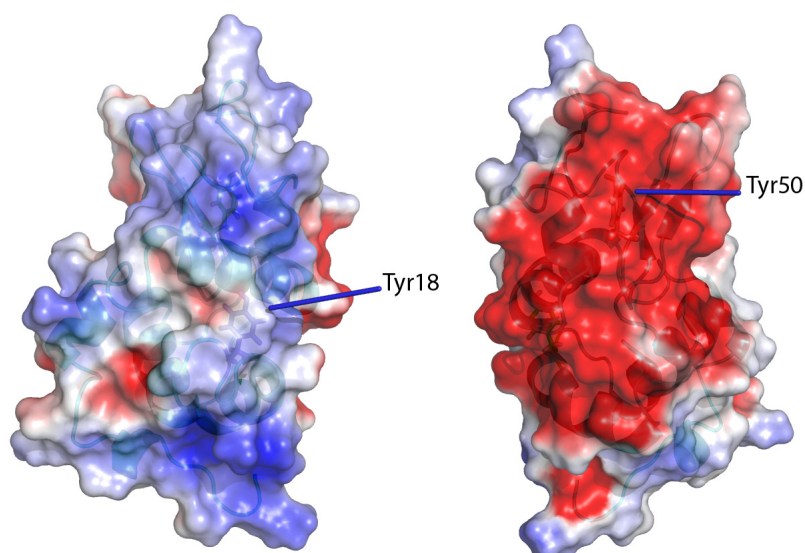
### ***Other modifications in the cross-linked $\alpha$ -lactalbumin dimer fraction***

Besides the one-electron oxidation of tyrosine side chains and the subsequent formation of dityrosine links, HRP might also catalyze the oxidation of other amino acid residues (20, 25). Therefore, the digestion profiles of HRP-treated  $\alpha$ -lactalbumin monomer and cross-linked  $\alpha$ -lactalbumin dimer fractions were analyzed for other modifications, like oxidation, deamidation, reduction, and acetylation. The single methionine (Met90) was found to be

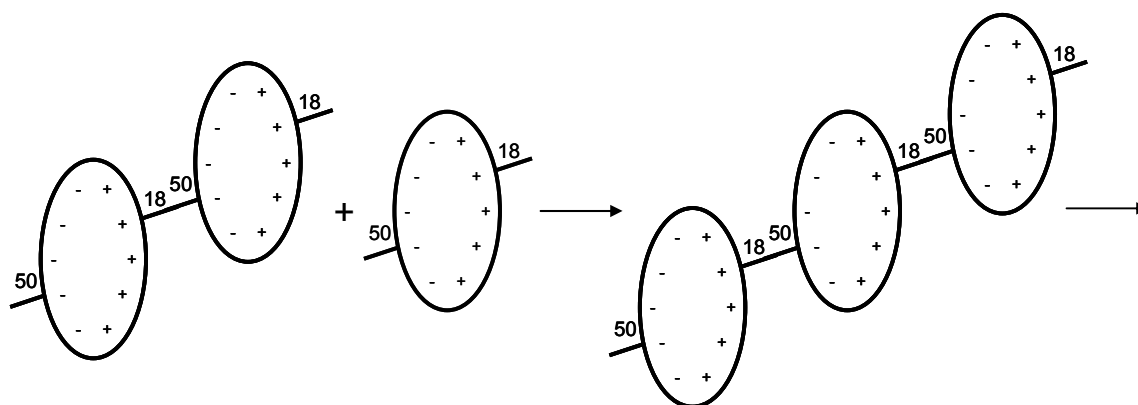
(a)



(b)



**Figure 5:** (a) Model of the apo  $\alpha$ -lactalbumin dimer with the Tyr18 and Tyr50 in close vicinity. (b) Surface charge distribution of the apo  $\alpha$ -lactalbumin monomer. Left: predominantly positive area around Tyr18 (blue) Right: mainly negative region around Tyr50 (red).



**Figure 6:** Model of the covalent apo  $\alpha$ -lactalbumin dimer reacting to higher oligomers.

oxidized for 60 % and for tryptophan, 3.5 % of all residues were dehydrogenated. Besides from the dityrosine link, these were the only  $\alpha$ -lactalbumin modifications found.

## Discussion

This research has identified the tyrosines involved in the peroxidase-catalyzed cross-linking of apo  $\alpha$ -lactalbumin. LC-FTMS of proteolytic digests revealed the unambiguous identification of a covalent link between Tyr18 and Tyr50. Tyr18 is located in the  $\alpha$ -helical domain and Tyr50 in the  $\beta$ -sheet domain (**Figure 1**), making the intramolecular conjugation between these residues rather unlikely. Previously, we showed that holo  $\alpha$ -lactalbumin is not sensitive to HRP-catalyzed cross-linking. This suggests that radical transfer from the peroxidase heme center to Tyr18 and/or Tyr50 is not efficient or that Tyr18 and Tyr50 of separate holo  $\alpha$ -lactalbumin molecules cannot come in close contact. The crystal structure of apo  $\alpha$ -lactalbumin reveals a more open structure at the location of Tyr103 in the  $\alpha$ -domain compared to the structure of holo  $\alpha$ -lactalbumin (**Figure 1**)(14). However, Tyr103 is not involved in the formation of the molecular dityrosine link. The structure determination was performed on crystals grown in high concentrations of ammonium sulfate (14), conditions that are clearly different from our cross-linking experiments. Previous research has shown the importance of ionic strength on the tertiary conformation of  $\alpha$ -lactalbumin (9, 26). Our results show that the reaction of HRP with apo  $\alpha$ -lactalbumin in low ionic strength is a rather specific reaction. Out of the available amino acids only tyrosine and methionine are extensively modified, with one other minor modification: tryptophan. Preliminary experiments show that the covalent  $\alpha$ -lactalbumin dimer displays strong fluorescence at 420 nm, indicative for a dityrosine rather than an isodityrosine bond (20).

The assignment of the Tyr18-Tyr50 cross-link fits with the results obtained from our previous research (8). In that study it was shown that the cross-linking of apo  $\alpha$ -lactalbumin is a consecutive process leading to the ultimate formation of polymers. The initial formation of a Tyr18-Tyr50 cross-linked dimer is in agreement with such sequential process. After formation of the covalent dityrosine bond there is always a spare Tyr18 and Tyr50 left for continuation of the cross-linking reaction. The sequential process is schematically presented in **Figure 6**. The cross-link formation can be described by a clustering and a reaction process. In the clustering process two  $\alpha$ -lactalbumin molecules form a complex through favorable charge interactions. In this complex, Tyr18 and Tyr50 are at about 4 Å distance (**Figure 5a**), which is close enough to form a cross-link in the reaction process. The HRP-mediated

oxidation of both tyrosine residues most likely occurs before complex formation. Thus we conclude that at low ionic strength, the flexible structure of apo  $\alpha$ -lactalbumin and charge-charge interactions are the driving force for the generation of the Tyr18-Tyr50 cross-link.

### Abbreviations used

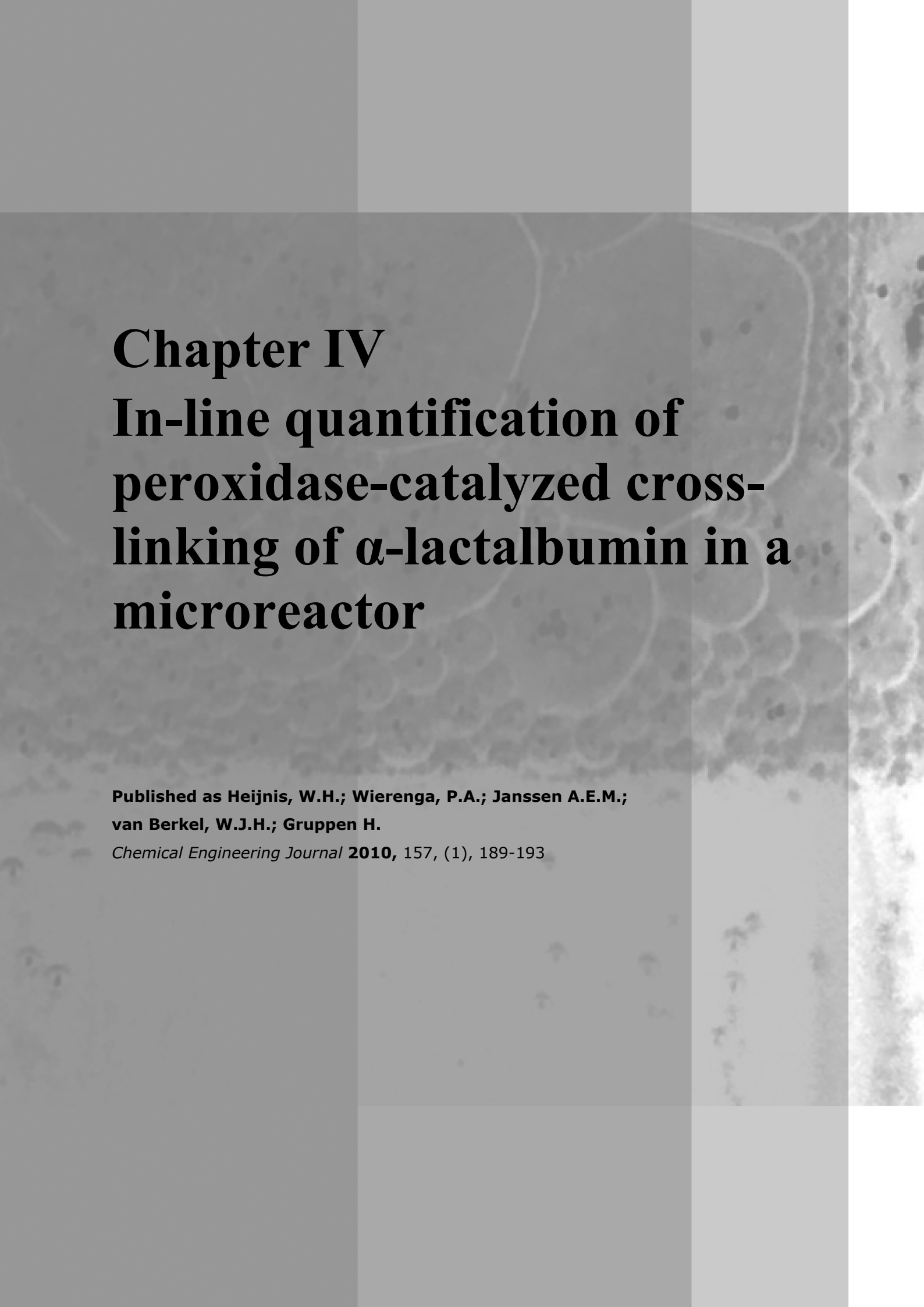
BSA, bovine serum albumin. HRP, horseradish peroxidase; FTMS, Fourier transform mass spectrometry; Glu-C, endoproteinase Glu-C.  $\beta$ -LG,  $\beta$ -lactoglobulin; HPLC, high-performance liquid chromatography, PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate. SEC, size-exclusion chromatography; TFA, trifluoroacetic acid.

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A grayscale microscopic image of cells, likely showing a network of thin, branching structures and some larger, more rounded cells, serving as a background for the text.

# Chapter IV

## In-line quantification of peroxidase-catalyzed cross- linking of $\alpha$ -lactalbumin in a microreactor

**Published as Heijnis, W.H.; Wierenga, P.A.; Janssen A.E.M.;  
van Berkel, W.J.H.; Gruppen H.**

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### **Abstract**

Horseradish peroxidase can induce the oxidative cross-linking of proteins through the radicalization of tyrosine residues and subsequent formation of dityrosine bonds. The dityrosine bond absorbs light at 318 nm which can be used to monitor in-line the peroxidase-catalyzed cross-linking of proteins in a microfluidic system. In this study calcium-depleted  $\alpha$ -lactalbumin is used as model protein. To quantify the progress of the reaction, the absorbance increase at 318 nm was monitored in-line and compared with the amount of reacted monomeric  $\alpha$ -lactalbumin as determined with size-exclusion chromatography (SEC) at various residence times. The increase in absorbance at 318 nm shows a logarithmic relation with the extent of reacted monomer. The logarithmic relation can be explained using a reaction model describing minimum and maximum formation of dityrosine cross-links to reacted monomer. Since the size distribution of reaction products was found to be reproducible, the absorbance increase at 318 nm can be used as a fast in-line screening method for the peroxidase-mediated cross-linking of proteins.



## Introduction

The cross-linking of food proteins is a tool to form potential new food ingredients. A quite novel approach in food protein cross-linking is enzymatic oxidation. The use of laccase and peroxidase to cross-link proteins has been well described (1-3). By catalyzing the conversion of tyrosine residues to phenolic radicals, peroxidase (EC 1.11.1.7) can initiate the formation of dityrosines (4) and trityrosines (*in vivo*) (5). This can be used to cross-link food proteins as was shown for  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin (3, 6).

Oudgenoeg (6) studied the importance of the protein conformation of  $\alpha$ -lactalbumin for the availability of tyrosine residues and the formation of dityrosine protein cross-links. Using calcium-depleted  $\alpha$ -lactalbumin (apo-form), the tyrosine residues became more exposed, resulting in a range of cross-linked protein oligomers and polymers. Within food chemistry increasingly, attention shifts towards understanding the functionality of individual molecules instead of that of mixtures of related components. For this a functionality profile of more specific products, on the molecular level, is required. To direct the formation of specific products with certain functionality, more control is required over the peroxidase-mediated protein cross-linking reaction than is currently achieved at lab scale reactions.

In recent years, the use of microfluidic systems in enzymatic reactions has increased considerably. Due to their small dimensions, localized control over concentration gradients, temperatures and separations can be obtained (7, 8). This increases the precise control of the system and the formation of unwanted side products can be prevented. The small dimensions and sample size enable control of enzymatic reactions in microfluidic devices and make them suitable for kinetic parameter determination (9, 10). Enzyme kinetics on microreactor scale do not significantly differ from lab scale reactions giving rise to the possible use for reaction optimization (7). Microreactors are ideal for directing complex enzymatic synthesis, like multienzyme catalysis (11, 12) and cascade reactions (13). With the possibility to place microreactors in parallel (14) enzymatic reactions in microreactors are easy to scale-up and show great potential in catalysis.

Here, we studied the peroxidase-catalyzed cross-linking of calcium-depleted  $\alpha$ -lactalbumin in a microreactor. To make better use of the possibilities of a microreactor, an in-line detection of the extent of formation of reaction products is needed. This would enable a direct detection of product formation, instead of the time-consuming subsequent off-line analysis. To our knowledge, there is, however, no in-line detection method to quantify the extent of oxidative cross-linking of proteins. As the dityrosine bonds formed can be monitored at 318

nm (15), an UV detector was connected in-line with a microreactor. Since the reaction products are heterogeneous (6), it is not clear whether UV detection can be used to quantify the extent of cross-linking of proteins. Therefore, the aim of the present study is to investigate a possible correlation of absorbance at 318 nm and extent of cross-linking. Modifications of  $\alpha$ -lactalbumin were carried out in a microreactor and in lab scale, to obtain a wide range of extents of cross-linking. The UV-signals were compared with the conversion of monomeric  $\alpha$ -lactalbumin determined by size-exclusion chromatography (SEC).

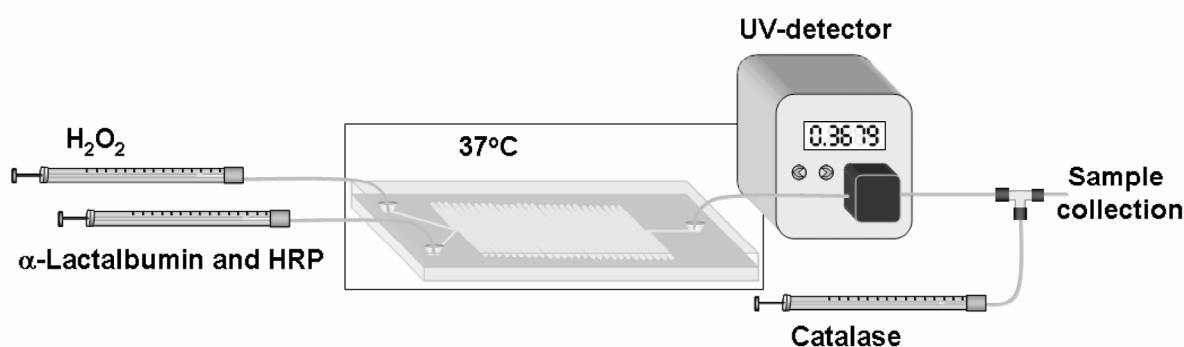
## Materials and methods

### Materials

A commercial  $\alpha$ -lactalbumin powder (BioPURE, Davisco Foods International Inc., Le Sueur, MN, USA) was used for the cross-linking experiments. According to the manufacturer, the powder's protein content was 95 % (w/w) (90 % (w/w)  $\alpha$ -lactalbumin) and 0.55 % (w/w) calcium. Horseradish peroxidase (HRP) type VI-a (P6782), catalase (C30) and  $\beta$ -lactoglobulin (L6879) were obtained from Sigma (Sigma Chemical CO, St Louis, MO, USA). All other (bio)chemicals were analytical grade and purchased from Sigma or Merck (Darmstadt, Germany)

### Microreactor experiments

Cross-linking of  $\alpha$ -lactalbumin was performed in a Y-shaped microfluidic chip (R150.676.1) of Micronit (Enschede, The Netherlands). The volume of the microreactor was 10.1  $\mu$ L, the channel is 150  $\mu$ m wide and deep, and 676 mm long. The microreactor was placed in a water bath at 37 °C and connected to an Ultimate UV-detector with an ULT-UZ-M10 flow cell (LC Packings, Sunnyvale, CA, USA), monitoring at 318 nm. The total reaction volume until the detector was 15.3  $\mu$ L. Reagents were inserted by Hamilton 250  $\mu$ L luer lock syringes (Reno, NV, USA), which were loaded onto a Harvard Apparatus Pico Plus 11 syringe pump (Holliston, MA, USA). All connections were made with 150  $\mu$ m ID fused silica capillaries and MicroTight connectors (P-772 and P-888, Upchurch Scientific, Oak Harbor, WA, USA) (**Figure 1**).



**Figure 1:** Schematic representation of the microreactor set-up.

Syringes were loaded with 2 mM H<sub>2</sub>O<sub>2</sub>, 9 µg/mL catalase, and a mixture of 2 % (w/v) α-lactalbumin and 1 mg/mL HRP, respectively. All solutions contained 0.1 M ammonium acetate. The oxidizing substrate was loaded separately from the enzyme to prevent the inactivation of HRP (16). The H<sub>2</sub>O<sub>2</sub> and enzyme-substrate mixture were guided through the microreactor. To quench the reaction, catalase was introduced in the capillary. This was done after the UV-detector to prevent disturbance of UV-signal by the formed oxygen. For the blank, the syringe with H<sub>2</sub>O<sub>2</sub> was replaced with a syringe containing only 0.1 M ammonium acetate. Reaction time was changed by changing flow rate (0.5 - 10 µL/min per syringe) equal for all three syringes. After each change in flow rate, the system was left to equilibrate for 150 µL. After equilibration, the UV absorbance was quantified and samples (60 µL) were collected at room temperature.

### ***Lab scale experiments***

A 1 % (w/v) protein solution of 1 mL was incubated at 37 °C with 1 mM H<sub>2</sub>O<sub>2</sub> and 0.5 mg/mL HRP. Catalase (3 µg/mL, final concentration) was added to quench the reaction at the desired incubation time. The absorption increase at 318 nm was measured after incubation using the Ultimate UV-detector.

### ***Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)***

The formation of covalently linked protein oligomers was analyzed with SDS-PAGE, using a Phast-system (GE Healthcare, Uppsala, Sweden) according to the supplier's instructions. PhastGels Gradient 8-25 (Amersham) were used for protein separation. Before application, protein samples were treated with β-mercapthoethanol and heated for 5 minutes at 100 °C in the presence of SDS. Proteins were stained with Coomassie Brilliant Blue and a protein molecular weight marker (article 17-0446-01; GE-Healthcare) was used for calibration.

### ***Size-exclusion chromatography (SEC)***

The conversion of monomeric α-lactalbumin to oligomers was determined by SEC. Diluted protein samples (20 µL, 5 mg/mL) in 0.1 M ammonium acetate were applied to a Superdex 75 10/300 GL column (GE Healthcare) with a Äkta Purifier system at room temperature. The column was equilibrated and eluted with 0.1 M ammonium acetate solution at a flow rate of 0.9 mL/min. The eluate was monitored at 280 nm. Calibration of the column was performed with a low molecular mass gel filtration calibration kit (GE Healthcare). The kit contained blue dextran (2000 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

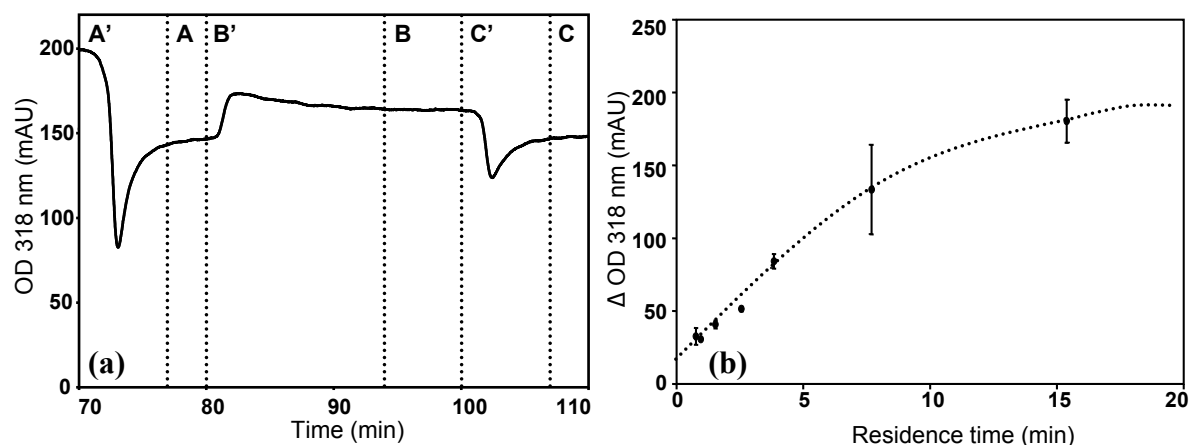
The areas (at 280 nm) of the peaks were calculated with Unicorn software (GE-Healthcare, Chalfont St. Giles, UK) divided over four sections. This calculation gave the proportions of decrease in monomeric α-lactalbumin and the increase in oligomers.

## Results and Discussion

### *Cross-linking in a microreactor*

To quantify, in-line, the cross-linking of  $\alpha$ -lactalbumin in a microreactor one needs a reliable and simple monitoring technique. As dityrosine formation results in an increase in absorbance at 318 nm (15), direct connection of a UV-detector to the microreactor provides a convenient method to measure the cross-linking. Different flow rates, and consequently residence times, were applied to monitor the extent of cross-linking in time.

**Figure 2a** shows a fragment of a typical in-line absorption trace recorded at 318 nm, consisting of three different residence times. The flow rate was increased at 70 and 100 minutes and decreased at 80 minutes. Each change in flow rate was followed by a two-step change in absorbance. Sections A', B' and C' represent the equilibration phase needed to stabilize signal intensity and pump pressure (17). Fractions were collected when the signal intensity was stable (Sections A, B and C) and the average signal intensities were calculated from the stable sections. In **Figure 2b**, signal intensities at different flow rates (1 - 20  $\mu$ L/min) are shown as a function of their corresponding residence time, which is inversely related to the flow rate. The absorbance relates linearly to the residence time up to 4 minutes. At longer residence times the amount of substrate becomes a limiting factor.

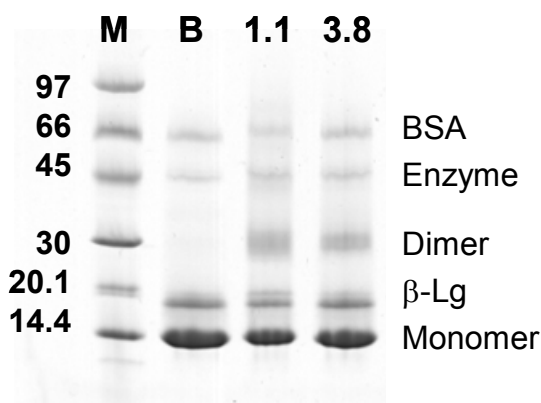


**Figure 2:** In-line UV-detection of the peroxidase-mediated cross-linking of  $\alpha$ -lactalbumin in a microreactor. **(a)** Fragment of a typical in-line absorption trace recorded at 318 nm. At 70, 80 and 100 minutes flow rate was changed. Sections A', B' and C' represent equilibration time. Sections A, B and C correspond to plateau values with residence times of 1.03, 2.20 and 1.10 min, respectively. **(b)** Values of the absorbance at 318 nm at different residence times.

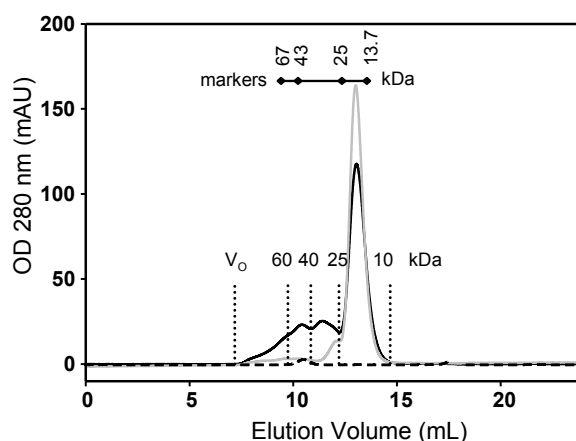
### *Detection of cross-linked product*

Quenched samples were analyzed by SDS-PAGE and SEC. The SDS-PAGE separation under reducing conditions is shown in **Figure 3**. The blank reaction (B), consists of HRP (45 kDa)

and the commercial  $\alpha$ -lactalbumin (a mixture of  $\alpha$ -lactalbumin (15 kDa),  $\beta$ -lactoglobulin (18 kDa) and BSA (66 kDa)). The enzymatic oxidation reactions with residence times 1.1 and 3.8 minutes show an additional protein band at  $\sim 30$  kDa, corresponding to dimeric  $\alpha$ -lactalbumin. Detection of trimer ( $\sim 45$  kDa) and tetramer ( $\sim 60$  kDa) formation is hindered by the presence of HRP and BSA. These results indicate the formation of covalently cross-linked oligomers of  $\alpha$ -lactalbumin.



**Figure 3:** SDS-PAGE of the peroxidase-mediated cross-linking of commercial  $\alpha$ -lactalbumin in a microreactor. M: Marker proteins with their molecular weights indicated at the left; B: Blank reaction without  $\text{H}_2\text{O}_2$ ; 1.1 and 3.8: Residence times of the corresponding sample in the microreactor; enzyme: HRP; monomer and dimer:  $\alpha$ -lactalbumin;  $\beta$ -Lg:  $\beta$ -lactoglobulin.



**Figure 4:** Size exclusion chromatogram of untreated  $\alpha$ -lactalbumin (grey line),  $\alpha$ -lactalbumin after peroxidase-mediated cross-linking (black line) and HRP (dashed line). The residence time of the cross-linking reaction was 15.4 minutes. Elution volumes of standard proteins (albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa)) are included. Sections A to D represent the area of integration to determine cross-linking.

**Figure 4** shows a typical size exclusion chromatogram of HRP (dashed line), untreated  $\alpha$ -lactalbumin and  $\alpha$ -lactalbumin incubated with HRP and  $\text{H}_2\text{O}_2$ . Untreated  $\alpha$ -lactalbumin shows one major peak eluting at 13.0 mL with a small shoulder at 12.3 mL, originating from

$\beta$ -lactoglobulin as determined via separate analysis (no further data shown). The minor peak eluting at 9.6 mL is originating from BSA, based on the results of SDS-PAGE analysis and HRP elutes at 10.5 mL. Incubated  $\alpha$ -lactalbumin shows di- and trimeric  $\alpha$ -lactalbumin eluting at 11.4 and 10.4 mL, based on the results of SDS-PAGE analysis. The recorded size exclusion chromatograms were used to determine the extent of cross-linking by integration of the separate peaks of the chromatogram (**Table I**). Besides oligomers, formed at short residence times, polymeric  $\alpha$ -lactalbumin (excluded volume 7.5 mL) is formed at longer residence times. This results in a broad range of molecules, as previously shown by Oudgenoeg (6). Similar size distribution in cross-linking patterns was observed with similar residence times (**Table I**), indicating a reproducible cross-linking.

**Table I:** Size distribution of cross-linked  $\alpha$ -lactalbumin products formed at different residence times. The relative concentration of product in each size range is given as percentage of the total (with standard deviations), measured by SEC (**Figure 4**).

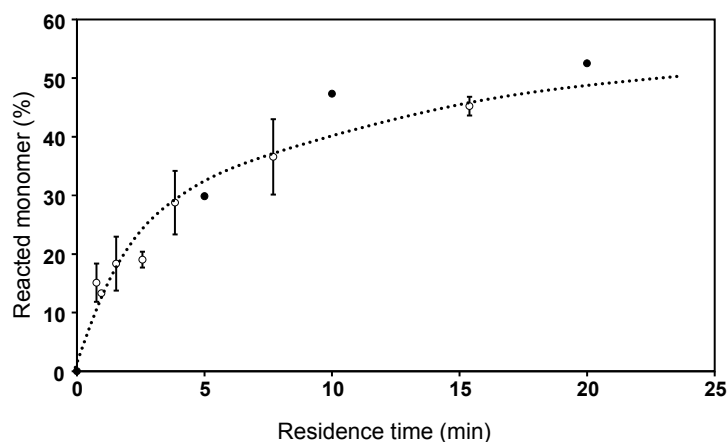
integration section (kDa)	residence time (min)						
	blank	0.77	1.54	2.57	3.85	7.70	15.39
>60	3.2 (0.6)	6.4 (0.5)	7.3 (1.1)	7.4 (2.5)	11.8 (2.3)	17.3 (4.1)	18.1 (2.9)
40-60	3.4 (0.2)	6.6 (0.4)	7.5 (0.6)	7.7 (1.3)	10.3 (0.9)	12.7 (1.3)	14.9 (0.6)
25-40	6.5 (0.4)	10.4 (0.6)	11.3 (0.9)	12.1 (1.6)	13.5 (0.1)	13.8 (0.6)	16.1 (1.8)
10-25	86.8 (0.9)	76.6 (1.5)	73.9 (2.6)	72.8 (5.4)	64.4 (3.3)	56.2 (6.0)	50.9 (1.8)

Besides  $\alpha$ -lactalbumin, traces of  $\beta$ -lactoglobulin are present in the commercial  $\alpha$ -lactalbumin preparation. As reported in previous research, peroxidase catalyzes the cross-linking of  $\beta$ -lactoglobulin in whey protein isolate solutions (3). In this research, however, purified commercial  $\beta$ -lactoglobulin did not show any tendency to cross-link, when treated with HRP and  $H_2O_2$  in lab scale reactions (SDS-PAGE and SEC results not shown). This indicates that only the cross-linking of  $\alpha$ -lactalbumin is causing the increase in absorbance at 318 nm.

#### ***Correlation between reacted $\alpha$ -lactalbumin and increase in absorbance at 318 nm***

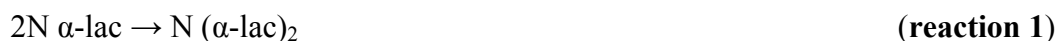
Because molar absorption coefficients of oligomeric  $\alpha$ -lactalbumin molecules are not known, the increase in absorbance at 318 nm due to dityrosine bond formation cannot directly be used for determining the extent of  $\alpha$ -lactalbumin cross-linking. Thus, the amount of reacted monomeric  $\alpha$ -lactalbumin was determined from the decrease in absorbance at 280 nm in SEC experiments. **Figure 5** shows the average extent of reacted monomer at their respective residence times. In agreement with the absorbance measurements (**Figure 2b**), the extent of reacted monomer levels off with increasing residence time. The extent of reacted monomer

is, as expected, quantitatively related to the absorbance at 318 nm. The cross-linking in a lab scale reaction (filled dots, **Figure 5**) shows comparable results as in microfluidic cross-linking. Both systems show a conversion of monomeric  $\alpha$ -lactalbumin up to a maximum of 50 %, upon a single addition of 1 mM  $\text{H}_2\text{O}_2$ . Similar observations for microreactor and lab scale reactions were made in lipase-catalyzed esterification (7). Because of good reproducibility and comparability the results obtained in both systems can be combined.



**Figure 5:** Average decrease in monomeric  $\alpha$ -lactalbumin upon peroxidase-mediated cross-linking at different residence times in a microreactor (○) and in a lab scale reaction (●).

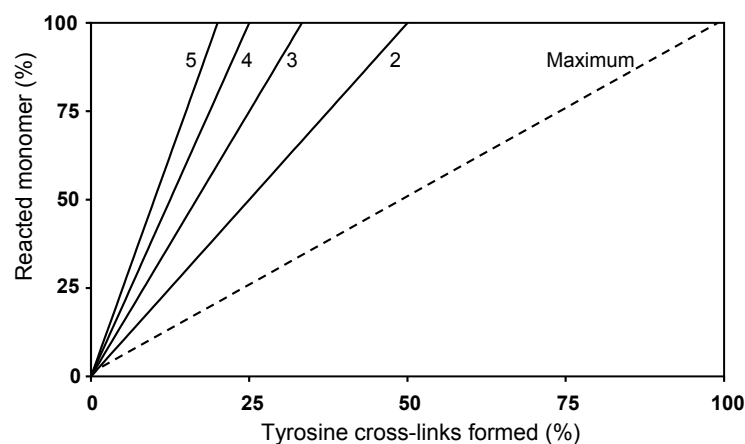
The peroxidase-catalyzed cross-linking of  $\alpha$ -lactalbumin can most simply be described by the following reaction sequences:



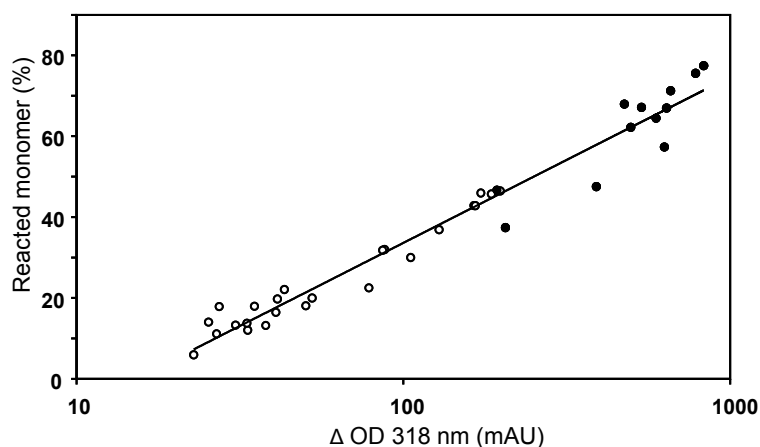
Minimum formation of tyrosine cross-links in relation to reacted monomer is achieved when only dimeric  $\alpha$ -lactalbumin is formed (**reaction 1**). The conversion of monomer to dimer and subsequent to trimer etc. describes maximum formation of cross-links in relation to reacted monomer (**reaction 2**).

$\alpha$ -Lactalbumin contains four tyrosine residues and with the theoretical ability to form trityrosines (5) a maximum of eight cross-links per molecule can be formed. **Figure 6** shows the correlation of reacted monomer to the proportion (%) of tyrosine cross-links formed. In a system with only two possibilities to form a dityrosine bond, a 100 % conversion of monomeric  $\alpha$ -lactalbumin will result, according to **reaction 1**, in a 50 % formation of the total amount of possible dityrosine bonds (line 2 in **Figure 6**). **Reaction 2**, in which

maximum formation of cross-links is described, will result in a 100 % formation of tyrosine cross-links (dashed line in **Figure 6**). Minimum formation of tyrosine cross-links is also shown in **Figure 6** for systems in which, arbitrarily chosen, 3 to 5 tyrosine cross-links per molecule can be formed. When the absorption increase at 318 nm is equal for all different tyrosine bonds the x-axis legend ‘tyrosine cross-links formed’ can be replaced by ‘ $\Delta OD$  318 nm’.



**Figure 6:** Tentative model for  $\alpha$ -lactalbumin cross-linking. Expected correlation for the reacted monomer and the formation of tyrosine cross-links. The minimal formation (solid lines) is indicated for  $\alpha$ -lactalbumin when two to five dityrosine bonds can be formed. The dashed line indicates the maximum formation of tyrosine cross-links.



**Figure 7:** Correlation of reacted monomeric  $\alpha$ -lactalbumin (measured with size-exclusion chromatography) and absorbance increase at 318 nm in a microreactor (○) with in-line UV-detection and in a lab scale reaction (●).

The correlation between the extent of reacted  $\alpha$ -lactalbumin and absorbance increase at 318 nm can be described well by a logarithmic function ( $R^2 > 0.96$ , **Figure 7**). This correlation is independent of the cross-linking system: lab scale reaction (filled dots) and microreaction (open dots) show the same trend. Cross-linking percentages up to 80 %, in batch reaction,



were obtained by adding aliquots of 1 mM H<sub>2</sub>O<sub>2</sub>. The logarithmic correlation is explained by the fact that at first only dimeric  $\alpha$ -lactalbumin is formed (**reaction 1**; solid lines in **Figure 6**). When the reaction continues dimers will react further and an increase in absorption at 318 nm is seen without a decrease in monomeric  $\alpha$ -lactalbumin. This results in a shift towards the maximum formation of tyrosine cross-links, following a logarithmic function, until the maximum has been reached. With the relation described, an in-line determination of extent of reacted monomer is obtained.

## Conclusion

The increase in absorption at 318 nm is a good tool to quantify in-line the peroxidase-catalyzed cross-linking of monomeric (calcium depleted)  $\alpha$ -lactalbumin in a microreactor. Since cross-linking was shown to be reproducible, with respect to both the amount of reacted monomer and to the size distribution of formed products, a fast validation of the peroxidase-catalyzed cross-linking of  $\alpha$ -lactalbumin in a microreactor is obtained. This is a first step in precisely controlling the oxidative cross-linking of food proteins.

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## **Chapter V**

# **Anti-foaming properties of peroxidase cross-linked $\alpha$ -lactalbumin**

**Heijnis, W.H.; Wierenga, P.A.; van Berkel, W.J.H.; Gruppen H.**

### **Abstract**

Process induced cross-linking or aggregation of proteins can change the foam stabilizing properties of these proteins. Recently, it was found that the peroxidase-mediated cross-linking of bovine apo  $\alpha$ -lactalbumin can be used to obtain protein oligomers of specific size. Cross-linked  $\alpha$ -lactalbumin preparations with different oligomer size distributions were obtained by varying the ionic strength and the number of  $\text{H}_2\text{O}_2$  additions. The influence of the oligomer size distribution on foam stability was studied by a foam test and the measurement of rupture times of thin liquid films in a capillary cell. Despite the similar surface properties of cross-linked and non-treated  $\alpha$ -lactalbumin, significant differences were found in their foam and film stabilizing properties. Extensively cross-linked  $\alpha$ -lactalbumin showed decreased stability in both foam stability and thin liquid film experiments, while with less extensively cross-linked proteins no significant changes in either experiments were observed. A foam destabilizing action of the extensively cross-linked  $\alpha$ -lactalbumin was observed in thin films exchange experiments with  $\beta$ -lactoglobulin stabilized films. This shows the potential for using such cross-linked proteins as anti-foam.

## Introduction

To change the foam properties of food proteins, the processing conditions of the proteins can be changed. Small changes in pH or the addition of EDTA already cause an altered surface behavior of the bovine whey protein  $\alpha$ -lactalbumin (1, 2). A change in pH, and thus the net charge of the protein, can cause an increase in flexibility of  $\alpha$ -lactalbumin, giving an optimum in adsorption speed at pH 4 (1). Removal of the calcium co-factor through EDTA chelation results in a more flexible protein that shows a faster adsorption to the air/water interface and more stable foam, compared to the non-treated  $\alpha$ -lactalbumin.

An alternative way to tune the protein foam properties is to pre-process the proteins. This can result in altered properties, without the need to change the conditions. Examples of pre-processing conditions and their effects on the foam properties of protein isolates can be found in literature (3-11). The typical effect of preprocessing is the formation of protein aggregates. Several articles have been published on the effects of aggregates on foam stability, as recently discussed (12). Aggregates formed by heating WPI or  $\beta$ -lactoglobulin solutions clearly affect foam stability (3, 6, 8-10). It appeared from these studies that large aggregates, in the absence of monomers, have a negative effect on foam stability, while small aggregates improved foam stability. An improved foam stability was also observed for large aggregates in the presence of monomers. Thus, control over the aggregate size might help to control foam properties of specific protein preparations.

Foam prepared from an aggregated protein solution needs monomeric proteins at the air-water interface to form an adsorbed layer and to function as anchor points (10). The protein aggregates can form bridges between two interfaces via extended interaction through protein unfolding of the adsorbed monomers at the interface. Another mechanism by which aggregates can increase the foam stability is that large aggregates become entrapped in the Plateau borders, thereby preventing drainage (10). These are all foam enhancing effects, while the mechanism behind the negative effect of large protein aggregates on foam stability has not been described in detail. A negative effect on foam stability can be regarded as an anti-foaming property (13, 14). In general, anti-foams are particles (proteins or aggregated proteins) with a high hydrophobicity. Therefore, they induce an air/water contact angle  $>90^\circ$  and cause the 'bridging-dewetting' mechanism (12) that leads to rupture of the films between neighbouring bubbles, known also as coalescence.

Recently, it was demonstrated that the horseradish peroxidase-catalyzed cross-linking of calcium-depleted bovine  $\alpha$ -lactalbumin can be used to produce protein preparations with a

relatively specific oligomer apparent molecular mass distribution (15). By varying the ionic strength a shift in the mass distribution occurred and a series of differently cross-linked  $\alpha$ -lactalbumin species were produced. In the present study, these cross-linked  $\alpha$ -lactalbumin preparations were used to study the effect of oligomer size on foam stability. The foam stability was determined by a traditional foam test and by measuring the rupture times of thin liquid films in a capillary cell.

## Materials and Methods

### *Materials*

Commercial apo  $\alpha$ -lactalbumin powder (BioPURE) was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). According to the manufacturer, the sample contains 95 % (w/w) protein (of which 90 % (w/w)  $\alpha$ -lactalbumin) and 0.055 % (w/w) calcium. Besides  $\alpha$ -lactalbumin the powder contains traces of bovine serum albumin and  $\beta$ -lactoglobulin (15). Horseradish peroxidase (HRP) type VI-a (P6782) was obtained from Sigma (Sigma Chemical CO, St Louis, MO, USA). All other (bio)chemicals were analytical grade and purchased from Sigma or Merck (Darmstadt, Germany).

### *Cross-linking of $\alpha$ -lactalbumin*

To test the effect of protein cross-linking on foam stability four different batches of modified  $\alpha$ -lactalbumin were produced. The peroxidase-mediated cross-linking reactions were performed as described in previous research (15) by varying the ionic strength (100, 10, and 0.1 mM ammonium acetate) and the number of H<sub>2</sub>O<sub>2</sub> additions (1 and 6 times). The cross-linked protein preparations were stored at -20 °C and thawed before use.

### *Size Exclusion Chromatography (SEC)*

Cross-linked protein samples (20  $\mu$ L, 5 mg/mL) in 0.1 M ammonium acetate, pH 6.8 were applied to a Superdex 75 10/300 GL column connected to an Äkta Purifier system (GE Healthcare, Uppsala, Sweden) at room temperature. The column was equilibrated and eluted with 0.1 M ammonium acetate, pH 6.8 at a flow rate of 0.9 mL/min. The eluate was monitored at 280 nm. Calibration of the column was performed with a low molecular mass SEC calibration kit (GE Healthcare). The kit contained blue dextran (2000 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

The SEC chromatogram was divided into four regions of different apparent molecular masses (>60 kDa, 40-60 kDa, 25-40 kDa, and 10-25 kDa) and the relative amount of each fraction was calculated as described before (15). Trivial names are attributed to the samples based on the relative amount of polymerized (>60 kDa)  $\alpha$ -lactalbumin (pLAN), with n varying from 5 to 55 % (Table I).

### *Drop Tensiometry*

Surface tension of the cross-linked proteins at the air/water interface was measured using an automated drop tensiometer (IT Concept, Longessaigne, France). Before each measurement a new air bubble (7  $\mu$ L) was formed in the protein solution (10  $\mu$ g/mL in 10 mM ammonium acetate pH 6.8). Rheological measurements were

performed with the same set-up using oscillations with an amplitude of 0.75 mm<sup>2</sup> over a period of 10 s. Five oscillating periods were alternated with the same number of resting periods.

### ***Foam stability***

Foam stability was tested with foams prepared in a foam beaker (radius 3.1 cm, height 13 cm) as described previously (7). The beaker was screwed onto a metal bottom containing a metal frit. In this frit a square grid of small pores is made (internal diameter 0.02 mm, at 0.85 mm intervals) for gas passage. Air was sparged at 50 mL/min through this frit and the protein solution, until the foam reached a height of 11 cm. After stopping of the gas flow the foam height is recorded as a function of time. When foam height was decreased to 6 cm the foam collapse became too uneven to obtain further reliable recordings and the experiment was stopped. All samples were measured at least in duplicate and each measurement was performed with a fresh protein solution (0.25 mg/mL; 40 mL 10 mM ammonium acetate pH 6.8). To correct for day-to-day variations, the half-life time of foams were calculated relative to the average half life time of pLA20, which was measured each day as a reference.

### ***Stability of thin liquid films***

The rupture times of thin liquid films were used to obtain indications for the effect of cross-linking on the foam stability. These thin liquid films were made in a capillary cell and observed under reflecting light, using the setup described previously (16, 17). The conditions of the experiment were optimized in order to have measurable rupture times (within practical time-scales). Therefore, the ageing time (time that the protein solution is placed in the cell before a film is formed) and the protein concentration were varied (0-20 minutes and 1.5 – 150  $\mu$ g/mL in 10 mM ammonium acetate pH 6.8, respectively). Based on these results, it was decided to allow the protein solutions (15  $\mu$ g/mL) to equilibrate for 20 minutes in the thin film cell. After this ageing time, a thin film (radius is 100  $\mu$ m) was formed without dimple formation. The rupture times, up to 600 s, of the films were registered, for each sample at least 50 films were measured. The data were used to plot the life time distribution.

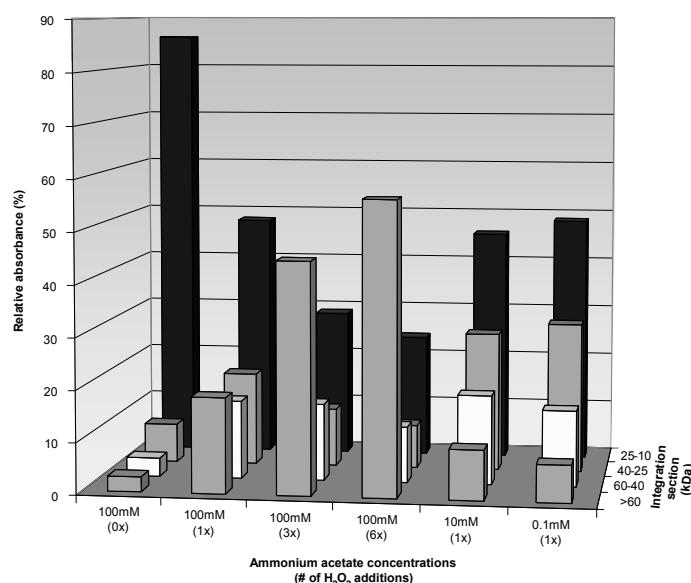
In the above experiments, the thin film stability of cross-linked  $\alpha$ -lactalbumin was studied. Another set of experiments was performed to see how these cross-linked proteins affect the stability of thin films of another protein ( $\beta$ -lactoglobulin). For this, the thin-liquid film exchange experiments were performed as described previously (18). A modified capillary cell is used, where two capillaries are connected to the thin film cell. First, one capillary is filled with the exchange liquid (buffer, non-treated  $\alpha$ -lactalbumin solution, or cross-linked  $\alpha$ -lactalbumin solution (pLa45)). Then a  $\beta$ -lactoglobulin solution is placed in the thin film cell and a small amount is sucked into the side capillary (to act as a buffer zone between the solutions in the cell and in the capillary). The solution is then equilibrated for 20 minutes to allow the formation of an adsorbed layer. After this time, the liquid between the two adsorbed layers is replaced by the solution from the first capillary (*e.g.* buffer). To realize this, liquid from the cell is sucked into the second (empty) capillary, while fresh solution from the first capillary is added. In this way, the liquid between the adsorbed layers is exchanged for the solution in the first capillary (*e.g.* buffer). During the exchange the volume in the cell (40  $\mu$ L) is exchanged approximately 10 times by the liquid in the first capillary (400  $\mu$ L), in a time-span of approximately 30 s. After

the exchange, a thin film is formed as described above, and the rupture time is measured. All experiments were repeated at least three times.

## Results and Discussion

### *Size distribution of cross-linked $\alpha$ -lactalbumin*

Four different batches of cross-linked apo  $\alpha$ -lactalbumin with different molecular weight distributions were produced. The SEC chromatograms (not shown) are integrated over four sections of which the first section contains mainly monomeric  $\alpha$ -lactalbumin (25-10 kDa) (**Figure 1**). The other sections contain mainly dimeric (40-25 kDa), trimeric (60-40 kDa) and polymeric (>60 kDa)  $\alpha$ -lactalbumin, respectively (15). For the non-treated  $\alpha$ -lactalbumin, small traces of  $\beta$ -lactoglobulin, BSA, and added HRP (shown by SDS-PAGE (15)) result in small signals at the molecular weight fractions; 40-25 kDa, >60 kDa, and 60-40 kDa, respectively. For all samples modified by a single addition of  $\text{H}_2\text{O}_2$  the same conversion of monomers to oligomers (50 %) is observed. At low ionic strength relatively more dimers, and at high ionic strength relatively more polymers, are formed.



**Figure 1:** Apparent molecular mass distribution of cross-linked  $\alpha$ -lactalbumin products formed at different incubation conditions. The relative absorbance of proteins in each size range is given as percentage of the total absorbance at 280 nm, as measured by SEC.

At 100 mM ionic strength, multiple additions of  $\text{H}_2\text{O}_2$  (at 30 min intervals) result in a higher degree of conversion and an increased proportion of polymeric material than at a single addition of  $\text{H}_2\text{O}_2$ . After six additions of  $\text{H}_2\text{O}_2$  the amount of dimeric  $\alpha$ -lactalbumin (40-25

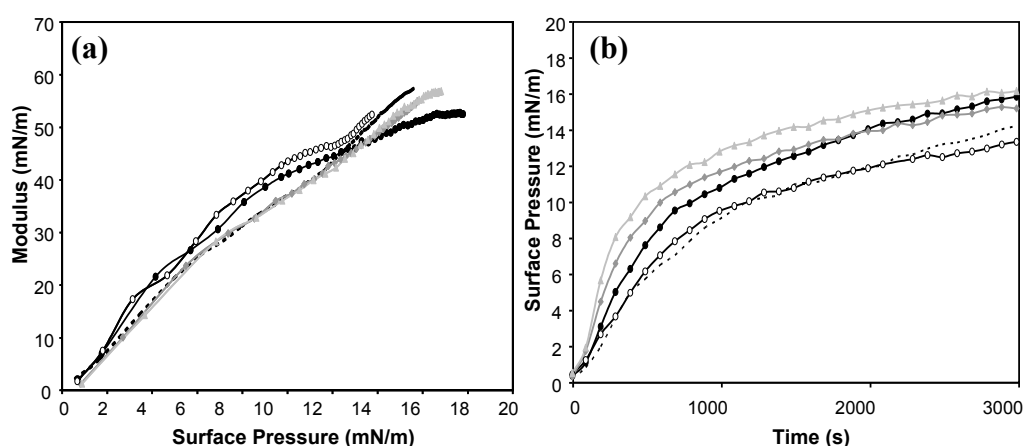


kDa) has decreased to negligible amounts, the residual signal in this fraction is due to  $\beta$ -lactoglobulin already present in the untreated  $\alpha$ -lactalbumin.  $\beta$ -Lactoglobulin was shown previously not to be susceptible to the cross-linking reaction (19).

The cross-linked protein products all contain 20-50 % residual monomers, which is sufficient, at the total protein concentration, to form an adsorbed protein layer at the air/water interface (9). This means that changes in foam behavior are due to changes in the degree of cross-linking, rather than changes in monomer concentration. Therefore, we attribute trivial names to the samples based on the percentage of polymerized ( $>60$  kDa)  $\alpha$ -lactalbumin (pLAN) present in the sample, with n varying from 5 to 55 % (Table I).

### *Interfacial properties of cross-linked $\alpha$ -lactalbumin*

To characterize the interfacial properties of the cross-linked  $\alpha$ -lactalbumin, the surface pressure as well as the dilatational modulus were monitored as a function of time. The dilatational modulus ( $E_d$ ) is shown as a function of the surface pressure ( $\Pi$ ) (**Figure 2a**). As can be seen, there are no significant differences between all samples. The relation between the dilatational modulus and surface pressure is directly related to the relation between the surface pressure and the adsorbed amount ( $\Pi$ - $\Gamma$ , or equation of state). The absence of differences in the plot of  $E_d$ - $\Pi$ , therefore, indicates similar equations of state. Consequently, it may be assumed that at each value of  $\Pi$  the amount of protein material adsorbed is equal and, therefore,  $\Pi(t)$  can be interpreted as adsorption kinetics.



**Figure 2:** The dilatational modulus versus the surface pressure (a) and surface pressure as function of time (b) for (—) non-treated and (( $\blacktriangle$ ) pLA5, ( $\blacklozenge$ ) pLA10, ( $\bullet$ ) pLA20, ( $\circ$ ) pLA55) cross-linked  $\alpha$ -lactalbumin.

The adsorption kinetics, represented by  $\Pi(t)$ , at the air/water interface is shown in **Figure 2b**. Both the adsorption kinetics and the surface pressure, reached after 3000 s (14 mN/m), do not significantly differ between the different samples. From these measurements it is concluded that the composition and properties of the interfacial layer formed by  $\alpha$ -lactalbumin do not change upon cross-linking. Therefore, any differences in foam properties should be attributed to the presence of the aggregates in the liquid lamellae separating the bubbles in the foam. The maillardation of lysozyme has been described as a similar system of modified proteins with different foaming properties, but with similar interfacial properties (7).

**Table I:** Surface and size properties of native (LA) and cross-linked (pLA) apo bovine  $\alpha$ -lactalbumin.

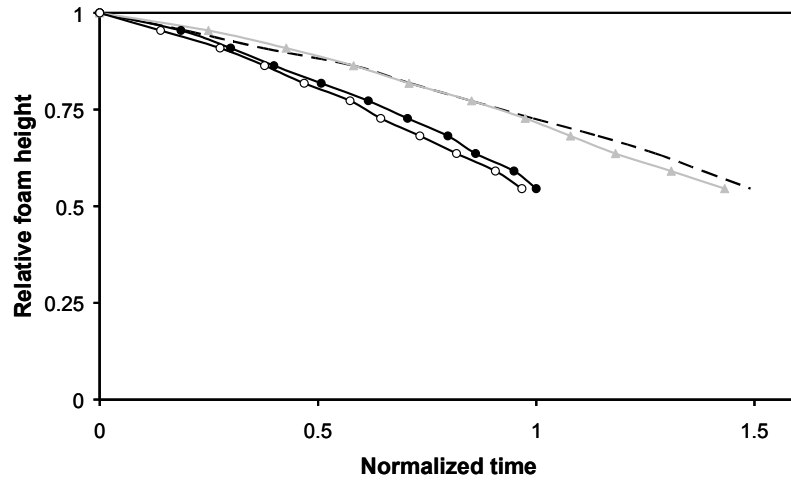
Incubation conditions <sup>a</sup> (# of H <sub>2</sub> O <sub>2</sub> additions)	Trivial name	$\Pi$ (mN/m)	Relative $t_{1/2}$ (-)	Thin liquid film		
				Mean rupture time (s)	$\mu$	$\sigma$
100 mM (0)	LA	14.3	1.49 ( $\pm$ 0.05)	76	1.84	0.40
0.1 mM (1)	pLA5	16.2	1.43 ( $\pm$ 0.25)	66	1.79	0.64
10 mM (1)	pLA10	15.2	ND <sup>b</sup>	92	1.89	0.62
100 mM (1)	pLA20	15.9	1.00 ( $\pm$ 0.12)	69	1.81	0.51
100 mM (3)	pLA45	ND	ND	37	1.57	0.38
100 mM (6)	pLA55	13.4	0.97 ( $\pm$ 0.20)	22	1.32	0.22

<sup>a</sup>Concentration ammonium acetate, pH 6.8. <sup>b</sup>ND: Not determined

### ***Foaming properties***

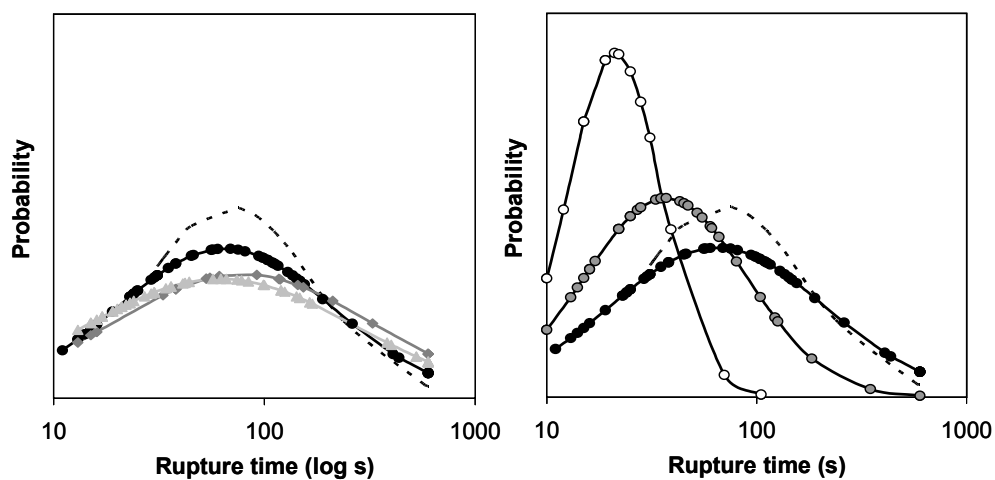
The foam stability was determined from the decrease of foam height in time and plotted in **Figure 3**. Here the relative half time in foam height can be observed for foams stabilized by treated and non-treated  $\alpha$ -lactalbumin preparations. It can be seen that both preparations containing a considerable amount of polymeric  $\alpha$ -lactalbumin (pLA20 and pLA55) formed a less stable foam compared to pLA5 and non-treated  $\alpha$ -lactalbumin. For non-treated apo  $\alpha$ -lactalbumin the foam height decreased to half its original volume in a relative half time of  $1.72 \pm 0.06$  (**Table I**). With increasing aggregate size (pLA5 to pLA55), a significant decrease (33 %) in foam stability is observed. Instead of a decrease, an increase in foam stability was expected as previous reports (8-10) show an increase in foam stability of heat aggregated  $\beta$ -lactoglobulin with monomer proportions down to 10 %. In our system the amount of monomer is at this concentration and should be sufficient for film stabilization at

the total protein concentration used. This indicates a destabilizing effect of the cross-linked  $\alpha$ -lactalbumin.



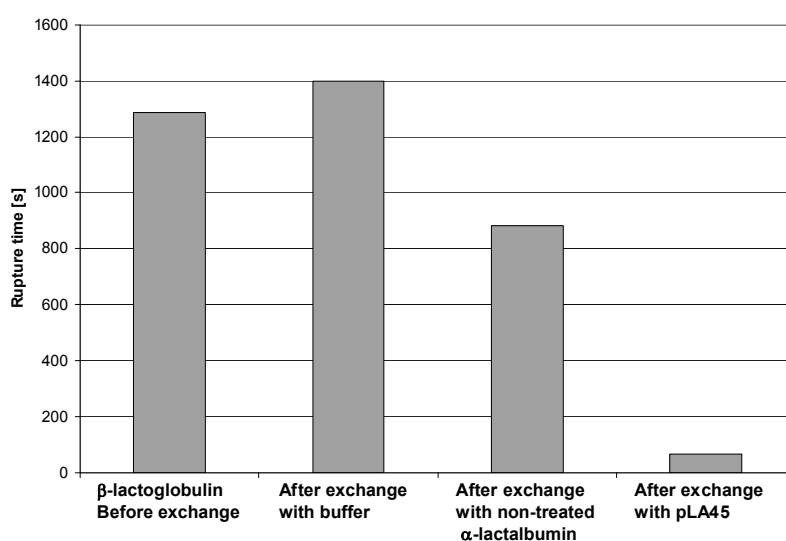
**Figure 3:** Foam stability curves of (—) non-treated and (( $\blacktriangle$ ) pLA5, ( $\bullet$ ) pLA20, ( $\circ$ ) pLA55) cross-linked  $\alpha$ -lactalbumin.

To both simplify the system and to decrease the amounts of sample needed, the rupture times of thin liquid films were measured as indicators for foam stability. The lifetime distribution curves of these films follow normal distribution curves as shown in **Figure 4**. For non-modified  $\alpha$ -lactalbumin a mean lifetime of 76 s is determined. pLA5, pLA10, and pLA20 show similar life-time distributions as the non-treated monomer (**Figure 4a**). Despite the difference in size distribution of the cross-linked  $\alpha$ -lactalbumin preparations no significant difference in mean rupture time was observed (**Table I**). This is in contrast to the foam stability experiments in which pLA20 was less stable than pLA5.



**Figure 4:** Rupture time of films stabilized by (cross-linked)  $\alpha$ -lactalbumin. A: cross-linked  $\alpha$ -lactalbumin with different size distribution of comparable amounts of oligomers. B: cross-linked  $\alpha$ -lactalbumin with increasing amount of polymers. (—) Non-treated  $\alpha$ -lactalbumin, ( $\blacktriangle$ ) pLA5, ( $\blacklozenge$ ) pLA10, ( $\bullet$ ) pLA20, ( $\bullet$ ) pLA45, and ( $\circ$ ) pLA55.

Pronounced differences in rupture time are observed for the samples with high proportions of polymeric  $\alpha$ -lactalbumin (pLA45 and pLA55). As shown in **Figure 4b** a decrease of the mean rupture time is seen with a large increase in aggregate size. This is in line with our foaming experiments and illustrates that even though the interfacial properties of these samples are comparable, large effects on both foam and thin film properties are obtained. From these experiments it becomes evident that cross-linked  $\alpha$ -lactalbumin destabilizes the thin films and consequently the foam. Additional experiments on the destabilizing effect of pLA45 were made using the modified capillary cell described previously (18). In these experiments first a solution of  $\beta$ -lactoglobulin is placed in the thin film cell. After adsorption of the proteins has taken place, the liquid between the adsorbed layers is exchanged by either buffer, non-treated  $\alpha$ -lactalbumin or pLA45 (**Figure 5**). The rupture time of  $\beta$ -lactoglobulin films was not changed very much after exchange with buffer (1300 s, and 1400 s respectively). Exchange with non-treated  $\alpha$ -lactalbumin resulted in a decrease (30 %) in the rupture time, but a significant, drastic decrease in rupture time was observed when the liquid was exchanged with pLA45 (65 s). These observations show that the  $\alpha$ -lactalbumin polymers present in pLA45 can destabilize thin films even if a preformed adsorbed layer (of  $\beta$ -lactoglobulin) is present. This indicates that, in the foam experiments, the residual amount of monomeric  $\alpha$ -lactalbumin is not determining the foam stability. And the mechanism by which these aggregates work may be similar to the mechanism by which other anti-foaming agents have been reported to work (13, 14).



**Figure 5:** Rupture times of  $\beta$ -lactoglobulin films before, and after exchange with buffer, non-treated  $\alpha$ -lactalbumin, or pLA45 (standard error  $\pm 10\%$ )

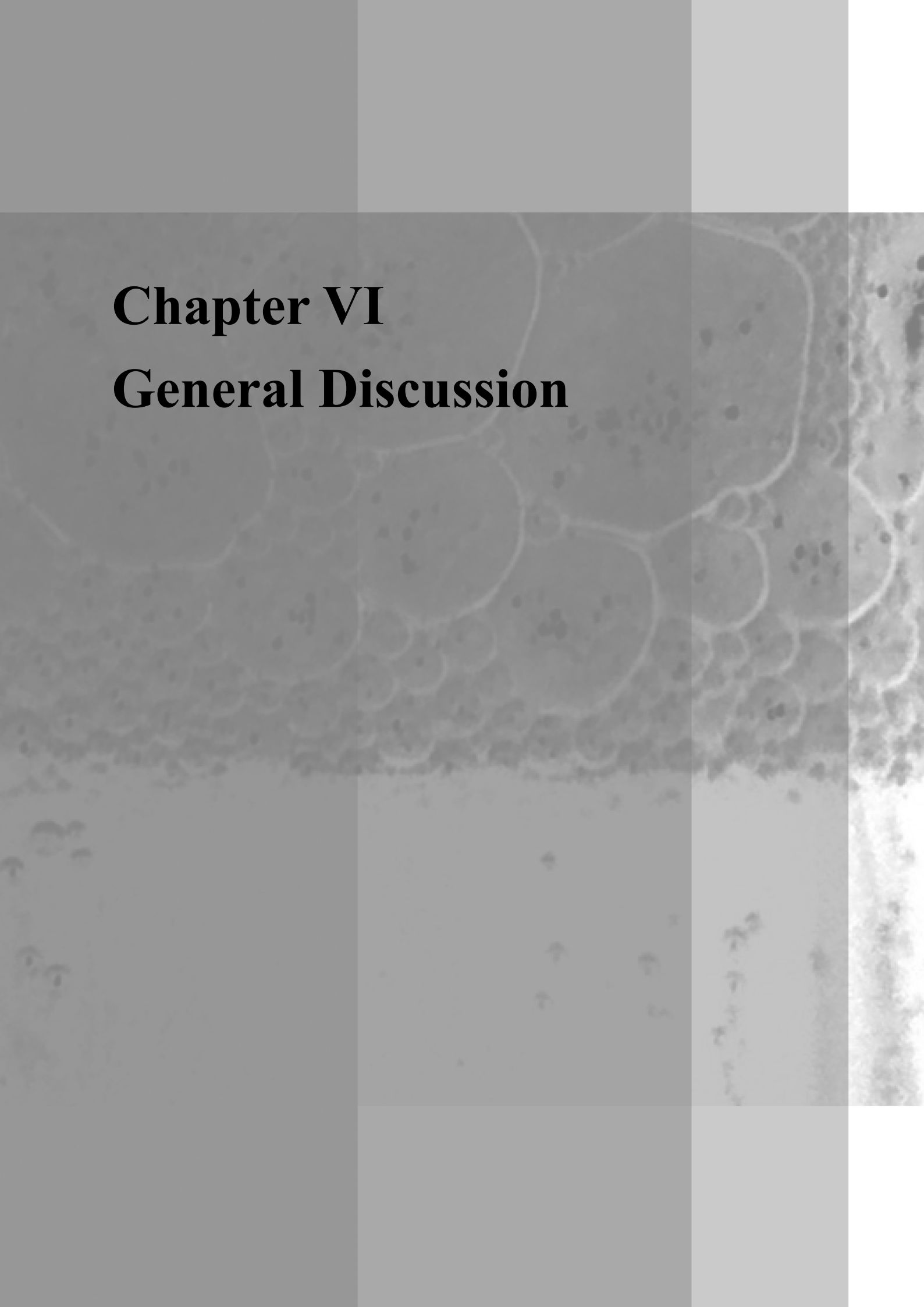
***$\alpha$ -Lactalbumin as anti-foam***

The peroxidase-mediated cross-linking of  $\alpha$ -lactalbumin does not change its interfacial properties but has a large effect on foam stability. Even in small quantities highly cross-linked  $\alpha$ -lactalbumin shows foam destabilizing abilities on  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin stabilized thin films. This anti-foam property of cross-linked proteins has not been reported before. In contrast, aggregated  $\beta$ -lactoglobulin shows at higher concentrations and a higher hydrodynamic radius (*e.g.* 197 nm) stabilized foams (10). One of the crucial differences considering anti-foams is the exposed hydrophobicity.  $\alpha$ -Lactalbumin is a highly hydrophobic protein with a score of 0.70 on a scale of 0-1.0, where  $\beta$ -lactoglobulin scores 0.38 (20). This hydrophobicity of  $\alpha$ -lactalbumin could result in a non-favorable wetting angle at the air/water interface. The extensive cross-linked  $\alpha$ -lactalbumin is proposed to be large enough to cause the bridging-dewetting mechanism and therefore, induce film rupture in a similar way as traditional anti-foaming agents. Further studies are needed to ascertain how generic the anti-foam properties of cross-linked  $\alpha$ -lactalbumin are.

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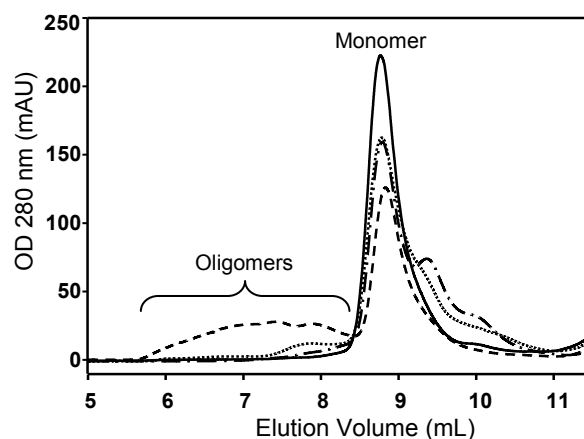
The background of the page is a grayscale microscopic image. The upper half shows a cluster of large, rounded cells with distinct, thin cell walls, characteristic of plant parenchyma or epidermal cells. Below this cluster, there is a layer of smaller, more densely packed cells, possibly representing a different tissue type or a different layer of the same tissue. The overall texture is granular and organic.

# **Chapter VI**

## **General Discussion**

## General Discussion

In this thesis we studied the homo cross-linking of  $\alpha$ -lactalbumin by horseradish peroxidase (HRP). Out of all peroxidase sources and their isozymes available for cross-linking (1), HRP type VI-a was selected to catalyze the cross-linking of  $\alpha$ -lactalbumin. In a preliminary study HRP type VI-a, HRP type I, and soybean peroxidase (SBP) were tested for their cross-linking capabilities (formation of oligomers) of apo  $\alpha$ -lactalbumin under the same reaction conditions (100 mM ammonium acetate pH 6.8, 1 hour at 37 °C) and enzyme/substrate ratio. The results (**Figure 1**) showed minor amounts of cross-linking with HRP type I (dotted line), no cross-linking with SBP (dashed/dotted line), while HRP type VI-a (dashed line) incubation yielded significant covalent cross-linking. Most markedly, for both HRP type I as well as SBP the formation of products with a smaller apparent molecular mass than the  $\alpha$ -lactalbumin monomers was observed. This phenomenon has been described before and is believed to be caused by oxidative peptide bond cleavage (2). This hydrolysis reaction makes both these commercial enzymes unsuitable for the cross-linking reaction and it shows the different cross-linking behavior of peroxidase enzymes. Since no such side reactions were observed for HRP VI-a, this enzyme was chosen for the studies presented in this thesis.



**Figure 1:** Size exclusion chromatograms of  $\alpha$ -lactalbumin oligomers formed by different peroxidase (iso)enzymes (Shodex 803 column; eluent 6 M Urea, 30 % (v/v) ACN, 0.1 % (v/v) TFA). Untreated  $\alpha$ -lactalbumin (—), and incubations with; horseradish peroxidase type VI-a (---), horseradish peroxidase type I (····), soybean peroxidase (- · - ·).

## Means to direct $\alpha$ -lactalbumin cross-linking

The folding state of  $\alpha$ -lactalbumin, and proteins in general, is of importance for the cross-linking reaction (3, 4). In (**Chapter IV**) and also by Oudgenoeg (5) it was clearly shown that holo  $\alpha$ -lactalbumin is insensitive to HRP VIa-mediated cross-linking, and that the removal of



the calcium cofactor is key to obtain  $\alpha$ -lactalbumin oligomers. The inability of HRP VIa to cross-link holo  $\alpha$ -lactalbumin is independent of ionic strength (0.1-100 mM ammonium acetate; no data shown). Apo  $\alpha$ -lactalbumin shows structural changes upon a change in pH and ionic strength (6, 7). These structural changes influence the cross-linking process and result in different size distributions of the oligomers generated.

The addition of the chelating agent EDTA increased the conversion of  $\alpha$ -lactalbumin by transglutaminase (4, 8). In our studies a commercial apo  $\alpha$ -lactalbumin preparation was used, which contains 0.55 % (w/w)  $\text{Ca}^{2+}$ -ions. Therefore, about 20 % of the  $\alpha$ -lactalbumin is expected to be in the holo-form. This perfectly coincides with the fact that an 80 % conversion of monomer was observed after extensive cross-linking upon multiple additions of  $\text{H}_2\text{O}_2$ . Indeed, the addition of EDTA increased the conversion of monomeric  $\alpha$ -lactalbumin by peroxidase (10-15 %), but this increase was only observed after extensive cross-linking (no further data shown).

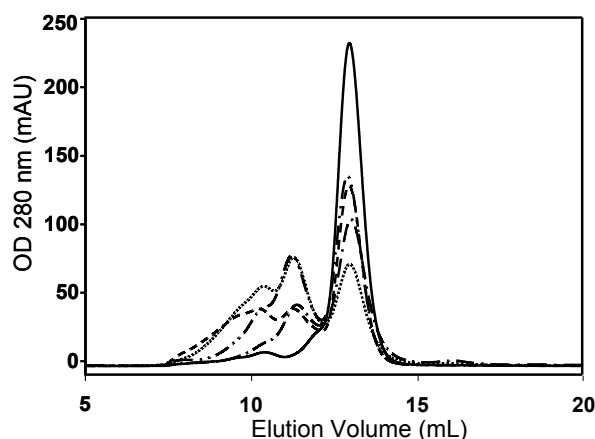
An advantage of the use of peroxidase instead of laccase or tyrosinase is that it enables an easy control of the amount of co-substrate. HRP needs  $\text{H}_2\text{O}_2$  as co-substrate while molecular oxygen is used by the other two enzymes.  $\text{H}_2\text{O}_2$  should be added in small quantities to prevent enzyme inactivation, but multiple additions of the small quantities can increase the conversion of  $\alpha$ -lactalbumin (**Chapters II and IV**). In **Chapter II** we showed that the added amount of  $\text{H}_2\text{O}_2$  does not inactivate the enzyme as we can increase  $\alpha$ -lactalbumin conversion upon multiple additions of  $\text{H}_2\text{O}_2$ . A single addition of  $\text{H}_2\text{O}_2$  resulted in a high dimer/oligomer ratio. The dimer/oligomer ratio gives an indication of control over the cross-linking reaction. In case of a high dimer/oligomer ratio this value can always be decreased by multiple additions of  $\text{H}_2\text{O}_2$ , while the increase of this ratio is not possible.

Other factors than the number of  $\text{H}_2\text{O}_2$  additions, like temperature, pH, and ionic strength affect the peroxidase-catalyzed cross-linking of  $\alpha$ -lactalbumin (**Chapter II**). These factors will be summarized below and (later on) examples will be given how these factors can be applied.

### ***Protein conformation***

The HRP-mediated cross-linking of apo  $\alpha$ -lactalbumin is dependent on the reaction conditions (**Chapter II**). At pH 5.9 the same conversion of monomers resulted in an increased ratio of polymers compared to an incubation at pH 6.8. This pH-dependence of  $\alpha$ -lactalbumin cross-linking might be due to differences in charge distribution of surface residues or structural changes in the local environment of tyrosine residues (6). Differences in

charge distribution might influence the ability of  $\alpha$ -lactalbumin tyrosine radicals to come into close proximity, which is needed to form a cross-link (**Chapter III**). Between pH 6.8 and pH 5.9, no significant change in surface charge distribution is expected as only 3 histidines are located near the protein surface. Local changes in the conformation of the  $\alpha$ -lactalbumin monomer or initially generated covalent dimer might, therefore, be of more importance in directing the cross-linking reaction, than the changes in surface charge distribution.



**Figure 2:** Size exclusion chromatograms of  $\alpha$ -lactalbumin oligomers formed by different enzymes at different ionic strength. Untreated  $\alpha$ -lactalbumin (—), Horseradish peroxidase type VI-a 100 mM ammonium acetate pH 6.8 (---), 10 mM ammonium acetate pH 6.8 (- · - ·), Tyrosinase 100 mM ammonium acetate pH 6.8 (····), Tyrosinase 0.1 mM ammonium acetate pH 6.8 (- - -).

The minor differences in the folding state of  $\alpha$ -lactalbumin at different salt concentrations, as determined by CD (**Chapter II**), enable differences in the size distribution of the oligomers formed by HRP. Another enzyme, which was already described as an  $\alpha$ -lactalbumin cross-linker is mushroom tyrosinase (9). Under the given conditions (pH 6.8), an effect of ionic strength was also observed with mushroom tyrosinase (**Figure 2**). Just as for HRP, at low salt concentrations (0.1 mM ammonium acetate), the tyrosinase-induced cross-linking resulted in the formation of more less higher-order oligomers compared to high salt concentrations. Interestingly, in contrast to HRP, the rate of conversion of monomer increased at higher ionic strength for tyrosinase.

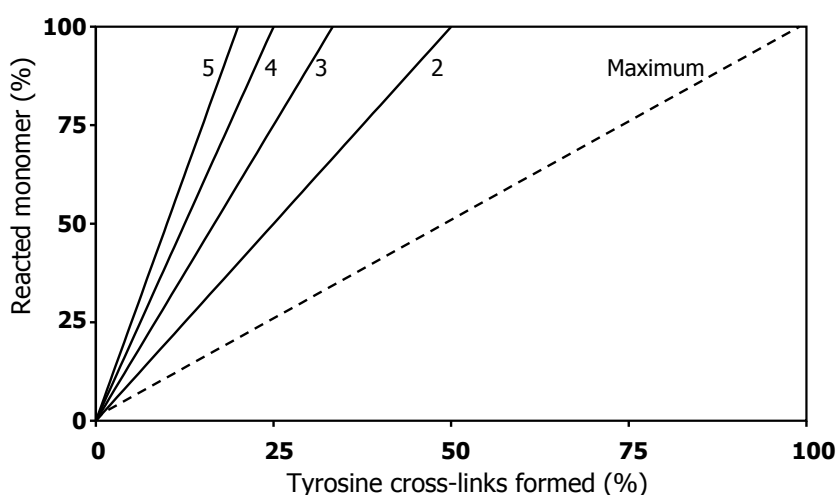
In reports about laccase and transglutaminase the influence of the reaction conditions on the cross-linking of  $\alpha$ -lactalbumin has also been described (4, 10). With these systems, the addition of DTT resulted in a more unfolded protein (7) and cross-linking was enhanced or even induced. This all implies the possibility, for all enzymes, to control or direct the cross-linking of  $\alpha$ -lactalbumin via changes in its conformation.

## Number and sites of cross-links

To be able to direct enzymatic protein cross-linking it is necessary to unravel the location and the extent of the cross-link(s). The difference in apparent molecular mass distribution observed upon various incubation conditions can only be fully understood if the target residues involved in the cross-linking process are known.

### *Quantification of the number of dityrosine cross-links*

In **Chapter IV** the absorption maximum of dityrosine at 318 nm was used to monitor the peroxidase-catalyzed cross-linking of  $\alpha$ -lactalbumin. It appeared that the increase in absorption is correlated to the decrease in monomeric  $\alpha$ -lactalbumin. This correlation is well described by a logarithmic function. With the assumption that the increase in 318 nm is linearly related to the number of dityrosines formed an explanation for this behavior is given.

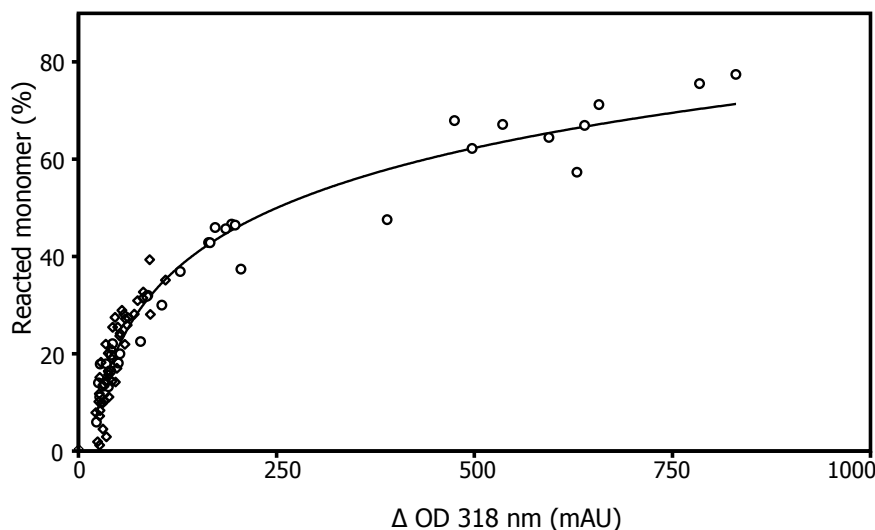


**Figure 3:** Expected correlation between the amount of reacted monomer and the formation of tyrosine cross-links. The minimal formation (solid lines) is indicated when two to five dityrosine bonds can be formed. The dashed line indicates the maximum formation of tyrosine cross-links (11).

In a system where only dimeric  $\alpha$ -lactalbumin is formed by a single dityrosine cross-link (**reaction 1**) a 100 % conversion of monomer with only two tyrosine residues available for cross-linking (line 2; **Figure 3**) will result in the formation of 50 % of the maximum dityrosine cross-links possible. The percentage of dityrosine cross-links formed upon

complete dimerization depends on the maximum number of tyrosine cross-links possible (1 to 8 for  $\alpha$ -lactalbumin (**Chapter IV**)). For 3, 4 or 5 possible cross-links this percentage is 33, 25 or 20 % (lines 3-5). The system in which the monomers react to dimers and subsequently to trimers etc. describes maximum formation of cross-links in relation to reacted monomer (**reaction 2**) and this results in a maximum formation of cross-links (dashed line).

The increase of  $A_{318\text{nm}}$  in relation to the increase in reacted  $\alpha$ -lactalbumin, as determined by the decrease of monomeric  $\alpha$ -lactalbumin, is shown in **Figure 4**. The reaction observed exhibits a combination of both **reactions 1 and 2**. The number of cross-links formed will be in between a solid and the dashed line, starting from the solid and moving to the dashed line (**Chapter IV**).

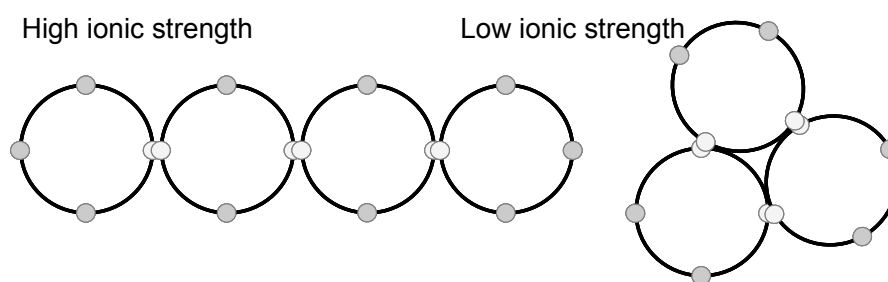


**Figure 4:** Correlation of reacted  $\alpha$ -lactalbumin monomer and absorbance increase at 318 nm at 100 mM (o) and 10 mM (◊) ammonium acetate pH 6.8 upon cross-linking with HRP according to the conditions in Chapter IV.

This correlation was established at a salt concentration of 100 mM ammonium acetate. The same relation was found for a salt concentration of 10 mM ammonium acetate (**Figure 4**) showing that this relation holds over a range of experimental salt concentrations. Despite the difference in apparent molecular mass with the same conversion of monomers at both ionic strengths (**Chapter II**), the correlation of reacted monomer and  $A_{318\text{ nm}}$  is comparable in both systems.

The assumption that the increase in  $A_{318\text{ nm}}$  is linearly related to the increase in the number of dityrosine cross-links formed implies that the same number of cross-links is formed at both salt concentrations. Related to the difference in the distribution of apparent molecular masses, it is hypothesized that at a low salt concentration relatively more cross-links are formed per

molecule than at a high salt concentration. This could be due to the formation of intramolecular cross-links, trityrosine or extra intermolecular cross-links. Although both the *in vivo* and *in vitro* formation of trityrosine has been shown (3, 12), the extra cross-links formed are most likely not originating from trityrosine. The *in vitro* formation of trityrosine was shown for the oligomerization of coactosin with laccase. For that particular protein substrate, the tyrosine residue was freely accessible on a flexible tail of the protein, and the protein structure hindered the formation of higher oligomers than a tetramer. With the mild reaction conditions used in our studies, it is expected that the structure of apo  $\alpha$ -lactalbumin will hinder trityrosine formation. Therefore, our model for the peroxidase-catalyzed cross-linking of apo  $\alpha$ -lactalbumin (Figure 5), only takes an increase in the number of dityrosine cross-links into account. Local unfolding of the tertiary structure of apo  $\alpha$ -lactalbumin induces an increased number of tyrosine residues available for cross-linking and the same number of cross-links can be observed for oligomers with different molecular mass.



**Figure 5:** Model of  $\alpha$ -lactalbumin oligomers with three dityrosine cross-links, formed at high and low salt concentration. Tyrosine residues are represented by grey circles.

### *Site of cross-linking*

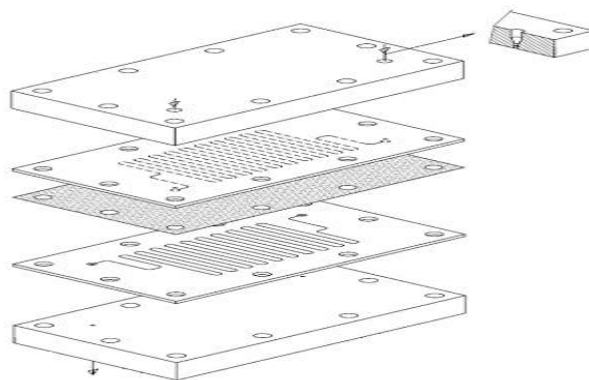
To our knowledge, the identification of the intermolecular Tyr18-Tyr50 cross-link in  $\alpha$ -lactalbumin is the first identification of a peroxidase-catalyzed cross-link in a client protein (**Chapter III**). Based on the identification of the dityrosine cross-link and the surface charge distribution of the  $\alpha$ -lactalbumin monomer, we propose that the formation of the covalent  $\alpha$ -lactalbumin dimer is a two-step process (**Chapter III**). After initial HRP-induced oxidation of the reactive tyrosine residues, the oxidized tyrosines should come together via protein complex formation. Transient non-covalent dimerization of  $\alpha$ -lactalbumin is stimulated by favorable charge-charge interactions bringing Tyr18 and Tyr50 in close vicinity. The formation of the Tyr18-Tyr50 dityrosine link also suggests that the subsequent cross-linking of  $\alpha$ -lactalbumin to higher oligomers obeys a sequential mechanism involving the remaining Tyr18 and Tyr50 residues (Figure 5, left).

The sequential covalent oligomerization process does not explain the shift in oligomer size distribution with constant monomer conversion and  $\Delta A_{318\text{ nm}}$  at low ionic strength (Chapter II). The sequential process, which leads to linear oligomers, is expected to take place at high ionic strength, where the structure of apo  $\alpha$ -lactalbumin is rather compact (7). However, the identified intermolecular cross-link between Tyr18 and Tyr50 was produced in 10 mM ammonium acetate where the protein adopts a partial unfolded state (7). This and the possibility that cross-linking might rearrange the protein structure (5) can result in a change in availability of tyrosine residues other than Tyr18 and Tyr50 (**Figure 5**, right).

## Application of microreactors to the peroxidase-catalyzed cross-linking of $\alpha$ -lactalbumin

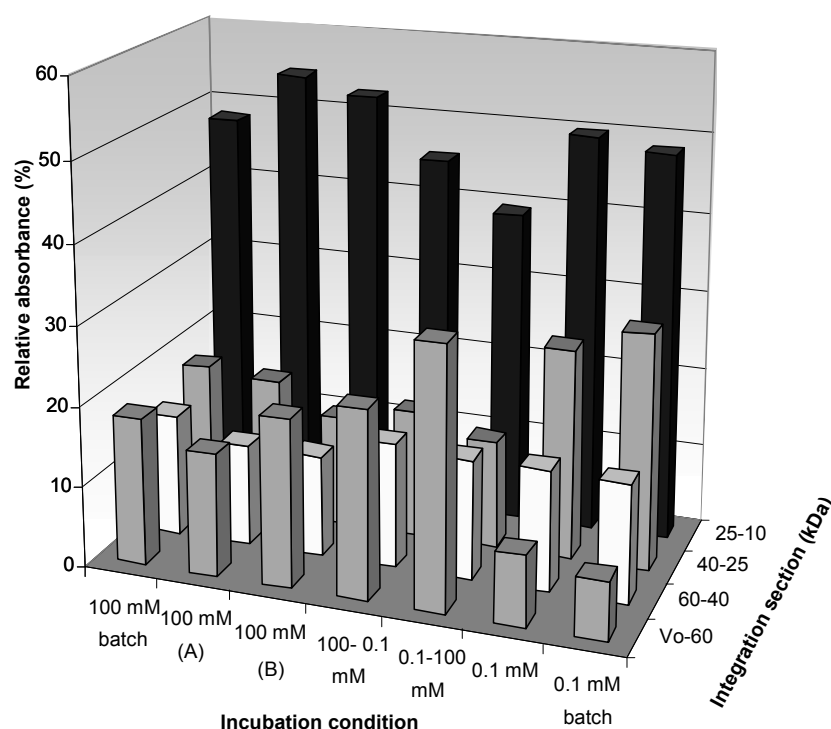
### *Reaction optimization*

The effect of the ionic strength on the mode of cross-linking of  $\alpha$ -lactalbumin (**Chapter II**) opens possibilities to apply a salt gradient in a membrane microreactor. A gradual change in salt concentration will result in a variety of  $\alpha$ -lactalbumin folding states and thus, a different reactivity of tyrosine residues. To realize such a microreactor, a membrane microfluidic device with a counter flow continuous system (**Figure 6**) was set-up to achieve a concentration gradient of 0.1 to 100 mM ammonium acetate.



**Figure 6:** Schematic drawing of the membrane microfluidic device. From top to bottom: polycarbonate (PC) layer, polyvinylchloride (PVC) layer with microchannels, membrane, PVC layer with microchannels, and PC layer. The arrows near the PC layers indicate the inlets and outlets (13).

This concentration gradient should enable the formation of new types of oligomers. It was hypothesized that at low salt concentration multiple cross-links were formed per oligomer resulting in different types of oligomers. Depending on the salt gradient conditions, the order and degree of formation of different type of oligomers can then be controlled.



**Figure 7:** Apparent molecular mass distribution of  $\alpha$ -lactalbumin oligomers formed inside and outside (batch) the membrane microreactor. The salt concentrations and gradients are indicated at the incubation conditions. (A) and (B) indicate a single and a double  $\text{H}_2\text{O}_2$  addition, respectively.

The apparent molecular mass distribution as obtained by size exclusion chromatography (SEC) of  $\alpha$ -lactalbumin oligomers is shown in **Figure 7** and is expressed per integration section of the SEC (cf Materials and Methods **Chapter II**). The different incubation conditions are shown on the x-axis and annotated as the salt concentration. The results obtained in the membrane reactor (100 mM, and 0.1 mM) are compared with the results of the batch reactions (batch) at their respective salt concentrations. For both 100 and 0.1 mM ammonium acetate the oligomer apparent molecular mass distribution obtained in the microreactor is quite comparable to that in the batch reaction. The small increases in polymeric  $\alpha$ -lactalbumin observed in the microreactor at both 100 mM (B) and 0.1 mM, is due to an extra counter flow addition of  $\text{H}_2\text{O}_2$ . This extra addition is absent in the 100 mM (A) sample and this results resemble more with the batch reaction. A concentration gradient 100–0.1 mM and 0.1–100 mM results in the formation of polymeric  $\alpha$ -lactalbumin up to 25 and 30 % (**Figure 7**). Based on the apparent molecular mass distribution obtained at 0.1 mM and 100 mM an apparent molecular mass distribution in between those distributions was expected when applying the concentration gradient in the microreactor.

The main factor influencing the apparent molecular mass distribution is the actual salt gradient. To determine this gradient, a reaction without protein was performed in a microreactor, which was connected to a conductivity meter. The syringe representing the protein solution was kept at a constant flow rate while the counter flow was varied. With this data it would be possible to determine the diffusional constants and the concentration gradient. Unfortunately, we did not succeed to obtain representative data of the concentration gradient. It is not clear at what point in the reactor the concentration reaches this level. Nevertheless, it was observed that at a residence time of 15 min the salt diffusion was complete. The salt concentrations at both outlets were at the same level as the concentrations of the counter inlets.

The data shown in **Figure 7** represent preliminary results. More attention has to be paid to the influence of this salt gradient on the formation of the  $\alpha$ -lactalbumin folding states. Nevertheless, these results indicate an influence of salt gradient on the oligomer apparent molecular mass distribution of cross-linked  $\alpha$ -lactalbumin in a microreactor.

### ***In-line product analysis***

One of the first bottlenecks in the application of microreactors to peroxidase-catalyzed cross-linking of proteins was the absence of an in-line detection system. To overcome time consuming analysis after production (sampling and analysis via SEC and/or SDS-PAGE), such an in-line detection method is a necessity. In **Chapter IV** the use of UV-detection has shown its value in the quantification of the reaction. The absorption of dityrosine at 318 nm showed a good correlation with the amount of reacted monomer. A similar approach can be used for processes with no change in UV absorption. This reaction can be monitored with, for instance, fluorescence and refractive index detectors placed in-line with the microreactor, provided that the chromophores are present and also for these detection methods a good correlation is found.

The determination of techno-functional properties of products formed in the microreactor is more difficult. Most techno-functional analysis systems require several milligrams of material, which takes hours to produce, while in a batch (lab scale) system this only takes minutes. At the current technological level, microreactors are more suitable for analysis applications, in which micro amounts of sample are used. Nevertheless, trends in food technology research are to minimize sample amounts to determine techno-functional properties. Thin films assays, as shown in this thesis (**Chapter V**) can be used for partially purified protein preparations, of which typically little material is available, and are especially

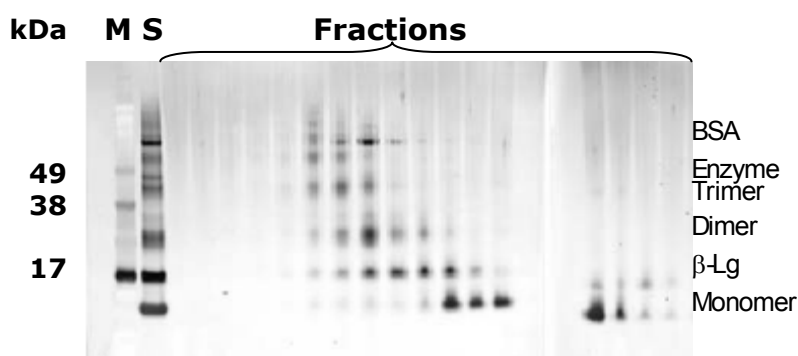


useful in combination with microreactor technology. Also, for the determination of gelling properties a minimization of the functionality assays has been described (14).

### *In-line product separation*

Theoretically, production and yield can be increased when a separation system is included in the microreactor system. Unreacted species can be separated and redirected into the reaction channel. One of the purification systems mentioned in the introduction, free flow electrophoresis (FFE), was tested for its possibility to separate the protein oligomers. A separation based on electrophoretic mobility was obtained by capillary electrophoresis (CE), in which a decreased mobility was observed for  $\alpha$ -lactalbumin oligomers (data not shown). Based on this a minimized electrophoretic system, FFE was tested.

With FFE the sample is separated by latitudinal electronic field on a longitudinal flow (15). The latitudinal electronic field separates molecules on basis of their mobility based on charge distributions and molecular size. The longitudinal flow creates a continuous system and separation, resulting in fractions which can be used for further analysis. The SDS-PAGE analysis of the FFE-separated  $\alpha$ -lactalbumin oligomer mixture is shown in **Figure 8**. The oligomer mixture fractionated in this study was formed at 100 mM ammonium acetate, pH 6.8. Although most fractions, obtained by FFE, still contain multiple protein species, the results clearly show that FFE is capable in separating and purifying protein oligomers.



**Figure 8:** SDS-PAGE of cross-linked  $\alpha$ -lactalbumin fractions as separated by FFE. M: Marker proteins with their molecular mass indicated at the left. The complete oligomer sample (S) and its fractions (Fractions). Enzyme: peroxidase.  $\beta$ -LG:  $\beta$ -lactoglobulin. BSA: Bovine serum albumin. Monomer, dimer, and trimer:  $\alpha$ -lactalbumin.

## Food applications of cross-linked $\alpha$ -lactalbumin

### *Functional properties*

Cross-linked  $\alpha$ -lactalbumin showed typical anti-foaming properties (**Chapter V**). The stability of  $\beta$ -lactoglobulin stabilized thin was decreased upon the addition of cross-linked  $\alpha$ -lactalbumin polymers. These polymers were most prominently created by six additions of  $H_2O_2$ . In continuing work  $H_2O_2$  additions up to fifty times, resulted in an extended polymerization (16).

Techno-functional analysis of enzymatic cross-linked proteins has mainly been focused on changing gelling properties, as shown in Chapter I. It has been described that the gelling of WPI was improved upon cross-linking within limits (17, 18). For both gelling and foaming an optimum in molecular size was described. Cross-linked  $\alpha$ -lactalbumin already showed a decrease in foam stability upon relatively small polymerization (**Chapter V**). Therefore, it would be interesting to compare the foaming and gelling properties of peroxidase-catalyzed cross-linked  $\alpha$ -lactalbumin.

### *Nutritional value*

Cross-linked proteins are less susceptible to proteolysis and essential amino acids are modified, resulting in a decrease in nutritional value (2, 19). The dityrosine cross-link is resistant to proteolysis (20). The dityrosine itself may also cause a steric unfavorable bond in the protein and the protein may not be fully hydrolyzed. A non complete hydrolysis may result in a reduced uptake of amino acids. Interestingly, hetero cross-linking of proteins may also result in an improved digestibility (21) as soy glycinin cross-linked with flavonoids showed an increased susceptibility for tryptic digestion.

The peroxidase treatment of  $\alpha$ -lactalbumin causes the oxidation of methionine and tyrosine, and to a lesser extent, the dehydrogenation of tryptophan (**Chapter III**). Milk proteins are considered a rich source of (semi) essential amino acids (22, 23). The number of essential amino acids of common food proteins, theoretically susceptible to modification by HRP are shown in **Table I**. A single dityrosine cross-link will result in a loss of 25 % of the tyrosine residues in  $\alpha$ -lactalbumin, but for BSA this number is lower (5 %). Control over the reaction can result in a single dityrosine cross-link for dimeric proteins, instead of more cross-links. This will reduce the number of amino acids modified and will reduce the loss in nutritional value.

**Table I:** Food proteins and their number of Tyr, Trp, and Met residues (expasy.org).

Protein	# of Tyr, Trp, and Met	Total number of AA
$\alpha$ -Lactalbumin <sup>a</sup>	4, 4, 1	123
$\beta$ -Lactoglobulin <sup>a</sup>	4, 2, 4	162
$\alpha_{S1}$ -Casein <sup>a</sup>	10, 2, 5	199
$\beta$ -Casein <sup>a</sup>	4, 1, 6	209
BSA <sup>a</sup>	20, 2, 4	583
Ovalbumin <sup>b</sup>	10, 3, 17	386
Lysozyme <sup>b</sup>	3, 6, 3	147
Glycinin <sup>c</sup>		
(G1 subunit)	11, 4, 7	495
$\beta$ -Conglycinin <sup>c</sup>		
( $\beta$ -subunit)	12, 0, 2	439

<sup>a</sup> Bovine; <sup>b</sup> Ovine; <sup>c</sup> Soy

### *Continuation of directing protein cross-linking*

A main objective of this thesis was to control the peroxidase-mediated cross-linking of  $\alpha$ -lactalbumin. We have uncovered several possibilities to obtain various oligomer size distributions. Because  $\alpha$ -lactalbumin is a model protein with regard to the (acid) molten globule state, apo form, holo form, etc. (**Chapter I**), these approaches can be useful for other proteins.

Oligomerization of proteins changes the structure and functional properties of the molecules involved (**Chapter V**). Proteins can also be cross-linked with other food constituents like carbohydrates and phenolic compounds. This type of cross-linking will result in hetero conjugates, which might have novel technofunctional properties. The cross-linking of whey proteins with plant phenols results in a decreased solubility within a wide pH range. The decrease in solubility of the whey proteins has been ascribed to an increase in hydrophobicity (24). This gives polyphenol-protein complexes new potential as for instance emulsion stabilizers. The enzymatic production of protein-carbohydrate conjugates has been shown before with feruloylated carbohydrates for peroxidase, laccase, and tyrosinase (25, 26), and is currently performed with linker-modified carbohydrates (27). Most important in these reactions is the molar ratio of carbohydrate/protein. The reactive species in these carbohydrates (ferulic acid) are oxidized more easily than the tyrosine residues in proteins and will form homo cross-links (28). To overcome this bottleneck the carbohydrate is added step-wise to the reaction mixture. This is a laborious process with demands for ‘new’ technologies. The membrane microreactor is an example of such a new approach. Diffusion-

based addition of the carbohydrates might allow a more controlled supply. Future experiments must show the benefit of the membrane reactor in these reactions.

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The background of the slide is a grayscale microscopic image of plant tissue, showing large, rounded cells with prominent cell walls and internal structures. The image is overlaid with a grid of semi-transparent gray rectangles. The title 'Summary en Samenvatting' is centered in a bold, black, serif font.

# Summary en Samenvatting

### **Summary**

In this thesis, the optimization of the horseradish peroxidase-catalyzed cross-linking of the bovine whey protein  $\alpha$ -lactalbumin is addressed. The enzymatic cross-linking of food proteins is a promising tool to change their functionality. Protein cross-linking may result in different solubilities, foam stabilities, whippabilities, gelation abilities, and in different emulsification properties.

In **Chapter I** an overview is presented of the enzymes currently used in food protein cross-linking (transglutaminase, laccase, tyrosinase, and peroxidase). The heme-containing enzyme horseradish peroxidase is known as a potent cross-linker of calcium-depleted  $\alpha$ -lactalbumin. Bovine  $\alpha$ -lactalbumin is an attractive target for protein cross-linking studies because its folding states are well known and can be changed. The calcium-free apo protein, for instance, has a native-like structure, which is more flexible and sensitive to changes in pH and ionic strength than the holo protein. This flexibility facilitates the electron transfer from reactive (tyrosine) side chains to the porphyrin radical cation of the peroxidase and subsequently results in a broad spectrum of cross-linked oligomers. To prevent the formation of a broad spectrum of  $\alpha$ -lactalbumin oligomers the peroxidase-catalyzed cross-linking needs to be controlled. It is proposed to use microreactors in obtaining control over the enzymatic cross-linking reaction. Our aims are to control and define the cross-linking reaction of  $\alpha$ -lactalbumin and to define the foam stabilizing properties of the cross-linked oligomers.

In **Chapter II** the first step in controlling the peroxidase-mediated cross-linking reaction is shown. The apparent molecular mass distribution of the cross-linked protein changes with the ionic strength and pH of the reaction conditions. At low ionic strength, more dimers of  $\alpha$ -lactalbumin are formed than at high ionic strength, at which higher oligomers are formed, while the same conversion of monomers is observed. Similarly, at pH 5.9 more higher oligomers are formed than at pH 6.8. This is proposed to be caused by local changes in apo  $\alpha$ -lactalbumin conformation as indicated by circular dichroism spectroscopy. A gradual supply of hydrogen peroxide improves the conversion of monomeric  $\alpha$ -lactalbumin and increases the proportion of higher oligomers. Just as in the first step of the cross-linking reaction, also after extensive cross-linking the ionic strength is a key factor in the apparent molecular mass distribution. These results show that the size distribution of the cross-linked  $\alpha$ -lactalbumin can be directed towards the protein oligomers desired.

In **Chapter III**, the mode of cross-linking of apo  $\alpha$ -lactalbumin is addressed in further detail. The initially formed covalent  $\alpha$ -lactalbumin dimer obtained at low ionic strength, at



which the formation of small oligomers dominates, was separated by ion-exchange chromatography and studied for its site of cross-linking. Liquid Chromatography, Fourier-Transform Mass Spectrometry (LC-FTMS) of proteolytic digests revealed for the first time the unambiguous identification of a covalent link between Tyr18 and Tyr50. This shows that, although the radical reaction is often regarded as a random reaction, the initial product formation is specific. Protein structural modeling indicated that the conjugation reaction between these tyrosines is sterically favored and involves initial non-covalent protein-protein complex formation through charge compensation, proving an intermolecular cross-link. The identification of the Tyr18-Tyr50 cross-link supports the view that the peroxidase-mediated oxidation of apo  $\alpha$ -lactalbumin is a sequential process, involving the formation of linear trimers and higher-order oligomers.

As a second step in controlling the reaction, a microreactor was used to control the process conditions. The first requirement is the development of a suitable tool to monitor the extent of cross-linking. **Chapter IV** describes the use of the intrinsic absorption properties of the dityrosine bond to monitor in-line the peroxidase-catalyzed cross-linking of  $\alpha$ -lactalbumin in a microfluidic system. The dityrosine bond absorbs light at 318 nm, which can be correlated to the conversion of monomeric protein. To quantify the progress of the reaction, the absorbance increase at 318 nm was monitored in-line and compared with the extent of monomeric  $\alpha$ -lactalbumin reacted, as determined by size-exclusion chromatography (SEC), at various residence times. The increase in absorbance at 318 nm showed a logarithmic correlation with the extent of reacted monomer. The logarithmic correlation can be explained using a reaction model describing minimal and maximal formation of dityrosine cross-links. Since the size distribution of reaction products was found to be reproducible at constant ionic strength, the absorbance increase at 318 nm can be used as a fast in-line screening method for the peroxidase-mediated cross-linking of proteins.

In **Chapter V**, the  $\alpha$ -lactalbumin oligomers obtained were tested in foam and thin film stability assays. Despite the similar surface properties of cross-linked and non-treated  $\alpha$ -lactalbumin, significant differences were found in their foam and film stabilizing properties. Extensively cross-linked  $\alpha$ -lactalbumin showed a decreased stability in both foam stability and thin liquid film experiments. The foam destabilizing action of the cross-linked  $\alpha$ -lactalbumin was also observed in thin films exchange experiments of  $\beta$ -lactoglobulin stabilized films. This shows the potential for using such cross-linked proteins as anti-foaming agent.

The last chapter (**Chapter VI**) discusses the major outcomes of the previous chapters. It is discussed why horseradish peroxidase type VI-a was chosen as the oxidative enzyme to cross-link  $\alpha$ -lactalbumin. Furthermore, the implications of the different folding states of  $\alpha$ -lactalbumin on the cross-linking with tyrosinase are discussed. The discussion about the accessibility of tyrosine residues is continued. On the basis of the single dityrosine cross-link found in the  $\alpha$ -lactalbumin dimer a model is proposed for the mode of cross-linking at high and low ionic strength. Apart from these mechanistic issues some applications to improve the cross-linking of  $\alpha$ -lactalbumin in a microreactor are discussed as well. Finally, implications of the cross-linked proteins, with respect to functionality and nutritional properties, and the extension of the cross-linking reaction with other substrates present in foods, are discussed.

## Samenvatting

In dit proefschrift wordt het optimalisatieproces van de door mierikswortelperoxidase (HRP) gekatalyseerde koppeling van het wei-eiwit  $\alpha$ -lactalbumine besproken. Het enzymatisch koppelen van voedingseiwitten is een veelbelovende methode om de functionaliteit van eiwitten te veranderen. De koppeling van eiwitten kan resulteren in een verschil in oplosbaarheid, schuimstabiliteit, (op)klopbaarheid en in veranderde gelerings- en emulsificatie eigenschappen.

In **Hoofdstuk I** wordt een overzicht gegeven van de huidige enzymen die gebruikt worden voor het koppelen van voedingseiwitten (transglutaminase, laccase, tyrosinase en peroxidase). Het heem-groep bevattende enzym HRP staat bekend om zijn potentie in het koppelen van calcium-vrij  $\alpha$ -lactalbumine. Buiten dat is  $\alpha$ -lactalbumine een aantrekkelijk eiwit in koppelingsstudies, omdat de verschillende vouwingsstaten goed bekend zijn en ook veranderd kunnen worden. Het calcium-vrije apo-eiwit, bijvoorbeeld, heeft een structuur die lijkt op het natieve eiwit, maar deze structuur is flexibeler en gevoeliger voor veranderingen in pH and ionsterkte dan de structuur van het calcium-bevattende holo-eiwit. Deze flexibiliteit faciliteert de elektronenovergang van de reactieve (tyrosine) zijgroepen naar het porfyryne kation radicaal van HRP. Dit leidt dan weer tot een breed spectrum aan gekoppelde oligomeren. Om te voorkomen dat een breed spectrum aan  $\alpha$ -lactalbumine oligomeren gevormd wordt, moet de door peroxidase gekatalyseerde koppelingsreactie gecontroleerd worden. Een mogelijkheid tot het verkrijgen van controle over de  $\alpha$ -lactalbumine koppeling is het gebruik van microreactoren. De doelen van het beschreven onderzoek zijn: het verkrijgen van controle over en het definiëren van de  $\alpha$ -lactalbumine koppelingsreactie en het beschrijven van de schuimeigenschappen van de gekoppelde oligomeren.

In **Hoofdstuk II** wordt de eerste stap in de controle van de peroxidase-gemedieerde koppelingsreactie weergegeven. De moleculaire massaverdeling van de gekoppelde eiwitten is afhankelijk van de ionsterkte en pH van de reactie omstandigheden. Bij een lage ionsterkte worden meer dimeren van  $\alpha$ -lactalbumine gevormd dan bij een hoge ionsterkte, waar hogere oligomeren worden gevormd. In beide gevallen is met een verschillende productvorming dezelfde omzetting van monomeren waargenomen. Hetzelfde is het geval bij pH 5.9, waar meer hogere oligomeren worden gevormd dan bij pH 6.8. Dit wordt waarschijnlijk veroorzaakt door lokale veranderingen in de apo  $\alpha$ -lactalbumine conformatie. Deze lokale veranderingen zijn gemeten met circulaire dichroïsme spectroscopie. Een verhoging van de omzetting van monomeer  $\alpha$ -lactalbumine wordt verkregen door een geleidelijke toevoeging

van waterstofperoxide en dit verhoogt tegelijk de vorming van hogere oligomeren. Net als in de eerste stap van de koppelingsreactie is de ionsterkte, ook na een uitgebreide koppeling, een belangrijke factor in de moleculaire massaverdeling. Uit deze resultaten blijkt dat de grootte verdeling van het gekoppelde  $\alpha$ -lactalbumine kan worden gestuurd in de richting van de gewenste eiwitoligomeren.

In **Hoofdstuk III** wordt dieper ingegaan op de vorming van de apo  $\alpha$ -lactalbumine koppeling. Hiervoor wordt het in eerste instantie gevormde covalente dimeer gescheiden via ionwisseling chromatografie en verder onderzocht op de exacte locatie van de koppeling. Dit dimeer is gevormd bij omstandigheden met een dominante vorming van kleine oligomeren. Liquid Chromatography, Fourier-Transform Mass Spectrometry (LC-FTMS) van het gehydrolyseerde dimeer onthulde een overduidelijke covalente koppeling tussen Tyr18 en Tyr50. Dit laat dat zien dat, ondanks dat de radikaal reactie omschreven staat als een willekeurige reactie, de initiele vorming van het dimeer een specifieke reactie is. Modelstructuren van het eiwit wijzen op een sterisch favoriete koppeling tussen deze tyrosines. Hierbij wordt er eerst een niet-covalent eiwit-eiwit complex gevormd door ladingscompensatie. Deze modellen bewijzen de vorming van een intermoleculaire koppeling. De identificatie van de Tyr18-Tyr50 koppeling ondersteunt het idee dat de peroxidase-gekatalyseerde oxidatie van apo  $\alpha$ -lactalbumine een continue proces is waarbij lineaire trimeren en hogere oligomeren gevormd worden.

Als tweede stap in het verkrijgen van controle over de reactie is een microreactor gebruikt. De eerste benodigheid, in deze, is een geschikte methode om de mate van koppeling te volgen. **Hoofdstuk IV** beschrijft het gebruik van de intrinsieke absorptie eigenschappen van de dityrosinebinding om de peroxidase gekatalyseerde koppeling van  $\alpha$ -lactalbumine te volgen. De dityrosinebinding absorbeert licht bij 318 nm, wat gekoppeld kan worden aan de omzetting van monomeer eiwit. Om de reactie te quantificeren, werd de toename in absorptie bij 318 nm gevolgd in serie en deze waarden werden vergeleken met de hoeveelheid gereageerd monomeer, bepaald met size-exclusion chromatography (SEC), bij verschillende verblijftijden. De toename in absorptie bij 318 nm liet een logaritmisch verband zien met de hoeveelheid gereageerd monomeer. Het logaritmische verband kan verklaard worden door middel van een reactie model, waarbij de minimale en maximale vorming van dityrosine koppelingen beschreven worden. Omdat de massaverdeling van de reactieproducten reproduceerbaar is bij een constante ionsterkte, kan de toename in absorptie bij 318 nm gebruikt worden als een snelle, in serie staande screenings methode voor de door peroxidase gevormde koppeling van eiwitten.

In **Hoofdstuk V** worden de verkregen  $\alpha$ -lactalbumine oligomeren getest in schuim- en dunne film stabiliteitsassays. Ondanks de overeenkomsten in oppervlakte eigenschappen van het gekoppelde en onbehandelde  $\alpha$ -lactalbumin, waren er significante verschillen gevonden in de schuim- en filmstabiliserende eigenschappen. Extensief gekoppeld  $\alpha$ -lactalbumine liet afnemende stabiliteiten zien in zowel schuim- als dunne film experimenten. De schuim destabiliserende actie van het gekoppelde  $\alpha$ -lactalbumine werd ook geobserveerd bij dunne film experimenten met  $\beta$ -lactoglobuline gestabiliseerde films. Dit laat de potentie zien van peroxidase gekoppelde eiwitten als anti-schuimmiddel.

Het laatste hoofdstuk (**Hoofdstuk VI**) bespreekt de belangrijkste uitkomsten van de vorige hoofdstukken. Er wordt onder andere uitgelegd waarom mierikswortelperoxidase type VI-a is gekozen als het oxidatieve enzym om  $\alpha$ -lactalbumine te koppelen. Verder worden de gevolgen van de verschillende vouwingsstaten van  $\alpha$ -lactalbumine op de koppeling met tyrosinase besproken en wordt de discussie over de toegankelijkheid van de tyrosine zijgroepen voortgezet. Op basis van de specifieke dityrosine koppeling gevonden in het  $\alpha$ -lactalbumine dimeer wordt er een model getoond voor de koppeling bij zowel hoge als lage ionsterkte. Buiten deze mechanistische discussies worden er ook wat mogelijkheden besproken om de koppelingsreactie in een microreactor te verbeteren. Daarnaast zijn kort de gevolgen van het koppelen van eiwitten op de functionele en nutritionele eigenschappen in voedsel alsook van koppelingen met andere substraten beschreven.



The background of the page is a grayscale microscopic image of plant tissue, showing large, rounded cells with prominent cell walls and smaller, darker structures within. The image is partially obscured by a grid of four vertical bars of varying shades of gray, which are positioned on the left and right sides of the page.

# **Dankwoord**

## **Dankwoord**

Daar zit je dan, je hebt met hulp van een  $x$ -aantal mensen een proefschrift in elkaar gezet en het enige wat nog rest is het dankwoord. Hiermee impliceer ik niet dat dit onderdeel de minste aandacht krijgt van mij. Integendeel, het dankwoord is immers het meest gelezen stuk van het hele proefschrift.

In de afgelopen vier jaar heb ik een achtbaanrit meegemaakt waarin een hoop hoogte- en dieptepunten zaten. Om eerlijk te zijn heb ik me op sommige momenten weleens afgevraagd of ik de hele rit wel uit zou moeten zitten. Nu het na een flinke beproeving van mijn uithoudingsvermogen dan toch zover is dat ik mijn werk mag gaan verdedigen tegenover hooggeleerde opponenten, komt er een gevoel van trots bij mij naar boven. Zoals ik al schreef zijn er een  $x$ -aantal mensen die me tijdens die vier jaar hebben geholpen dit proefschrift succesvol af te ronden. Naar een aantal hiervan wil ik mijn speciale dank uitspreken.

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People who have seen me frequently are of course my roommates from room 508. Alice, Tai, Yvonne V., Simone, Tere, Melliana, Maaïke, Moniek, Anita, Annemieke, and Raluca. Thank you for all the good times and laughter we have had together.



De kamer waar ik eveneens veel tijd heb doorgebracht is kamer 514. Laura, Ruud en Martine, zonder het dagelijkse wandelingetje naar kamer 514 had ik toch andere manieren moeten vinden om mijn verhaal en energie kwijt te raken en daar waren waarschijnlijk een hoop mensen niet zo blij mee geweest. Ondanks dat jullie bij Time Management een manier hadden geleerd om aan te geven dat je druk was en geen tijd had om te praten (handen op het toestelbord houden), heb ik bijna altijd wel het idee gehad dat ik welkom was voor een gesprek of om mijn hart te luchten. Ditzelfde geldt natuurlijk voor René, Marijke en Marijn, gelukkig dat jullie in een andere kamer zaten, zo kon ik verschillende bezoeken over de dag spreiden, of zo'n bezoekje combineren met een wandeling in het arboretum.

During my PhD-project I have always stated that I wanted to supervise a student. However, the only student who was interested in my project, Dhanavel was hardly supervised by me. Sorry for the inconvenience Dhanavel, but I still learned a lot from supervising at a distance. Fortunately, my inability to supervise you didn't have a negative impact on your performance, because other people were willing to help you out! That is also one of the strengths of Food Chemistry as a Laboratory: The willingness to help other people. Keep up the good work!

Jolanda, als de nood het hoogst was kon ik altijd bij jou aankloppen voor een oplossing op verschillende problemen. Vooral de korte taxiritjes in je rode autootje waren mij enorm tot steun.

Ook buiten het werk waren verschillende collega's ook een belangrijk onderdeel van mijn sociale leven: Avonden & nachten in de Radstake, Scheveningen, Nijmegen en Hoofddorp. Hartelijk dank voor al deze mooie momenten Marijke, Ruud en Martine. Ondanks de grote woorden hebben we hier helaas geen resultaten geboekt. Dit mocht de pret niet drukken, want we hebben immers goede dansbewegingen geleerd.

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Doodle, je krijgt een knuffel en een mooi bot!

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Walter

The background of the page is a grayscale microscopic image of plant tissue, showing large, rounded cells with prominent cell walls and internal structures. The image is partially obscured by a grid of four vertical bands of varying shades of gray, which are wider on the left and right sides and narrower in the center.

# About the author

## ***Curriculum Vitae***

Walter Hendrik Heijnis is geboren op 26 januari 1983 te Heelsum. In 2001 heeft hij zijn VWO diploma behaald op het *Dorenweerd College* te Doorwerth. In datzelfde jaar begon hij aan de opleiding Levensmiddelentechnologie aan de *Wageningen Universiteit*. Binnen deze studie specialiseerde hij zich in ‘Product Functionality’. Deze specialisatie werd afgesloten met een stage bij de *Vurense Snack Industrie* (VSI, Leerdam) en daarnaast een groot en klein afstudeervak bij de leerstoelgroep Levensmiddelenchemie. Dit resulteerde, in september 2006, in het behalen van zijn ingenieurs-diploma. Na het afronden van de opleiding Levensmiddelentechnologie is Walter zijn promotie-onderzoek gestart bij de *Wageningen Universiteit*, eveneens bij de leerstoelgroep Levensmiddelenchemie. De resultaten van dat onderzoek staan in dit proefschrift beschreven. Walter ambieert een carrière in het hoger- of middelbaaronderwijs, hierop is hij zich momenteel aan het oriënteren.

**List of publications**

Kabel, M.A.; Heijnis, W.H.; Bakx, E.J.; Kuijpers, R.; Voragen, A.G.J.; Schols, H.A., Capillary electrophoresis fingerprinting, quantification and mass-identification of various 9-aminopyrene-1,4,6-trisulfonate-derivatized oligomers derived from plant polysaccharides. *Journal of Chromatography A* **2006**, 1137, (1), 119-126.

Heijnis, W.H.; Wierenga, P.A.; Janssen, A.E.M.; van Berkel, W.J.H.; Gruppen, H., In-line quantification of peroxidase-catalyzed cross-linking of  $\alpha$ -lactalbumin in a microreactor. *Chemical Engineering Journal* **2010**, 157, (1), 189-193.

Heijnis, W.H.; Wierenga, P.A.; van Berkel, W.J.H.; Gruppen, H., Directing the oligomer size distribution of peroxidase-mediated cross-linked bovine  $\alpha$ -lactalbumin. *Journal of Agricultural and Food Chemistry* **2010**, 58, (9), 5692-5697.

Heijnis, W.H.; Dekker, H.L.; de Koning, L.J.; Wierenga, P.A.; Westphal, A.H.; de Koster, C.G.; Gruppen, H.; van Berkel, W.J.H., Identification of the peroxidase-generated intermolecular dityrosine cross-link in  $\alpha$ -lactalbumin. *Submitted for publication*.

## ***Overview of completed training activities***

### **Discipline specific activities**

#### ***Conferences and meetings***

PoaC symposia 08, Geleen and Lunteren, The Netherlands, 2008-10

BC study group meeting, NWO/CW, Lunteren, The Netherlands, 2009

OxiZymes Symposium, Leipzig, Germany, 2010

#### ***Courses***

Industrial Proteins, VLAG, Wageningen, The Netherlands, 2006

Lab-on-a-Chip, ANAC, Enschede, The Netherlands, 2007

Food Enzymology, VLAG, Wageningen, The Netherlands, 2008

### **General courses**

Scientific Writing, CENTA, Wageningen, 2007

Career Assessment, WGS, Wageningen, 2009

Orientation internship at high school, SPRINT-UP, Zetten, 2010

Learning and Instruction Theory, ECS, Wageningen, 2010

### **Optionals**

Preparation of the PhD research proposal, 2006

PhD trip Food Chemistry, Belgium, England and France, 2006

Study trip to Ghent University, 2009

Food Chemistry Seminars, 2006-10

Food Chemistry Colloquia, 2006-10

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# Persoonlijke noot



## ***Notities***



***Persoonlijke noot***



